Pollinators of the Rocky Mountain columbine: temporal variation, functional groups and associations with floral traits

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INTRODUCTION

There is ample evidence that pollinators prefer some floral traits (Fulton and Hodges, 1999) and can select for floral traits (Nilsson, 1988; Campbell, 1989; Galen, 1996; Johnson and Steiner, 1997; Maad, 2000; Alexandersson and Johnson, 2002). For example, hawkmoths affect the spur or tube length of flowers (Nilsson, 1988; Johnson and Steiner, 1997; Maad, 2000; Alexandersson and Johnson, 2002) although a lack of correlation between spur length and seed production has also been observed (Herrera, 1993). Pollinators affect flower colour in various plant species (Schemske and Bradshaw, 1999; Jones and Reithel, 2001; Irwin and Strauss, 2005; Hoballah et al., 2007). In Mimulus species, Bradshaw and Schemske (2003) have shown how flower colour was dependent upon alternative alleles at a locus and that flower colour strongly influenced visitation rates by bumble-bees or hummingbirds. More recently, Hoballah et al. (2007) used genetic introgression and transgenics to show how ANTHOCYANIN2 (AN2), a well-defined myb-type transcription factor that affects flower colour variation between Petunia integrifolia and Petunia axillaris, also influenced pollinator attraction as changes in a single gene caused a shift in pollinators. Additional ongoing studies are examining the genetic basis of floral traits that are influenced by pollinators.

Many plant species are visited by a large number of pollinator species and the abundance and composition of pollinator species often vary over space and time (Herrera, 1988; Waser et al., 1996; Johnson and Steiner, 2000; Thompson, 2001; Fenster and Dudash, 2001). Different pollinators can select for distinct floral features and the temporal and spatial fluctuations in the pollinator fauna can create a heterogeneous selective regime that may weaken selection on floral traits (Schemske and Horvitz, 1989; Herrera, 1996). The large number of pollinators of a given plant species and their potential variation over space and time led Fenster et al. (2004) to propose that the diverse pollinators should be grouped according to function rather than taxonomy, e.g. pollen-collecting pollinators of a plant species. Such practice can reduce the diversity of floral visitors to one functional pollinator group containing many species that exert similar selective pressures on floral traits (Fenster et al., 2004). When they grouped pollinators based on function, Fenster et al. (2004) found one specialist functional pollinator group in about 75% of plant species examined. The concept of functional pollinator group helped explain how diverse pollinators could exert similar selection pressures on floral traits.
Besides pollinators, other biotic factors that can influence selection of floral traits include herbivory, nectar robbing and competition (Levin and Brack, 1995; Strauss et al., 2004; Irwin, 2006). Drought and heat stress have been shown to favour flowers producing anthocyanins (reviewed in Strauss and Whittall, 2006). In Linanthus parryae, the flower colour polymorphism is maintained by a fluctuating environment whereby the blue morph is favoured in years of drought and the white morph in years of abundant precipitation (Schemske and Bierzychudek, 2001). Drought stress may also affect flower size, at times differentially via male and female functions (Galen, 2000). In fact, floral traits may often represent a compromise between selection caused by pollinators and selection by other biotic or abiotic factors (reviewed in Strauss and Whittall, 2006).

Pollinators appear to have played a significant role in the diversification of the plant genus Aquilegia and in the evolution of the spur in this group (Hodges and Arnold, 1995; Whittall and Hodges, 2007). Studies of hummingbird-pollinated A. formosa and hawkmoth-pollinated A. pubescens demonstrated that hawkmoths prefer upright white flowers and that shortening of the spurs in A. pubescens flowers did not affect hawkmoth visitation but significantly reduced pollen removal (Fulton and Hodges, 1999). Miller (1981) observed that blue flowers of A. coerulea set more seeds than white flowers during years in which bumble-bees are abundant and hawkmoths absent, suggesting a preference of blue flowers by bumble-bees. Moreover, Miller (1981) reported bluer flowers in Colorado associated with greater bumble-bee abundance and whiter flowers in south-west Utah and northern Arizona where hawkmoths prevailed. In A. canadensis, herkogamy has been associated with mating system and in A. coerulea, various floral traits and pollinator types seem to affect outcrossing (Brunet and Eckert, 1998; Brunet and Sweet, 2006; Herlihy and Eckert, 2007). Such associations with mating systems may influence the impact of pollinators on floral traits. Selection can only act on genetically encoded traits, and a strong genetic component to key floral traits has been identified in Aquilegia species (Hodges et al., 2002). Finally, the genome sequence of Aquilegia (A. coerulea ‘Origami’, a horticultural variety) will soon be released, thereby enhancing studies of the genetic basis of adaptation in this plant genus.

The present study reports on patterns of variation in pollinator abundance, floral traits and abiotic factors among populations of the Rocky Mountain columbine, A. coerulea, and look for associations between pollinators or abiotic factors and floral traits. I do not claim cause and effect but the highlighted associations suggest future research directions for the study of the evolution of floral morphology in this plant species. Populations were selected within the range examined by Miller (1981), concentrating on regions reported to differ in their predominant flower colours and pollinators. I report 3–4 years of pollinator abundance data for most populations and examine how pollinator abundance and the percentage of the two functional pollinator groups, one that collects pollen and a second that collects nectar, vary over space and time. Additionally, I describe the variation in floral traits among 12 populations, investigating spur length, flower colour and sepal length (flower size) both because these traits have been shown to influence pollinators in Aquilegia or other plant species (Johnston and Steiner, 1997; Fulton and Hodges, 1999) and because they are often affected by abiotic factors (reviewed in Strauss and Whittall, 2006). Finally, the following associations between pollinators or abiotic factors and floral traits were tested. I predicted that: (1) populations with hawkmoths present every year would have whiter flowers than populations in which hawkmoth presence was variable from year to year; (2) populations visited by both Hyles lineata and Sphinx vashti would have flowers with longer spurs relative to populations visited only by Hyles lineata; (3) populations with a greater proportion of nectar-collecting pollinators (hawkmoths) would have whiter flowers with longer spurs; and finally (4) populations at higher altitudes would have whiter flowers. The reasoning behind each hypothesis is presented in the Materials and Methods section. Data on the variation in pollination and floral traits among wild populations of A. coerulea and associations between these factors should greatly benefit future studies of genetic adaptation in this plant species.

MATERIALS AND METHODS

Study species

The Rocky Mountain columbine, Aquilegia coerulea (Ranunculaceae), is a perennial herbaceous plant that occupies montane and subalpine habitats at elevations of 2100–3700 m in Colorado, New Mexico, Utah, Idaho, western Wyoming and northern Arizona (Whitemore, 1997). The plant is self-compatible and does not reproduce clonally. The strongly protandrous flowers open sequentially on inflorescences, with distal flowers opening first (Brunet, 1996). The radially symmetrical flowers have five petals consisting of an upper flattened lamina and an elongated spur with a nectary located at the base of each spur. The female reproductive organ is surrounded by 50–130 stamens and consists of five to ten unfused carpels each of which can develop into a mature follicle. The onset of stigma receptivity is indicated by the swelling of papillae on the stigmatic surface.

Study sites

Floral measurements were made in 12 populations, three in each of four geographical areas (Fig. 1). The geographical areas included the North Rim division of the Grand Canyon National Park, the Dixie National Forest east of Cedar city in south-west Utah, the Gunnison National Forest in central Colorado, and the Routt National Forest in north-west Colorado. Pollinator observations were made in six of the 12 populations with at least one population per geographical area. Pollinator abundance data previously published in Brunet and Sweet (2006) were combined with two to three additional years of data for a total of three to four years in each population, except in central Colorado where only one year of data were gathered for each of two populations.

Pollinator abundance, composition and behaviour

Pollinator observations were made on at least three different days in each population for an average of 18.7 h of day
observations and 7 h of dusk observations per population each year (totalling 318 h of day observations and 120 h of dusk observations; Table 1). Within each population, pollinators were observed over four 1-h periods: 0930–1030 h, 1230–1330 h, 1530–1630 h and the approximately 2030–2130 h of dusk, depending on time zone. The only exception was Falls Creek in summer 2001, for which pollinators were observed during two time periods: midday (1330–1430 h) and dusk (2030–2130 h). These time periods accommodated the distinctive diurnal activity patterns exhibited by the different types of pollinators including hawkmoths, which come out at dusk.

During every 1-h observation period, one to three observers (depending on site and year) each examined a separate patch which typically included 10–30 flowers. Before each observation period, the number of plants in the patch and the number of open flowers on each plant were registered. During an observation period, every pollinator entering the patch of flowers, the number of flowers visited in succession per foraging bout, and the time spent visiting flowers during the foraging bout were recorded. A foraging bout started when a pollinator entered a patch of flowers and ended when the same pollinator left the patch. Specimens of each type of insect observed visiting *Aquilegia coerulea* flowers were collected and later identified. Hawkmoths and bumble-bees were identified at the species level, solitary bees and syrphid flies at the genus level, and house flies at the family level as Muscidae.

Floral visitors were grouped into hawkmoths, bumble-bees, solitary bees, syrphid flies and Muscidae. Pollinator abundance was calculated as the number of visits per flower per hour for each pollinator group. This was also measured for each bumble-bee and hawkmoth species visiting *Aquilegia coerulea* flowers. Bumble-bees, solitary bees, syrphid flies and Muscidae were present mostly during the day while...
hawkmoths were present mostly at dusk. Thus, dusk abundance data were used for hawkmoths and day abundance data were used for the other pollinators, both to determine the spatial and temporal (yearly) patterns of variation in pollinator abundance for the different groups using ANOVAs (Proc GLM, SAS 2003, Version 9-1, Cary, NC: SAS Institute Inc.) and to contrast the abundance of the different species of hawkmoths and bumble-bees in each population.

The time spent per flower and the number of flowers visited during a foraging bout among pollinator groups were compared in the different populations using ANOVAs and Duncan multiple range tests for mean comparisons (Proc GLM, SAS 2003). These analyses were performed first including then excluding Muscidae as this group did not play an important role as pollinators (my personal observations). The time spent per flower was obtained by dividing the time spent in a foraging bout by the number of flowers visited during that bout. Data for these analyses came from the 2004 field season because the same observers visited all geographical areas that summer.

Relative contribution of functional pollinator groups

Pollinators were grouped into their respective functional group and the percentage of the two functional groups in each population each year was calculated. The pollen-collecting functional group included bumble-bees, solitary bees and syrphid flies. Muscidae were not included in that group because they spent a long time in a flower, rarely moved between flowers and therefore did not play much of a role as pollinators (my personal observations). The second functional pollinator group consisted solely of hawkmoths, the only pollinators able to collect nectar produced at the base of the spurs in this plant species. Because of the interest in fly pollination in alpine habitats (Kearns and Inouye, 1994), I report the percentage of Muscidae as a separate category together with the percentage of each of the two functional pollinator groups in each population each year.

Floral characteristics

On average, 53.7 flowers were measured (range 33–88; one flower per plant) in each of 12 populations in 2004. For each flower at the beginning of stigma receptivity, sepal colour was recorded and the length of the longest spur and sepal length were measured to the closest 0.01 mm using digital calipers. Spur length was measured as the distance between the base of the spur and the beginning of the petal blade (as shown in fig. 1 in Hodges et al., 2002) and sepal length as the distance between the tips of two opposite sepals in a flat plane. Although floral traits varied among regions, ANOVAs and Duncan’s multiple range tests were used to compare spur length and sepal length among the 12 populations (Proc GLM, SAS 2003). I was interested in whether populations differed from each other irrespective of their region of origin.

Flower colour, for non-white flowers, was categorized in the field using paint colour charts 59–64 (purples to blues) from Ace Hardware (Oak Brook, IL). Within each colour chart, colour was classified between letters A and G where G corresponded to very pale blue, E–F to pale blue, C–D to blue and A–B to dark blue. Next, a value of 1 was assigned to white, 2 to very pale blue, 3 to pale blue, 4 to blue and 5 to dark blue in order to quantify colour, a non-parametric ANOVA was then used to compare colour among populations (Proc NPAR1WAY, SAS 2003). To determine differences between the 12 populations, I first determined the rank for each color value (Proc RANK, SAS 2003) and performed ANOVAs and Duncan’s multiple range tests on these ranks (Proc GLM, SAS 2003). Finally, I examined the distribution of flower colour within each population and calculated Spearman rank correlations between flower colour, spur length and sepal length (Proc CORR, SAS 2003).

Associations between pollinators or altitude and floral characteristics

Hawkmoths are present every year in Arizona and Utah but occur on average every other year in north-west Colorado and much less frequently in central Colorado (see Discussion). The presence and absence of hawkmoths has been linked to geographical areas in this plant species (Miller, 1981; my personal observations). Moreover, in areas where hawkmoth occurrence varies from year to year, bumble-bees tend to be present each or most years and are important pollinators of this plant species (Miller, 1978). I therefore compared floral traits between populations with constant and variable hawkmoth presence to determine whether the yearly presence of hawkmoths influenced floral traits. First, because hawkmotths tend to be crepuscular pollinators typically attracted to whiter flowers and bumble-bees prefer blue flowers in this plant species (Miller, 1981), I predicted that populations with annual hawkmoth activity would have whiter flowers relative to populations with less frequent hawkmoth activity. Second, hawkmoths are the only pollinators that can collect the nectar produced at the base of the spurs. I hypothesized that spur length would not change between populations with annual vs. variable hawkmoth presence as long as hawkmoths were present in some years. Third, I predicted that flower size would not vary between these two groups as there is no evidence suggesting that bumble-bees and hawkmoths prefer distinct flower size in this plant species.

Two hawkmoth species have been observed visiting A. coerulesa flowers, Sphinx vashti and Hyles lineata. Whereas H. lineata has been observed at least in some years in all geographical areas used in this study (see Discussion), S. vashti has not been observed in central (Miller, 1981) or north-west Colorado (my personal observations). The latter occurs in northern Arizona and has been observed in two of the three populations in south-west Utah: it was not seen at Cedar Breaks, the population at the highest elevation (3195 m). Individuals of S. vashti tend to have longer tongues than H. lineata individuals [5-8 cm for S. vashti (n = 3) vs. 4-0 cm for H. lineata (3-8 for n = 25 and 4-1 for n = 15; Miller, 1981)]. If tongue length influences spur length, we would expect populations in which S. vashti is present at least in some years to have longer spurs relative to populations visited only by H. lineata. On flowers with spurs shorter than the tongue of the hawkmoth, nectar is taken but no pollination occurs because the hawkmoth does not come into contact with anthers or stigmas. This is disadvantageous
to the plant, due to lack of pollination, and disadvantageous to the short-tongued hawkmoths because hawkmoths with a longer tongue deplete the nectar in visited flowers. Therefore, if their frequency significantly affects pollen transfer and nectar availability to shorter-tongued hawkmoths, the presence of hawkmoths with longer tongues should select for longer spurs in the population. The increased spur length should force contact of longer-tongued hawkmoths with anthers and stigmas while allowing shorter-tongued hawkmoths to obtain the nectar as they reach deeper into the flower (Miller, 1981). To test whether spurs were longer in populations visited by S. vashti, I compared this floral trait between populations only visited by H. lineata and populations where S. vashti was present at least in some years. I also contrasted flower colour and sepal length between these two groups of populations, although I expected no difference in flower colour and only a difference in sepal length should a strong correlation exist between spur length and sepal length (indirect selection). For the previously described tests, ANOVAs were used to compare groups of populations (Proc GLM for spur length and sepal length and Proc ANOVAs were used to compare groups of populations (Proc GLM for spur length and sepal length and Proc NPAR1WAY for flower colour, SAS 2003).

To examine how the two functional pollinator groups influenced floral traits, I examined the regressions between the percentage (proportion × 100) of nectar-collecting pollinators in a population and spur length or sepal length (Proc GLM, SAS 2003) and Spearman correlations between the percentage of nectar-collecting pollinators and flower colour (Proc CORR, SAS 2003). Percentages were arcsine transformed to stabilize the variance. I predicted a negative relationship between an increase in nectar-collecting pollinators and flower colour but a positive relationship between nectar-collecting pollinators and spur length or sepal length. For these analyses, the pollinator percentages were averaged for populations over the multiple years of observations.

Finally, I examined correlations between altitude and floral traits. Higher altitudes are associated with lower temperatures and with greater water availability in this plant species (my personal observations). Because drought and heat stress tend to favour flowers producing anthocyanins (reviewed in Strauss and Whittall, 2006), I expected whiter flowers at higher altitudes. Similarly, if maintaining flowers is costly in terms of water use I predicted larger flowers at higher altitudes. Finally, if larger flowers are correlated with longer spurs then I predicted longer spurs at higher altitudes. Therefore, I tested for correlations between altitude and whiter, larger flowers with longer spurs (Proc CORR, SAS 2003) (Spearman’s rank correlation for flower colour and Pearson product moment correlation coefficients for spur length and sepal length).

RESULTS

Pollinator abundance

Pollinator observation data were not available for the same years for all populations, so I compared yearly variation within each population. In addition, because I was interested in the variation of the different pollinator groups over time, I examined temporal variation within a population separately for each pollinator group. Over time, each group of pollinators varied in abundance in some populations and distinct groups of pollinators varied in the different populations (Fig. 2). For example, hawkmoth abundance varied over years in the Original, Cedar Breaks and Falls Creek populations (P < 0.05; Fig. 2A–E), while syrphid abundance varied significantly in the Original and Falls Creek populations (Fig. 2F–J). Whereas hawkmoth but not bumble-bee abundance varied significantly over time at the Falls Creek population (Fig. 2D), the reverse occurred at the Archery population (Fig. 2C). In addition to this temporal variation, the abundance of each group of pollinators varied significantly among populations (spatially) at least in some years (P < 0.05; Table 2). Bumble-bees were most abundant in Cedar Breaks in 2000 and in Falls Creek in 2003 (Table 2). Hawkmoths were most abundant in the Original population in 2000 and in Cedar Breaks in 2003 (Table 2). Therefore, the abundance of the different groups of pollinators to A. coerulae flowers varied both spatially and temporally.

Despite this strong temporal and spatial variation in abundance of pollinators, the presence or absence of hawkmoths and bumble-bees showed a clear pattern at each location (Fig. 2) and supported previous findings (Brunet and Sweet, 2006). Hawkmoths were the major pollinator every year in northern Arizona (Original); both bumble-bees and hawkmoths were present every year in south-west Utah (Archery and Cedar Breaks); bumble-bees were present every year in north-west Colorado although hawkmoths were abundant in some years and rare or absent in other years (Falls Creek); and only bumble-bees were observed in central Colorado (Emerald Lake and Flat; Fig. 2). By contrast, the presence or absence of the other pollinator groups (syrphid flies, solitary bees and Muscidae) was not predictable for any population, geographical area or year (Fig. 2).

Pollinator composition

Between one and four species of bumble-bees and a maximum of two hawkmoth species were observed in each population in a given year (see Supplementary Data Table S1, available online). When multiple species of bumble-bees or hawkmoths were present in a population, one species always predominated (Supplementary Data Table S1). In 2000, Bombus occidentalis was the most common bumble-bee in all populations examined from north-west Colorado to northern Arizona (Supplementary Data Table S1). However, from 2001, Bombus flavifrons became the dominant species and B. occidentalis was no longer observed after 2001. H. lineata was the most common hawkmoth species in all populations except northern Arizona in 2000, for which only S. vashti was observed visiting A. coerulae flowers that year.

Within solitary bees, sweat bees (Halictidae) were recorded from the genera Lasiosglossum, Dialictus and Halictus; mining bees (Andrenidae) from the genus Andrena; and leaf cutter and resin bees (Megachilidae) from the genera Megachile, Latinanus and Osmia. Syrphid flies were largely from the genera Chrysotoxum and Syrphus.
Pollinator behaviour

The effect of pollinator group on the number of flowers visited per foraging bout did not vary among populations and this was true whether Muscidae were included or not in the analyses (including Muscidae: interaction term, $F_{7,1524} = 0.77$, $P = 0.62$; excluding Muscidae: interaction term, $F_{6,1471} = 0.80$, $P = 0.57$; Table 3A). Hawkmoths visited...
Table 2. Spatial variation in pollinator abundance (number of visits per flower per hour) within pollinator group within year

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<thead>
<tr>
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<tr>
<td>Bumble-bees</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cedar Breaks</td>
<td>1.10</td>
<td>Falls Creek</td>
<td>1.08</td>
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<td>Archery</td>
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<td>Archery</td>
<td>0.90</td>
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<td>0.07</td>
<td>Cedar Breaks</td>
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<td>Grand Canyon</td>
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<td>Hawkmoths</td>
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<td>Grand Canyon</td>
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Lower-case letters indicate statistically significant differences among populations as indicated by Duncan’s multiple range tests.

Table 3. Two-factor analysis of variance examining the impact of pollinator group and population on number of flowers visited per foraging bout and time spent per flower in the Rocky Mountain columbine in 2004, Muscidae excluded

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>MS</th>
<th>F</th>
<th>P</th>
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<tr>
<td>(A) No. of flowers visited per foraging bout</td>
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<td></td>
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<tr>
<td>Pollinator group</td>
<td>3</td>
<td>49.4</td>
<td>13.61</td>
<td>&lt;0.0001</td>
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<tr>
<td>Population</td>
<td>4</td>
<td>4.4</td>
<td>1.21</td>
<td>0.306</td>
</tr>
<tr>
<td>Population × Pollinator</td>
<td>6</td>
<td>2.9</td>
<td>0.80</td>
<td>0.570</td>
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<tr>
<td>Error</td>
<td>1471</td>
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<td></td>
<td></td>
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<tr>
<td>(B) Time spent per flower</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pollinator group</td>
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<td>39.765</td>
<td>42.91</td>
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</tr>
<tr>
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<td>&lt;0.0001</td>
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<tr>
<td>Population × Pollinator</td>
<td>6</td>
<td>11.628</td>
<td>12.55</td>
<td>&lt;0.0001</td>
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<tr>
<td>Error</td>
<td>1386</td>
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</tbody>
</table>

*Muscidae excluded.

Flat population, the interaction term (population × pollinator group) and the population term were not statistically significant when Muscidae were included ($F_{4,1394} = 1.9, P = 0.112$ for the interaction; $F_{3,1394} = 1.95, P = 0.119$ for the population) but remained significant when Muscidae were excluded (interaction: $F_{4,1364} = 3.69, P = 0.0054$; population: $F_{3,1364} = 3.84, P = 0.0054$) mainly because solitary bees and syrphid flies spent more time per flower at Archery relative to the other populations (52.2 vs. 24.35 s for solitary bees and 29.3 vs. 19.3 s for syrphid flies). Overall, hawkmoths and bumble-bees spent the least time per flower (3.5 and 4.6 s, respectively) followed by syrphid flies and solitary bees (20.1 and 26.1 s, respectively) and finally Muscidae (122.4 s; Fig. 3B).

Relative contribution of functional pollinator groups

Each year, the majority of pollinators in the Original population in northern Arizona were nectar collectors (Supplementary Data Fig. S1, available online). In the Archery and Cedar Breaks populations in south-west Utah, both nectar- and pollen-collecting pollinators were common each year, although their relative importance fluctuated from year to year (Supplementary Data Fig. S1). In the Falls Creek population in north-west Colorado, the prevalence of nectar-collecting pollinators strongly fluctuated by year. Nectar-collecting pollinators were absent in 2004 and rare in 2002 but played a significant role in 2001 and 2003 (Supplementary Data Fig. S1). The relative importance of nectar- or pollen-collecting pollinators varied among the...
different geographical regions and over time within some of these populations, with the strongest temporal pattern observed in north-west Colorado (Falls Creek).

**Floral characteristics**

Flower colour, spur length and sepal length all varied significantly among populations (for flower colour d.f. = 11, 615, $\chi^2 = 185.59$, $P < 0.0001$; for spur length $F_{11,615} = 63.29$, $P < 0.0001$; and for sepal length $F_{11,616} = 38.73$, $P < 0.0001$; Fig. 4A, B and C, respectively). The Archery and Hiking Trail populations in south-west Utah had the whitest flowers (Fig. 4A, B) and a high frequency of very pale blue flowers (Fig. 5A, B). The Cedar Breaks population in south-west Utah had bluer flowers relative to the other two Utah populations (Fig. 4A; Fig. 5A–C). The Cedar Breaks population had a frequency distribution of flower colour more closely resembling populations in north-west Colorado (Fig. 5A–C, G–I). The populations in northern Arizona had a large percentage of both blue and very pale blue flowers and a more bimodal distribution of flower colour relative to the other examined populations (Fig. 5D–F). Populations in central Colorado had the bluest flowers (Fig. 4A; Fig. 5J–L). The shortest spurs were found in populations in north-west Colorado while the Hiking Trail and Archery populations in south-west Utah and the Point Imperial population in northern Arizona had the longest spurs (Fig. 4B). The Cedar Breaks population in south-west Utah had spurs significantly shorter than the other two populations from this same geographical region (Fig. 4B). Spur length varied significantly ($P < 0.05$) among at least some of the populations from each geographical region. Sepal length was less variable among populations relative to the other floral characteristics. The Emerald lake and Flat populations in central Colorado had the largest flowers (estimated by sepal length), while Mount Crested Butte from this same geographical region had the smallest flowers (Fig. 4C). Sepal length was negatively correlated with flower colour (Spearman $r = -0.11$, $P = 0.005$) and was positively correlated with spur length (Spearman $r = 0.60$, $P < 0.001$) and flower colour was negatively correlated with spur length (Spearman $r = -0.23$, $P < 0.0001$; $n = 616$). Therefore, whiter flowers tended to be larger with longer spurs.

**Associations between pollinators or altitude and floral characteristics**

As predicted, populations where hawkmoths were present every year had whiter flowers (2.9 vs. 3.5, $n = 6$ vs. 6; d.f. = 1, $\chi^2 = 6.6$; $P = 0.01$). These populations also had longer spurs (50.3 vs. 40.9 cm, $n = 6$, 6; d.f. = 1, $F = 10.79$, $P = 0.008$) but did not have larger flowers (75.9 vs. 69.2 mm, $n = 6$, 6; d.f. = 1, $F = 2.15$; $P = 0.17$) than populations where hawkmoths were variable from year to year. These data suggest that annual hawkmoth presence selected for white flowers with longer spurs. In the same vein, populations visited by both *H. lineata* and *S. vashti* also had whiter flowers (2.9 vs. 3.5, $n = 5$ vs. 7; d.f. = 1, $\chi^2 = 4.82$, $P = 0.028$) and longer spurs (51.6 vs. 41.3 cm, $n = 5$ vs. 7; d.f. = 1, $F = 14.6$, $P = 0.003$) and tended to have bigger flowers although this trait was not statistically significant (77.4 vs. 69.1 mm, $n = 5$ vs. 7; d.f. = 1, $F = 3.63$, $P = 0.09$). These latter data suggest that the presence of *S. vashti* may select for whiter flowers with longer spurs. However, except for the Cedar Breaks population where hawkmoths were observed every year but *S. vashti* was not seen over three years of pollinator observations, all other populations with *S. vashti* were also populations with annual hawkmoth presence. The most likely explanation for the longer spurs is hence the presence of the longer tongued *S. vashti* rather than the annual presence of hawkmoths. Conversely, the whiter flowers are unlikely to result from the presence of *S. vashti* but are most probably the consequence of having hawkmoths every year in the population.

No statistically significant relationships were observed between the percentage of nectar-collecting pollinators in a population and larger, whiter flowers with longer spurs (regression for spur length $y = 2.28x + 44.2$, $r^2 = 0.04$, $P = 0.75$; and for sepal length $y = 1.67x + 74.7$, $r^2 = 0.01$, $P = 0.86$; Spearman correlation for flower colour $r = -0.60$, $P = 0.28$). The percentage
of nectar-collecting pollinators in the population explained a very small portion of the variance in a floral trait (1–4 %). Similar results were obtained when the percentage of pollen-collecting pollinators was used as the independent variable (results not shown).

Contrary to expectations, higher altitude was correlated with bluer flowers (Spearman $r = 0.68$, $P = 0.015$). Higher altitude was not correlated with larger flowers (Pearson $r = –0.12$, $P = 0.71$) or longer spurs (Pearson $r = –0.45$, $P = 0.14$).

A summary of the tests for associations between pollinators or abiotic factors and floral traits is presented in Table 4.

**DISCUSSION**

**Pollinators**

Numerous pollinator groups visited *A. coerulea* flowers and their abundance varied over space and time. However, there was a clear pattern in the presence and absence of hawkmoths and bumble-bees at each of these sites, confirming the pattern previously reported for these different populations (Brunet and Sweet, 2006). The present data supported Miller’s (1981) suggestion that hawkmoths are more common and more reliable in Utah and Arizona. Although I only observed a temporal

**Table 4. Summary of the tests for associations between pollinators or abiotic factors and floral traits**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Whiter flowers</th>
<th>Longer spurs (mm)</th>
<th>Larger flowers (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Populations with hawkmoths present every year (ANOVA)</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>2.9 vs. 3.5</td>
<td>50.3 vs. 40.9</td>
<td>75.9 vs. 69.2</td>
<td></td>
</tr>
<tr>
<td>$P = 0.01$</td>
<td>$P = 0.008$</td>
<td>$P = 0.17$</td>
<td></td>
</tr>
<tr>
<td>Populations visited by <em>Sphinx vashii</em> (ANOVA)</td>
<td>Yes</td>
<td>Yes</td>
<td>Borderline</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.9 vs. 3.5</td>
<td>51.6 vs. 41.3</td>
<td>77.4 vs. 69.1</td>
<td></td>
</tr>
<tr>
<td>$P = 0.028$</td>
<td>$P = 0.03$</td>
<td>$P = 0.09$</td>
<td></td>
</tr>
<tr>
<td>Populations with greater % of nectar-collecting pollinators (correlation or regression)</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>$r = 0.04$</td>
<td>$r^2 = 0.04$</td>
<td>$r^2 = 0.01$</td>
<td></td>
</tr>
<tr>
<td>Populations at higher altitude (correlation)</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>0.68</td>
<td>$r = –0.12$</td>
<td>$r = –0.45$</td>
<td></td>
</tr>
<tr>
<td>$P = 0.015$</td>
<td>$P = 0.71$</td>
<td>$P = 0.14$</td>
<td></td>
</tr>
</tbody>
</table>
pattern of variation in hawkmoth abundance in north-west Colorado, with hawkmoths being common every other year on average, pollinator data collected by other researchers suggest that hawkmoth abundance may also vary temporally in central Colorado. Miller (1978, 1981) reported sporadic hawkmoth abundance around Gothic Colorado, with abundant hawkmoths in the Rocky Mountain Biological Laboratory area (RMBL) in 1970, 1971, 1973 and 1978 and no or rare hawkmoths in 1969, 1974, 1976 and 1979. No observations were made in 1972, 1975 or 1977. This pattern resembles the present observations in north-west Colorado although the frequency of hawkmoth presence in central Colorado appears to have declined over the last 20 years (N. Waser, University of California, Riverside, pers. comm.). Campbell et al. (2002) detected hawkmoth visits to Ipomopsis tenuituba in central Colorado in two years (1993 and 2001) between 1991 and 2001, suggesting hawkmoth presence every 8 years or so. In summary, hawkmoths seem to be present every year in northern Arizona and south-west Utah, biennially in north-west Colorado and much less frequently in central Colorado.

Although Miller (1981) expected common hawkmoths and rare bumble-bees throughout the south-west United States, we observed rare bumble-bees only in northern Arizona but annually abundant bumble-bees in south-west Utah. Bumble-bees were also abundant every year in north-west Colorado. They were uncommon in central Colorado from the present observations. By contrast, Miller (1978) reported that bumble-bees were common and important pollinators of this plant species in central Colorado, although he did observe some years of low bumble-bee abundance (Miller, 1978).

The present data also support the idea that bumble-bees and hawkmoths are the major pollinators of this plant species (Miller, 1978). Not only did the abundance of solitary bees, syrphid flies and muscidae fluctuate unpredictably both between years within a population and between populations, but syrphid flies and solitary bees spent significantly more time in a flower and visited fewer flowers per foraging bout relative to hawkmoths and bumble-bees. Muscidae spent long periods of time eating pollen within a flower and rarely moved between flowers. Although I do not have data on the number of pollen grains removed and deposited on a stigma per visit by each pollinator group (pollinator effectiveness), the evidence available suggests that bumble-bees and hawkmoths are more reliable pollinators of A. coerulea flowers. Moreover, Sahli and Conner (2007) have shown that pollinator abundance is often more important than pollinator effectiveness in explaining the reproductive success in wild radish.

The pollinators of this plant species can be grouped into two distinct functional pollinator groups: a pollen-collecting group that includes bumble-bees, solitary bees and syrphid flies and a nectar-collecting group that consists entirely of hawkmoths. The pollen collectors actively forage for pollen and cannot reach the nectar produced at the base of the spurs. By contrast, the nectar collectors actively forage for nectar and gather pollen in the process. Aquilegia coerulea is thus among the 25% of plant species with multiple functional pollinator groups, although by the 75% abundance rule of Fenster et al. (2004) a single functional pollinator group existed in some geographical areas. Moreover, in many of the populations a single species of bumble-bee or of hawkmoth made up the majority of that pollinator group each year. Despite the apparently large variation in floral visitors over time and space, one species of bumble-bee and/or hawkmoth predominated in each population each year. Whereas B. occidentalis was the most common bumble-bee visitor prior to 2001, it has been replaced by B. flavifrons. Miller (1978) also reports B. occidentalis as the most common bumble-bee visiting A. coerulea flowers. Interestingly, only B. occidentalis has been observed robbing nectar from spurs of A. coerulea flowers (Miller, 1978; my personal observations). The decline in B. occidentalis visitation coincided with its general decline throughout the western part of the United States (Thorp and Sheperd, 2005). The subsequent increase in B. flavifrons suggests previous interference by the former pollinator. From a separate paternity study done in one population, it is clear that both the pollen- and the nectar-collecting pollinators can influence the evolution of A. coerulea flowers via both male and female functions (J. Brunet and K. G. A. Holmquist, unpubl. res.). In fact, when two groups of plants were visited exclusively by nectar-collecting (hawkmoths) or by pollen-collecting pollinators (84% bumble-bees in the studied year), both groups of plants set similar numbers of seeds and sired significant numbers of seeds (J. Brunet and K. G. A. Holmquist, unpubl. res.).

Floral traits

Based on differences in flower colour and spur length, A. coerulea as a species has previously been divided into five varieties (Munz, 1946; Whittemore, 1997). The two varieties within the range of the present study include A. coerulea var. coerulea (south-west Utah and northern Arizona). The variety coerulea has been characterized by white petal blades, blue sepals and spurs (length 4.0–5.0 cm) and is said to be common in Colorado and northern Mexico; the variety pinetorum (Tidestrom) Kearney and Peebles has white petal blades, pale blue or white sepals and spurs (length 5.0–7.0 cm) and is common in southern Utah and northern Arizona. Interestingly, the state of Utah no longer recognizes the variety pinetorum and lumps it with var. ochroleuca Hooker (M. Bartow, Utah State University Herbarium, pers. comm.). The variety ochroleuca has white petals, sepals and spurs, with spur length similar to var. coerulea (4.0–5.0 cm) and is common in central and northern Utah, Idaho and western Wyoming. The present data do not support the presence of different varieties throughout the range examined but suggest substantial variation in flower colour and spur length within and among populations. Unlike Miller (1981), I did not observe a majority of white flowers in northern Arizona nor a majority of white and pale blue flowers around central Colorado. There were plenty of blue flowers in both of these areas. In central Colorado, the increase in blue flowers over time may be associated with a decrease in the frequency of hawkmoth visits in the area. The only population that could represent var. pinetorum is the Hiking Trail population in south-west Utah while the Archery population in south-west Utah best fits the description of var. ochroleuca. All other populations are better described by var. coerulea, although the spur length of the Point Imperial population in northern Arizona is within the range of var. pinetorum. The southern
populations sampled were east of Cedar City in south-west Utah and within the North Rim of the Grand Canyon National Park in northern Arizona, the same general areas Miller (1981) collected from 25 years earlier. Compared with Miller’s descriptions (1981), I observed an increase in the frequency of blue flowers and a decrease in spur length in these areas. It would be interesting to examine the current distribution of flower colour throughout the rest of the range examined by Miller to determine whether the concept of varieties should be maintained for this plant species.

Selective forces and floral traits

Floral traits varied among neighbouring populations, which suggests that gene flow does not homogenize the populations and that selection may be responsible for the observed differences in floral traits. Population sizes are large enough in most areas to limit the impact of genetic drift on population divergence (Brunet and Sweet, 2006). Pollinators are the biotic factor most likely to influence floral morphology in this plant species. Other biotic factors are either uncommon (nectar robbing has only been observed in central Colorado by B. occidentalis) or unaffected by floral form (deer herbivory). Of the abiotic factors likely to influence floral traits, populations of A. coerulea occur at different altitudes and latitudes so temperature and water availability vary. Populations of A. coerulea at higher altitudes are exposed to lower temperatures and higher water availability. In addition, populations in Colorado have more water available relative to populations in Utah and Arizona (Brunet and Sweet, 2006).

The present data suggest that the annual presence of hawkmoths in a population may select for whiter flowers, although I did not observe correlations between the average proportion of hawkmoths (or pollen collectors) in a population and any of our floral traits. In fact, Arizona populations where hawkmoths were the major pollinators had a significant proportion of blue flowers and more of a bimodal distribution of flower colours. Because bumble-bees were rarely seen visiting A. coerulea flowers in that area, preference of bumble-bees for blue flowers does not explain their high frequencies in the populations. Hawkmoths do visit A. coerulea flowers during the day in northern Arizona in some years (although they are much more abundant at dusk), a trend that was not observed in the other populations studied here but which has been reported in central Colorado (Miller, 1981; Campbell et al., 2002). Although white flowers are more visible to crepuscular visitors, their colour does not signal pollinators during the day. Hawkmoths may thus show a preference for white flowers at dusk but disregard flower colour during the day, which may explain the greater proportion of blue flowers in Arizona relative to Utah populations. The Arizona populations are also at lower latitude, with higher temperatures and lower water availability, which could also help to explain the maintenance of bluer flowers as anthocyanins have often been associated with tolerance to abiotic stresses (reviewed in Strauss and Whittall, 2006). Conversely, bluer flowers were found to be associated with higher altitude overall. For example, the Cedar Breaks population in Utah was at higher altitude and had bluer flowers relative to the Archery and Hiking Trail populations despite that fact that both hawkmoths and bumble-bees were common in all three populations and hawkmoths were not observed during the day in these populations. More research is needed to explain the prevalence of blue flowers at higher altitude in this plant species but both pollinators and environmental factors appear likely to influence A. coerulea flower colour.

The present data suggest that the presence of S. vashti may select for longer spurs as Miller (1981) had previously suggested. In other plant species hawkmoths tend to affect the spur or tube length of flowers (Nilsson, 1988; Johnson and Steiner, 1997; Maad, 2000; Alexandersson and Johnson, 2002) although here I report an association between one specific hawkmoth species and spur length. Clearly studies are needed to examine the impact of tongue length on reproduction via both male and female functions in this plant species. Spur length is a floral characteristic that is likely to have played an important role in the diversification of the genus Aquilegia (Hodges and Arnold, 1995), and I have shown that in A. coerulea it appears to be associated with a specific hawkmoth species.

I did not observe a significant effect of annual hawkmoth presence, of a specific hawkmoth species or of an abiotic factor on flower size. Given the fairly strong correlation between spur length and sepal length, selection on flower size may be indirect and result from the direct selection on spur length. In addition, the statistically significant correlations between spur length, flower colour and sepal length suggest some integration of floral traits such that A. coerulea flowers with longer spurs tend to be larger and whiter. Hodges et al. (2002) reported similar patterns of correlation and suggested integration between spur length and flower colour in F2 individuals derived from a cross between A. formosa and A. pubescens. Furthermore, in a review of genetic correlations between floral traits, Ashman and Majetic (2006) suggested that integration of floral traits may be a common feature of angiosperm reproduction. However, despite such floral trait integration, the present study indicated that distinct selective forces may influence each floral trait. The annual presence of hawkmoths and environmental factors linked to altitude affected flower colour while spur length was most affected by the presence of Sphinx vashti. In addition, there seemed to be a mosaic of selection regimes in the different populations (Thompson, 1999). However, I did not observe a majority of white flowers associated with hawkmoth abundance in south-west Utah and northern Arizona as I had originally anticipated based on previous studies (Miller, 1981). Future studies should examine the impact of the two hawkmoth species on male and female functions in A. coerulea as well as the impact of pollinators vs. abiotic factors on flower colour. Moreover, with the imminent release of the genome sequence of A. coerulea ‘Origami’, genetic studies identifying the genes that affect spur length and increasing our knowledge of genes that influence flower colour would help increase our understanding of floral evolution, especially when examined in the light of the selective forces highlighted here.

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.aob.oxfordjournals.org/. Table S1 describes the different species of...
bumb-bees and hawkmoths that visited *Aquilegia coerulea* flowers in the different populations each year together with their abundance (number of visits per flower per hour). Figure S1 summarizes the relative contribution of the distinct pollinator groups and the two functional pollinator groups in *Aquilegia coerulea* populations each year.

**ACKNOWLEDGEMENTS**

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**LITERATURE CITED**


