

The role of pollinators in maintaining variation in flower colour in the Rocky Mountain columbine, *Aquilegia coerulea*

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- **Background and Aims** Flower colour varies within and among populations of the Rocky Mountain columbine, *Aquilegia coerulea*, in conjunction with the abundance of its two major pollinators, hawkmoths and bumble-bees. This study seeks to understand whether the choice of flower colour by these major pollinators can help explain the variation in flower colour observed in *A. coerulea* populations.
- **Methods** Dual choice assays and experimental arrays of blue and white flowers were used to determine the preference of hawkmoths and bumble-bees for flower colour. A test was made to determine whether a differential preference for flower colour, with bumble-bees preferring blue and hawkmoths white flowers, could explain the variation in flower colour. Whether a single pollinator could maintain a flower colour polymorphism was examined by testing to see if preference for a flower colour varied between day and dusk for hawkmoths and whether bumble-bees preferred novel or rare flower colour morphs.
- **Key Results** Hawkmoths preferred blue flowers under both day and dusk light conditions. Naïve bumble-bees preferred blue flowers but quickly learned to forage randomly on the two colour morphs when similar rewards were presented in the flowers. Bees quickly learned to associate a flower colour with a pollen reward. Prior experience affected the choice of flower colour by bees, but they did not preferentially visit novel flower colours or rare or common colour morphs.
- **Conclusions** Differences in flower colour preference between the two major pollinators could not explain the variation in flower colour observed in *A. coerulea*. The preference of hawkmoths for flower colour did not change between day and dusk, and bumble-bees did not prefer a novel or a rare flower colour morph. The data therefore suggest that factors other than pollinators may be more likely to affect the flower colour variation observed in *A. coerulea*.

Key words: *Aquilegia coerulea*, Ranunculaceae, Rocky Mountain columbine, flower colour variation, pollinators, bumble-bees, *Bombus impatiens*, hawkmoths, *Hyles lineata*, learning behaviour, novelty, frequency dependence.

INTRODUCTION

Abiotic and biotic factors can both influence flower colour (Strauss and Whittall, 2006; Streisfeld and Kohn, 2007; Kulbaba and Worley, 2013). Anthocyanins are associated with tolerance to abiotic stresses and, in many plant species, these pigments influence flower colour (Strauss and Whittall, 2006; Whittall *et al.*, 2006). Water availability has been shown to maintain a flower colour polymorphism in populations of *Linanthus parryae*, where the blue morph set more seeds under drought conditions and the white morph under wet conditions, and where the pollinator does not prefer one of the two colour morphs (Schemske and Bierzychudek, 2001). Biotic factors, especially pollinators, affect flower colour in various plant species (Bradshaw and Schemske, 2003; Irwin and Strauss, 2005; Hoballah *et al.*, 2007; Hopkins and Rausher, 2012; Kulbaba and Worley, 2012, 2013). In *Mimulus* and *Petunia* species, a direct link has been established between the genetic basis of flower colour variation and the differential visitation rate by pollinators, and a change in a single gene has been shown to cause a shift in pollinator (Bradshaw and Schemske, 2003; Hoballah *et al.*, 2007).

The impact of insect pollinators on flower colour can be affected by their visual capacity. Bumble-bees prefer flowers within the blue spectrum of visible light (400–500 nm) as supported by direct observations of pollinator behaviour (Briscoe and Chittka, 2001; Raine *et al.*, 2006) and by examination of the spectral sensitivity of their photoreceptors (Skorupski and Chittka, 2010). The colour preference of non-Hymenoptera pollinators has received less attention, possibly because of the long-held belief that these nocturnal/crepuscular pollinators mostly depend on olfactory cues to find flowers (Brantjes, 1978). The importance of visual cues for these pollinators has, however, recently been identified (Raguso and Willis, 2002; Goyret *et al.*, 2007), and hawkmoths have been shown to have very keen vision (Kelber *et al.*, 2002). A close association between the most common flower colour morphs in Australia and the visual spectrum of the major Hymenopteran pollinators suggests that these pollinators may act as selective agents for flower colour in plant populations and influence the evolution of flower colour in angiosperms (Dyer *et al.*, 2012).

Different mechanisms, including innate preference, learning, novelty and frequency dependence, can influence the foraging decisions of pollinators. Bumble-bees have an innate preference

for blue flowers (Simonds and Plowright, 2004; Raine and Chittka, 2007a) and, when exposed to novel colours, they prefer to forage on a colour that is close to their innate preference (Gumbert, 2000). Bumble-bees do, however, learn to associate a reward with a flower colour, even colours for which they do not have an innate preference (Raine and Chittka, 2007b; Ings *et al.*, 2009). Bumble-bees are also known to forage on novel phenotypes, even if such phenotypes carry the risk of not being associated with a reward (Jersáková *et al.*, 2006). Such behaviour may help the bees track resource levels within populations (Heinrich, 1979; Keaser *et al.*, 2013). Finally, bumble-bees have been shown to forage in a frequency-dependent manner, preferring the common morph when a flower is rewarding (Smithson and MacNair, 1996; Smithson, 2001; Forrest and Thomson, 2009) and the rare morph when flowers are not rewarding (Real, 1990; Smithson and Macnair, 1997; Gigord *et al.*, 2001), although exceptions exist (Dormont *et al.*, 2010).

The proportion of blue and white flowers varies among populations of *Aquilegia coerulea*, and polymorphisms for flower colour exist within populations (Miller, 1981; Brunet, 2009). Blue flowers can vary in intensity, and populations in central Colorado have the highest frequency of darker flowers and populations in Utah the lowest (see Brunet, 2009, fig. 5). The abundance of the two major pollinators, hawkmoths and bumble-bees, also varies among populations. Hawkmoths are more abundant in northern Arizona where bumble-bees are rare, while the reverse is true in central Colorado where hawkmoths are absent in most years (Brunet, 2009). Within a population, one species of bumble-bee and/or one species of hawkmoth tend to dominate in a given year (Brunet, 2009). The species *Hyles lineata* is the most common hawkmoth visitor to *A. coerulea* flowers, but *Sphinx vashti* is present in some years in some populations (Miller, 1981; Brunet, 2009). The species *Bombus occidentalis* was the most common bumble-bee visitor to *A. coerulea* flowers prior to 2001, but has been replaced by *B. flavifrons* across most of the *A. coerulea* range (Brunet, 2009). Hawkmoths are mostly crepuscular and forage for nectar in *A. coerulea* flowers, while bumble-bees are diurnal, forage for pollen and cannot reach the nectar produced at the tip of the spurs (Miller, 1978).

The floral diversification of the genus *Aquilegia* in the New World appears to have been pollinator driven (Whittall and Hodges, 2007), whereas habitat specialization and abiotic factors may have played a more important role in Europe (Bastida *et al.*, 2010). In the North American species, *A. coerulea*, associations have been reported between hawkmoths and populations with white and lightly coloured flowers and between bumble-bees and populations with blue flowers (Miller, 1981). Brunet (2009) detected significant correlations between the annual presence of hawkmoths in a population and the abundance of lightly coloured flowers. Higher seed set of blue flowers in Central Colorado has been associated with years of high bumble-bee abundance, and higher seed set of white flowers with years of high hawkmoth abundance (Miller, 1981). The evidence accumulated to date, although indirect, suggests that pollinators may influence and select for flower colour in this plant species.

In the current study, we examine various hypotheses to determine whether and how the choice of flower colour by hawkmoths and bumble-bees could explain the variation in flower

colour observed within and among *A. coerulea* populations. We first determine whether a differential preference for flower colour by the two major pollinators, with hawkmoths preferring white and bumble-bees blue flowers, could explain the flower colour variation observed within *A. coerulea* populations. Such a differential preference may also explain the variation in flower colour observed among *A. coerulea* populations with different abundances of the two major pollinators. Secondly, we examine whether a flower colour polymorphism could be maintained by a single pollinator. A temporal variation in preference for flower colour by hawkmoths, with more visits to blue flowers during the day and to white flowers at dusk, could maintain variation in flower colour in populations where hawkmoths are present. Bumble-bees do not forage at dusk, but we examine how learning influences choice of flower colour and determine whether a preference for a novel or rare flower could help maintain a polymorphism for flower colour. Our goal is to elucidate the role played by the two major pollinators in the maintenance of flower colour variation in *A. coerulea* populations.

MATERIALS AND METHODS

Plant species

The Rocky Mountain columbine, *Aquilegia coerulea* (James) (Ranunculaceae), grows typically between 2100 and 3700 m in mountains in New Mexico, Arizona, Utah, Colorado, Idaho and Wyoming (Whittemore, 1997). It is found on mountain slopes, in mountain meadows, rocky areas or forest understoreys. Co-occurring flowering plants can include mountain bluebell, *Mertensia ciliata*, cow parsnip or *Heracleum maximum*, and species of *Castilleja* (Indian paintbrush), *Aconitum*, *Delphinium*, *Geranium*, *Ipomopsis*, *Lupinus*, *Penstemon*, *Pedicularis*, *Ranunculus* and *Thalictrum*.

Pollinators

The white-lined sphinx moth, *Hyles lineata* (Lepidoptera: Sphingidae), occurs in every state in the USA except Alaska (Tuttle, 2007). A colony of *H. lineata* was started from eggs collected from the wild and was maintained in a greenhouse at the University of Wisconsin in Madison, WI. Adults were kept in a 1.2 × 1.2 × 0.91 m flight cage and fed a 20 % sugar solution placed on artificial *A. coerulea* flowers. The adults laid eggs and the larvae were raised on *Oenothera* plants.

Colonies of *Bombus impatiens* were obtained from Koppert Biological Systems, and a colony was kept in a meshed 1.83 × 1.83 × 1.83 m enclosure containing flowering *A. coerulea* plants. Additional pollen was made available to the bees on artificial plastic *A. coerulea* flowers. The nectar source provided with the hive remained available to the bees throughout the experimental period.

Experimental flowers

Both natural and artificial flowers were used in the assays. Natural flowers came from *A. coerulea* plants grown in the greenhouse. Natural *A. coerulea* flowers do not emit UV light

(Miller, 1981; Supplementary Data Fig. S1). Artificial flowers had sepals and petals made of paper placed around a paint brush with bristles trimmed to the average height of natural anthers (Fig. S2). Because petals of *A. coerulea* flowers are typically white and sepals can vary in colour, the petals were painted with Decoart Americana-Snow (Titanium) white (DA01) and sepals were painted either white with Decoart Americana DA01 or blue using Decoart Americana Wistera (DA211). The spectrometer readings of these paints matched the readings of natural white and blue *A. coerulea* flowers (Ocean Optics USB4000, Orlando, FL, 350–1000 nm). Each natural flower was put in a floral tube filled with water and placed in a larger centrifuge tube. The paint brushes of artificial flowers were placed directly into the centrifuge tubes. A 5 mg aliquot of pollen was added onto the paint brush bristles of artificial flowers.

Flower colour preference of hawkmoths

We examined the preference of hawkmoths for flower colour under both day and dusk light conditions. We selected light conditions that corresponded to natural lighting conditions for day and dusk when hawkmoths tended to be active in the wild. The flower colour preference of hawkmoths during daylight conditions ($13 \mu\text{mol m}^{-2} \text{s}^{-1}$) (LI-1000 Datalogger, Li-Cor, Lincoln, NE, USA) was tested using dual choice assays. Pairs of natural *A. coerulea* flowers, one blue and the other white, were placed 15 cm from each other in the flight cage. Natural flowers were selected from the same plant and at the same sexual stage given the strongly protandrous flowers (Brunet, 1996). Observation of hawkmoth behaviour began when the flowers were placed in the flight cage. Typically 10–15 adult moths were present in the cage during the trials. Preliminary assays indicated that this number of moths maximized the chance of getting visits to the flowers. The first flower visited by a hawkmoth was recorded as a choice and, following each visit, the hawkmoth was captured and removed from the cage and a new pair of flowers was set up. If no choice had been made within 20 min, the flower pair was also changed. Hawkmoths that were still alive the next day became part of that day's trial.

To examine hawkmoth preference at dusk, we used experimental arrays of flowers and loss of nectar in tubes as a measure of hawkmoth visits. This method reduced the number of observation hours needed to complete the experiment. In these trials, visitation rates were measured on arrays of 18 artificial flowers under dusk light conditions ($0.0045 \mu\text{mol m}^{-2} \text{s}^{-1}$) (LI-1000 Datalogger, Li-Cor). The other environmental factors, besides lighting conditions, were kept constant. The flowers were placed 15 cm apart in three rows of six flowers where flower colour alternated within and between rows. A 1 mL aliquot of 20 % nectar solution was provided in a 1.2 mL nectar tube located in the centre of each flower, and moths were allowed to forage on the flowers for 6 h. At least three additional flowers, with nectar tubes covered with a wire mesh, were placed along the edge of the cage and served as control for ambient evaporation in each trial. All tubes were weighed prior to and at the end of each trial, and the loss of weight (mg) was calculated for each tube. The total weight loss for the white and blue flowers was calculated each day, and flower arrays were set up on six separate days. Only the flowers with a weight loss greater than

the average weight loss in the control flowers for the respective trial were used to calculate total weight loss for blue or white flowers because these flowers were more likely to represent visited flowers. Similar results were obtained if only flowers with a weight loss greater than the largest weight loss in the control flowers were used in the calculations.

Flower colour preference of bumble-bees

To understand how learning could influence the choice of flower colour by bumble-bees, we first examined the colour preference of naïve and experienced bees when pollen reward was similar between colour morphs. We then confirmed the ability of bumble-bees to associate a flower colour with a pollen reward. Lastly, we tested the impact of prior experience on choice of flower colour. To help understand the prior experience results and to determine whether bumble-bees alone could maintain both flower colours, we examined the role of novelty and of flower colour morph frequency on choice of flower colour. All trials used experimental arrays of flowers, except for the test of the impact of prior experience which used dual choice assays. Flowers were placed 15 cm apart and 24 cm from the hive. In experimental flower arrays, a foraging bout began when a bumble-bee started foraging on the flower array and ended once it left the array. The colour of each flower visited in succession within a foraging bout was recorded. Data were recorded for a minimum of 30 foraging bouts, typically over a 3 d period.

Pollen reward for naïve and experienced bees. Experimental arrays of 60 flowers (an 8×8 array with the four corner flowers removed) in a 50 blue:50 white (50B:50W) ratio were set up with, flower colour alternating within and between rows. For experiments with similar pollen rewards, the same amount of pollen was added to each flower. To associate a pollen reward with a flower colour, pollen was only added to one of the two flower colours. We first added pollen only to white flowers and in a second set of trials to blue flowers. Naïve bees had not previously visited the flower arrays. Bees were marked after their first foraging bout and then became experienced bees. We examined and contrasted the number of visits to blue and white flowers by naïve and experienced bees foraging on flowers with similar pollen reward and for naïve bees on flowers with different pollen rewards.

Prior experience. Bees were trained on either (1) an equal mixture (50B:50W) of blue and white flowers; (2) only white flowers; or (3) only blue flowers. The preference of bumble-bees for flower colour was tested with dual choice assays using either natural or artificial flowers (Table 1, prior experience). A choice between a white and a blue flower was recorded as the first flower visited by a bee. If no choice was made within 15 min, the bumble-bees flying in the cage were captured and removed from the enclosure and the flower pair was replaced with a fresh pair of flowers.

Novelty. To test whether bumble-bees preferentially visited a novel flower colour, bees were trained on only blue flowers and tested on experimental arrays containing 80B:20W, 50B:50W or 20B:80W flowers (Table 1, novelty). We used 30 flowers in

TABLE 1. Summary of the experiments examining the impact of prior experience, novelty and frequency dependence on the choice of flower colour in bumble-bees

Experiment	Trained on flowers	Tested on flowers
Prior experience	50B:50W	Natural painted
		Natural not painted
		Artificial
Novelty	Blue only	Natural painted
		Artificial
Frequency dependence	80B:20W 50B:50W 20B:80W	Natural painted
		Artificial
		80B:20W 50B:50W 20B:80W

For prior experience, bumble-bees were trained on all white, all blue or 50B:50W flower arrays and tested on pairs of one white and one blue flower using either natural or artificial flowers (dual choice assay).

For novelty, bees were trained on all blue flowers and tested on experimental arrays of 80B:20W (blue:white), 50B:50W or 20B:80W flowers.

For frequency dependence, bees were trained and tested on similar experimental arrays of either 80B:20W, 50B:50W or 20B:80W flowers.

the 50B:50W array but 60 flowers for the other two arrays to obtain enough low frequency flowers. If bees prefer novel flowers, we expect bees trained on only blue flowers to visit white flowers preferentially irrespective of their frequency in the array.

Frequency-dependent visitation. To test whether frequency dependence affected the choice of flower colour by bumble-bees, we trained and tested bees on similar frequencies in order to eliminate any potential bias due to prior training. We used arrays of 60 flowers in frequencies of 80B:20W, 50B:50W or 20B:80W flowers (Table 1, frequency dependence). If bumble-bees exhibit negative frequency-dependent visitation, we expect preference for the rare colour morph, i.e. the white morph in the 80B:20W flower array and the blue morph in the 20B:80W flower array. We do not expect to see a preference for a flower colour in the 50B:50W flower array. If bumble-bees exhibit positive frequency-dependent visitation, we expect a preference for the common colour morph, i.e. a blue morph in the 80B:20W flower array and a white morph in the 20B:80W flower array.

Statistical analysis

Dual choice assays were used to examine the choice of flower colour by hawkmoths during daylight conditions and the impact of prior experience on choice of flower colour by bumble-bees. Binomial tests with a null hypothesis of equal visitation to blue and white flowers were used to determine flower colour preference in these dual choice assays. The colour preference of hawkmoths during dusk light conditions was determined by comparing the weight loss of nectar from the beginning to the end of a trial between white and blue flowers using a mixed linear model (Proc Mixed, SAS 9.3; SAS Institute Inc., 2011). Nectar weight loss (mg) was log

transformed prior to analyses. All other tests used experimental arrays of flowers. A generalized linear mixed model (Proc Glimmix, SAS 9.3) with a binomial distribution and a logit regression function determined whether bumble-bees preferred a flower colour in these experimental arrays. Day and foraging bout (day) were used as random variables in the generalized mixed model. Significance was tested using an approximate normal (or Z) test in order to determine whether the number of visits to blue and white flowers differed significantly from the 80B:20W and 20B:80W frequencies used in some of the experimental arrays.

RESULTS

Flower colour preference of hawkmoths

In the choice assays performed under daylight conditions, hawkmoths selected blue more frequently than white flowers (22 visits to blue and ten visits to white flowers; Table 2). In the experimental arrays of flowers performed under dusk light conditions, blue flowers lost significantly more nectar relative to white flowers (total volume lost 6.15 ± 0.06 g for blue and 2.07 ± 0.04 g for white flowers, and average volume lost per flower 0.21 g for blue and 0.08 g for white flowers; Table 2). While the distribution of the amount of nectar removed per flower was similar for the blue and white flowers, the blue flowers had more instances of nectar removal. Hawkmoths preferentially visited blue flowers under both day and dusk light conditions (Table 2).

Flower colour preference of bumble-bees

Similar pollen reward. Naïve bumble-bees visited blue flowers more frequently than white flowers in the arrays (Table 2). Over 28 foraging bouts, naïve bees visited 114 blue and 71 white flowers. Experienced bees did not, however, exhibit a preference for a flower colour (Table 2). Over 35 foraging bouts, experienced bees visited 89 blue and 68 white flowers. Day and foraging bout did not influence the choice of flower colour for either naïve or experienced bees. In the model for naïve bumble-bees, parameter estimates of zero were obtained for both day and foraging bout (day) while in the model for experienced bees a parameter estimate of zero was obtained for day and 0.021 ± 0.093 for foraging bout (day). Although naïve bees preferred blue flowers, bees quickly learned to visit flower colour equally frequently in the 50B:50W flower array, when the two flower colours provided similar rewards.

Associating flower colour with pollen reward. Naïve bumble-bees visited white flowers significantly more often than blue flowers ($Z = 6.80$, $n = 202$, $P < 0.001$) when white flowers were associated with the pollen reward in these 50B:50W flower arrays (Table 2). Bumble-bees visited 25 blue and 177 white flowers over 39 foraging bouts. Neither of the parameters day or foraging bout (day) influenced the choice of flower colour in the model; a parameter estimate of zero was obtained for day and 1.00 ± 0.60 for foraging bout (day). When blue flowers were the only rewarding flowers, all visits were to blue flowers; no bees visited a white flower. In 35 foraging bouts, 122 visits were made to blue flowers and zero to white flowers

TABLE 2. Choice of flower colour by hawkmoths during day and dusk light conditions and by naïve and experienced bumble-bees on flowers with similar or different pollen rewards

Pollinator	Experiment	<i>n</i>	Preference	Test/Procedure	<i>P</i> -value
Hawkmoth	Day	32	Blue	Binomial	0.025
	Dusk	6	Blue	Proc Mixed	0.030
Naïve bees	Similar reward	185	Blue	$Z = -3.13$, Proc Glimmix	0.002
	Different reward	>120	Rewarding flower	Z-value (see text), Proc Glimmix	<0.0001
Experienced bees	Similar reward	157	None	$Z = -1.39$, Proc Glimmix	0.166

The Proc Mixed and Proc Glimmix are SAS procedures for Linear mixed models.

The variable *n* for the binomial test represents the number of trials, for the Proc Mixed the number of days, and for experimental arrays the number of flowers.

($Z = -4.78$, $n = 122$, $P < 0.001$) (Table 2). In order to run the test, we added a foraging bout with a single visit to a white flower. Because naïve bumble-bees quickly associated a flower colour with pollen reward, we did not examine the behaviour of experienced bees.

Prior experience. Bumble-bees preferred blue flowers when they were previously exposed to 50B:50W flowers, and this preference remained whether non-painted or painted natural flowers or artificial flowers were used in the choice assays (Table 3). When bumble-bees had previously foraged on all blue or all white flowers, however, they visited blue and white flowers equally frequently and this was true whether natural or artificial flowers were used in the choice assays (Table 3). Prior experience influenced bumble-bee choice of flower colour when pollen reward was similar between flower colours.

Novelty. Bumble-bees visited but did not prefer novel flowers. In the 80B:20W flower array, we observed 16 visits to white and 92 visits to blue flowers over 32 foraging bouts (Table 4A). In the 50B:50W flower array, bumble-bees visited 119 blue and 108 white flowers over 39 foraging bouts (Table 4A). Finally, in the 20B:80W flower array, bumble-bees made 90 visits to white and 26 visits to blue flowers over 28 foraging bouts (Table 4A). Day and foraging bout (day) did not affect the number of visits to white flowers in any of the trials; the parameter estimate for day was 0.66 ± 1.3 and for foraging bout (day) 1.29 ± 0.95 for the 80B:20W flower array model, 0 for day and 0.04 ± 0.09 for foraging bout (day) for the 50B:50W flower array model and 0.06 ± 0.21 for day and 0.04 ± 0.32 for foraging bout (day) for the 20B:80W flower array model. In all cases, novel flowers were visited in the frequency in which they were present in the array.

Frequency-dependent visitation. Bumble-bees did not prefer rare or common flowers but visited a flower colour as expected based on its frequency in the array (Table 4B). When trained and tested on the 80B:20W flower array, bees visited 48 white and 165 blue flowers over 41 foraging bouts. When trained and tested on 50B:50W flower arrays, bees visited 107 blue and 103 white flowers in 28 foraging bouts. Finally, when trained and tested on 20B:80W flower arrays, 85 visits were made to white and 24 to blue flowers over 27 foraging bouts. Day and foraging bout (day) did not affect the number of visits to a flower colour in any of the arrays. The parameter estimate for day was 0 and for foraging bout (day) 0.31 ± 0.33 in the 80B:20W flower array model; 0 for day and 0.04 ± 0.10 for foraging bout in the 50B:50W flower array model; and, finally,

TABLE 3. The influence of prior experience on bumble-bee flower colour choice

Prior experience	Flower type used	<i>n</i>	No. of blue flowers visited	<i>P</i> -value
50B:50W	Natural painted	30	22*	0.008
	Natural not painted	30	20*	0.049
	Artificial	40	26*	0.040
Blue only	Natural painted	30	18	0.181
	Artificial	40	22	0.318
White only	Natural painted	20	12	0.252
	Artificial	20	11	0.412

Bumble-bees were trained on either 50B:50W, all blue or all white flower arrays and tested using dual choice assays. Natural or artificial flowers were used in the dual choice tests. *n* indicated the number of trials.

The *P*-values are from binomial tests.

*Preference for blue flowers.

TABLE 4. Novelty, frequency dependence and flower colour choice by bumble-bees

Trained on flowers	Tested on flowers	<i>n</i>	Z- or <i>t</i> -test estimate	<i>P</i> -value
(A) Novelty				
Blue only	80B:20W	108	-0.39	0.70
Blue only	50B:50W	227	0.60	0.55
Blue only	20B:80W	116	-0.60	0.55
(B) Frequency dependence				
80B:20W	80B:20W	213	-0.11	0.91
50B:50W	50B:50W	210	-0.20	0.84
20B:80W	20B:80W	109	-0.87	0.39

The *P*-value is based on a Z-test where the estimates for the population mean and its standard error were obtained using a generalized linear mixed model with a binomial distribution and a logit regression function using Proc Glimmix in SAS 9.3.

0 for day and 0.59 ± 0.71 for foraging bout in the 20B:80W flower array model.

DISCUSSION

Despite the fact that light-coloured flowers are typically associated with hawkmoth and blue flowers with bee pollination, even in the genus *Aquilegia*, we did not observe a differential preference for flower colour by the two major pollinators, hawkmoths and bumble-bees, when both flower colours presented similar rewards (Miller, 1981; Chittka *et al.*, 2001;

Hodges *et al.*, 2004; Raine and Chittka, 2007a; Brunet, 2009). Hawkmoths preferred blue flowers under both day and dusk light conditions. Bumble-bees had an innate preference for blue flowers but quickly learned to visit flowers of both colours with equal frequency in the 50B:50W flower arrays. Bumble-bees visited flower colours in the frequency in which they were presented, a behaviour which will not change the frequency of a flower colour in a population.

A differential preference for a flower colour by the two pollinators would require associations between flower colour and reward. Hawkmoths, at least *Manduca sexta*, have been shown to learn to associate a flower colour with a nectar reward, and such an association may help explain their frequent visits to many species with crepuscular white flowers despite their innate preference for blue flowers (Goyret *et al.*, 2008; Balkenius and Balkenius, 2010).

In the experiments, even naïve bumble-bees quickly associated a flower colour with a pollen reward. Previous studies have indicated that bumble-bees can quickly discriminate and learn to forage on nectar-rewarding flowers using colour as their only cue (Raine and Chittka, 2012; Strand and Sherry, 2013). Unlike nectar, which is hidden from view at the bottom of the spurs, pollen is visible to the bees and they can discriminate between flowers with and without dehiscent anthers (Brunet and Sweet, 2006). Bees may therefore use pollen reward, rather than flower colour, as a direct cue when associating pollen reward with a flower colour in these experiments.

Associations between blue flowers and larger pollen reward and white flowers and larger nectar rewards could engender preference for blue flowers by bumble-bees and for white flowers by hawkmoths. We expect blue flowers to have not only more pollen available for bees but also less nectar available for hawkmoths, while white flowers should show the opposite pattern. These associations would create a negative correlation between pollen and nectar production within flowers. While associations between a flower colour and a reward, within a plant species, can easily be obtained experimentally, in natural populations such associations would require strong linkage between the genes responsible for flower colour and the genes for pollen and/or nectar production. While variation in pollen and nectar reward exist among *A. coerulea* individuals, statistically significant correlations between measures of flower colour obtained using a spectrometer and nectar volume or concentration have not been observed in plants grown from seeds in the greenhouse (Van Etten and Brunet, unpubl. res.). Associations between reward and flower colour may be easier to maintain among species rather than within a species. A preference by *H. lineata* for lighter flowers (Fulton and Hodges, 1999; Whittall *et al.*, 2006) was in fact detected in *Aquilegia* by comparing two species, *A. pubescens* with lighter yellow flowers and *A. formosa* with darker red flowers. A preference for lighter flowers was not detected in this study where *H. lineata* was exposed to white and blue colour morphs of *A. coerulea*. The evidence available to date suggests that associations between flower colour and reward are unlikely to be maintained in *A. coerulea* populations. In the absence of such correlations, a differential preference for flower colour by hawkmoths and bumble-bees is not expected to maintain the variation in flower colour observed in *A. coerulea* populations.

While our experiments considered only flower colour and reward, floral fragrances can also affect the feeding behaviour of hawkmoths (Raguso and Willis, 2002; Balkenius *et al.* 2006; Goyret *et al.*, 2007). Some nocturnal hawkmoths such as *M. sexta* seem to require the presence of both odour and colour before initiating feeding behaviour (Raguso and Willis, 2002), although the visual display may be the ultimate indicator of a nectar source (Goyret *et al.*, 2007). We observed nectar feeding with *H. lineata* in the absence of odour, although the presence of floral fragrance could increase feeding frequency, as has been observed in other hawkmoth species (Raguso and Willis, 2002). For fragrance to affect choice of flower colour in our system, we would expect a relationship between fragrance and flower colour, with white flowers being more fragrant than blue flowers or at least emitting more of the chemicals that are attractive to hawkmoths in the evening. Associations between flower colour and floral scent have been found in some plant species but not in others (summarized in Vereecken and Schiestl, 2009). In addition, white flowers should also be associated with larger nectar production as otherwise hawkmoths will be attracted to the white flowers but would learn to visit white and blue flowers equally frequently if nectar production was similar in the two floral morphs (Raguso and Willis, 2002; Goyret *et al.*, 2008; Balkenius and Balkenius, 2010). In fact, odour did not affect the association learning between flower colour and reward in the hawkmoth *M. sexta* (Balkenius and Balkenius, 2010). The fact that flower colour and reward do not appear to be associated in *A. coerulea* suggests that even if odour were associated with flower colour in this plant species it would not be expected to affect choice of flower colour by hawkmoths.

While our experiments examined *A. coerulea* in isolation, this species grows in a community and we did not examine whether the choice of flower colour by pollinators may be affected by the other species concurrently blooming in the area. The plant community in these higher altitude environments, briefly described in the Materials and Methods, does not contain many white-flowered hawkmoth-pollinated plants and therefore the presence of other plant species is unlikely to bias the preference of hawkmoths towards white flowers. The presence of species with blue flowers may influence bumble-bees, but pollen is visible to the bees and they may cue on pollen reward directly rather than flower colour. Moreover, bumble-bees quickly learn to visit flower colour at equal frequency when pollen reward is similar among colour morphs. Therefore, floral scent and the presence of other plants in the community are unlikely to create a differential preference for flower colour by hawkmoths and bumble-bees and help explain the variation in flower colour observed in *A. coerulea* populations.

In the absence of a differential preference by the two major pollinators, a single pollinator may be able to maintain a flower colour polymorphism within a population. For example, temporal variation in hawkmoth preference for flower colour, with a preference for blue flowers during the day and white flowers at dusk, could help maintain both flower colour morphs in populations visited by hawkmoths. Hawkmoths have very keen night vision and they can use colour vision rather than achromatic cues at night (Kelber *et al.*, 2002). The white-sphinx hawkmoth, *H. lineata*, can distinguish colour during both low (dusk) and high (day) light intensities, and it has been observed visiting

blue flowers while foraging during the day (Kelber *et al.*, 2002, 2003). Another sphingid hawkmoth, *M. sexta*, has been shown to prefer blue flowers, although it is commonly seen foraging on white flowers in the field (Goyret *et al.*, 2008). While hawkmoths may use a combination of visual and olfactory cues when selecting flowers (Raguso *et al.*, 1996; Raguso and Willis, 2002; Goyret *et al.*, 2007), both natural and artificial flowers were used in this study and the white-sphinx hawkmoths still preferred blue artificial flowers which did not emit floral scent. Flower colour alone can influence the foraging behaviour of *H. lineata*, and the evidence presented here indicates a preference for blue flowers by these hawkmoths both during the day and at dusk. Even if only white flowers emitted scent at dusk, odour does not appear to influence the association learning between flower colour and reward (Balkenius and Balkenius, 2010), and a preference for white flowers would still require greater nectar reward in these flowers. A change in flower colour preference between day and dusk by hawkmoths cannot explain the maintenance of a flower colour polymorphism in populations visited by hawkmoths.

A preference for a novel flower colour morph may help maintain a flower colour polymorphism by bumble-bees. Although bees preferred blue flowers when trained on 50B:50W flower arrays, they did not exhibit a preference for a flower colour when trained on only white or only blue flower arrays (prior experience). This lack of preference when trained on all white or all blue flowers suggested that bees may preferentially visit a novel colour morph. Because flowers are ephemeral and nectar and pollen rewards fluctuate, visiting novel flowers is likely to represent an advantageous strategy for bees (Goulson, 2003; Keasar *et al.*, 2013). Bees often forage on major and minor types when visiting different species, and such a strategy may allow them to forage on high rewarding flowers while exploiting other options (Heinrich, 1979). However, when pollen reward did not vary between colour morphs, we observed no preference for a novel colour morph in the experiments. Bees visited flowers with a novel colour but the number of visits to the novel flower colour was proportional to its frequency in the array. Moreover, a novel colour morph within a species would imply that some colour morphs are not present in some populations and the pollinators encounter the novel morph when moving into a population. Given that both light and dark colour morphs occur in *A. coerulea* populations, novelty is unlikely to play a strong role in maintaining flower colour polymorphism within populations.

Negative frequency-dependent visitation could lead to frequency-dependent selection, a mechanism known to maintain polymorphisms in populations (Chang and Rausher, 1998; Brunet and Mundt, 2000; Koskella and Lively, 2009; Joly and Schoen, 2011), including polymorphisms for flower colour (Smithson, 2001). Previous experiments investigating bumble-bee visits to a rare morph have indicated a preference for the rare morph when flowers are unrewarding (Smithson and MacNair, 1997; Gigord *et al.*, 2001) although this was not always the case (Dormont *et al.*, 2010). In contrast, bumble-bees can forage in a positive frequency-dependent manner when flowers present a reward (Smithson and MacNair, 1996, 1997; Forrest and Thomson, 2009). Positive frequency-dependent selection tends to create unstable equilibrium where one of the colour morphs gets fixed in the population (Charlesworth and

Charlesworth, 2010). The number of visits to a flower colour increased with the frequency of that colour in the array, which suggests that bumble-bees visited flower colour in a frequency-dependent manner. However, we detected no negative or positive frequency-dependent visitation. The bees did not prefer the rare flower colour which would indicate negative frequency-dependent visitation or the common flower colour phenotype which would support positive frequency-dependent visitation. The number of visits to the rare or common flower colour was proportional to its frequency in the array. Although we did not test frequency dependence over a wide range of frequencies, the lack of preference for the rare or common morph in the 20B:80W and 80B:20W flower arrays strongly suggest the lack of negative or positive frequency-dependent visitation in this system. The evidence suggests that the preference of a single pollinator for flower colour could not explain the variation in flower colour observed in *A. coerulea*.

While pollinators can play a role in maintaining floral colour diversity among plant species (Fulton and Hodges, 1999; Whittall *et al.*, 2006), our data suggest that neither a differential preference of flower colour by the two pollinators nor the preference of a single pollinator for flower colour could help explain the variation in flower colour observed within and among *A. coerulea* populations. This may seem surprising, especially given the potential importance of pollinators in the diversification of columbine species in North America (Whittall and Hodges, 2007). We do not expect our results to be influenced by the fact that the species *B. impatiens* is not a pollinator in wild *A. coerulea* populations. The different bee species tested so far have been shown to be trichromatic, with peaks of photoreceptor spectral sensitivity in the UV, blue and green regions of the spectrum (Skorupski *et al.*, 2007; Skorupski and Chittka, 2010). The minor differences observed in the sensitivity for the blue receptors among some bumble-bee species are not expected to influence the discrimination between blue and white flowers tested in this study (Skorupski and Chittka, 2010). Moreover, although the choice of flower colour by hawkmoths was tested under fewer conditions relative to bumble-bees, we could not envisage a pollinator scenario that would explain the variation in flower colour observed in *A. coerulea* populations. The discrepancy between the role of pollinators in explaining species diversity of North American columbines and the lack of associations between flower colour variation and pollinator choice within a columbine species may reflect the fact that associations between reward and flower colour are easier to maintain among rather than within a plant species. The behavioural data presented in this study suggest that factors other than pollinators, possibly abiotic factors, may be more likely to act as selective factors on flower colour and help explain the variation in flower colour observed within and among *A. coerulea* populations.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: UV reflectance spectra for flowers with very pale blue sepals and white petals, and flowers with white sepals and petals. Fig. S2: image

of a natural blue *Aquilegia coerulea* flower next to an artificial blue flower.

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