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Published in:

Journal of Plant Ecology

DOI:

[10.1093/jpe/rtt040](https://doi.org/10.1093/jpe/rtt040)

Publication date:

2014

Citation for published version (APA):

Kipling, R. P., & Warren, J. (2014). How generalists coexist; the role of floral phenotype and spatial factors in the pollination systems of two *Ranunculus* species. *Journal of Plant Ecology*, 7(5), 480-489.
<https://doi.org/10.1093/jpe/rtt040>

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How generalists coexist: the role of floral phenotype and spatial factors in the pollination systems of two *Ranunculus* species

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Abstract

Aims

Competition for pollinators between phenotypically similar flowers is believed to play an important role in floral trait diversification in the angiosperms. However, in many plant communities, species with apparently similar floral phenotypes and generalist pollination systems co-flower. Here, the pollination systems of *Ranunculus acris* L. and *Ranunculus repens* L. were investigated to determine the factors enabling the species to coexist within apparently overlapping pollination niches.

Methods

Sympatrically flowering populations of *R. acris* and *R. repens* were investigated at three study sites in West Wales. The floral phenotypes of the two species were compared using measurements of floral morphology and spectral analyses of petal reflectance, using principal component analysis and bee and fly colour-space models. Evidence of inter-specific discrimination by foraging insects was tested for in the field and using floral arrays. The relative roles of behavioural constancy and spatial patchiness in maintaining pollinator fidelity were estimated.

Important Findings

The floral phenotypes of *R. acris* and *R. repens* differed significantly. Social bees were highly constant when foraging at flowers of the two species and patchy floral distribution explained some of the observed fidelity. Dipterans visiting mixed floral arrays appeared to discriminate between the species, visiting more *R. acris* than *R. repens* flowers, but there was no difference in the number of visits to single-species arrays. Social bees were more likely to display constancy to flowers of *R. repens* in the field.

Patchiness in floral distribution, subtle differences in floral phenotype, pollinator preferences and behavioural constancy are all likely to contribute to the continued coexistence of *R. acris* and *R. repens*, despite apparent overlap in their pollination niches. Such differences have the potential to facilitate the maintenance of species diversity in plant communities, even where plants appear to share similar floral phenotypes.

Keywords: floral phenotype, pollination niche, *Ranunculus*, functional specialization, constancy

Received: 16 February 2013, Revised: 8 July 2013, Accepted: 11 July 2013

INTRODUCTION

In conditions of competition for pollinators, selection may be expected to produce character displacement, which pushes co-flowering plant species into better defined pollination niches (Armbruster *et al.* 1994; Levin and Anderson 1970; Waser 1978). Inter-specific pollen flow can affect reproductive success (Brown and Mitchell 2001; Galen and Gregory 1989; Morales and Traveset 2008) through stigma clogging or closure (Caruso 2000; Proctor *et al.* 1996; Waser and Fugate

1986), stilar clogging or inhibition (Cruzan 1990), pollen wastage and pollen layering on the pollinator (Kohn and Waser 1985) and pollen allelopathy (Kanchan and Chandra 1980; Murphy 2000; Murphy and Aarssen 1989) potentially leading to selection for divergent floral phenotypes and flowering phenology.

Despite the theoretical costs, many community level studies of flowering phenology have failed to find evidence of character displacement in a number of temperate plant communities (Arnold *et al.* 2009a; Gumbert *et al.* 1999; Kipling

2010). In these studies, a number of species with similar floral phenotype were found to co-flower with related species. This raises the question of how such species are able to coexist despite occupying apparently overlapping pollination niches. Studying the pollination systems of these species can shed light on the processes that drive floral diversification in some species but not in others (Gomez and Zamora 2006). It can help us to ascertain whether plants that appear to share a pollination niche are actually subtly differentiated from each other, as might be expected given the potential reproductive costs of coexisting in overlapping pollination niches.

When considering generalization and specialization in plant–pollinator interactions, it is important to define these terms clearly. A useful framework is provided by Ollerton *et al.* (2007a) who define three types of floral specialization. Phenotypic specialization refers to the level of floral adaptation displayed by a species and is often associated with functional specialization (referring to the broad taxonomic group of pollinators, which serves a flower). Ecological specialization (which describes the number of species that are effective pollinators of a plant in a particular environment) may or may not be associated with phenotypic or functional specialization.

Phenotypic specialization may occur through the type of character displacement described above. Levin and Anderson (1970) described how two plant species with a shared pollinator can coexist where differences in floral phenotype allow individual pollinators to express fidelity when foraging. Such phenotypic differences allow the partitioning of the shared pollinator resource, reducing inter-specific pollen flow and thereby reducing the theoretical disadvantage in reproductive fitness accruing to the minority species. Such processes may offer an explanation for findings such as those of Ollerton *et al.* (2007b), who discovered no differences in pollination niche to explain the continued coexistence of *Centaurea scabiosa* L. and *Orobancha elatior* Sutton but observed that individuals of their shared pollinator species, *Bombus pascuorum*, displayed constancy to one or other species.

Pollinator fidelity can only increase the chances of species coexisting if different pollinators (or different individuals of a shared pollinator species) also differ in their preferences, with some pollinating the minority species (Levin and Anderson 1970). Such differences can be identified through observations of the number of visits made by insects to flowers of each species. Where co-flowering plants compete for shared pollinators, other factors may also improve the chances of continued coexistence, including spatial patchiness in the distribution of flowers (which physically segregates the flowers of different species from each other and can thereby reduce inter-specific pollen flow when pollinators forage randomly) and differences in floral phenology (Levin and Anderson 1970; Ollerton *et al.* 2007b).

The aim of this study was to determine how two co-flowering species with apparently similar flowers, *Ranunculus acris* L. and *Ranunculus repens* L., are able to coexist while

apparently sharing pollinators as functional generalists. Inter-specific differences in floral phenotype between *R. acris* and *R. repens* have not previously been quantified, and the ability of floral visitors to discriminate between flowers of the two species has not been tested, although studies in alpine plant communities have shown that dipteran pollinators discriminate between flowers of *R. acris* on the basis of petal size, flower depth and flower-stalk height (Totland 1994) and show a preference for larger flowers (Totland 2004). Because *R. acris* and *R. repens* occur sympatrically in many plant communities, it was expected that some or all of the conditions proposed by Levin and Anderson (1970) for the coexistence of two species with shared pollinators would hold.

Specifically, it was expected that (i) there would be differences between the floral phenotypes of *R. acris* and *R. repens* that could reduce inter-specific pollen flow by increasing the fidelity of pollinators, (ii) floral visitors would discriminate between the two species, (iii) pollinator guilds or individual pollinators would differ in their preferences for flowers of either species, or (iv) flowers of the two species would be distributed patchily, reducing the number of inter-specific floral visits made by pollinators foraging randomly between them.

MATERIALS AND METHODS

The study species

Ranunculus repens and *R. acris* are native to the UK (Preston *et al.* 2002) and commonly co-occur in British grasslands (Harper 1957; Lamoureaux and Bourdot 2007), with *R. acris* in general occupying drier areas than *R. repens* (Harper 1957). Although their flowering phenologies are similar (with *R. repens* beginning to flower slightly earlier than *R. acris*), the two species are not known to hybridize (Harper 1957; Sarukhan and Harper 1973). Both are self-incompatible and can spread vegetatively (Fitter and Peat 1994; Harper 1957). To the human observer the yellow, disk-shaped flowers of *R. acris* and *R. repens* appear relatively undifferentiated from each other, with nectar reward offered at the base of the petals and pollen openly available when the anthers dehisce (Harper 1957). The flowers of both species attract a variety of pollinator guilds, such as flies, social bees, solitary bees and butterflies (Harper 1957; Power and Stout 2011; Totland 2001). The pollen of *R. acris* and *R. repens* has been discovered on bumblebees and honeybees (Harper 1957; Whittington *et al.* 2004), and visits by dipterans to flowers of *R. acris* have been shown to increase seed set in some populations (Totland 1994).

The study sites

Three field sites close to the Aberystwyth University Llanbadarn Campus, where *R. repens* and *R. acris* flower sympatrically, were visited during the summer of 2007. These semi-improved agricultural fields were chosen as they contained large populations of both study species, but few other insect-pollinated flowers (Table 1).

Table 1: floral density of insect-pollinated plant species found within transects at three study sites

| Species name | Site A (52° 24'43" N, 4° 02'51" W) | Site B (52° 24'41" N, 4° 02'52" W) | Site C (52° 24'31" N, 4° 03'11" W) |
|---------------------------------|------------------------------------|------------------------------------|------------------------------------|
| <i>Ranunculus acris</i> | 114 ^a | 139.8 | 38.8 |
| <i>Ranunculus repens</i> | 93.4 | 29.2 | 45.6 |
| <i>Cerastium fontanum</i> Baumg | 5.2 | 0.4 | 0 |
| <i>Hypochaeris radicata</i> L. | 0.4 | 0 | 0 |

^aAll figures are the numbers of open flowers per metre square (data derived from transect surveys described in text).

Comparative floral morphologies

In order to determine if there were phenotypic differences between the flowers of *R. acris* and *R. repens*, the floral depth, floral diameter, petal width, nectar guide length and whole petal length of flowers of both species were measured, as well as flower-stalk height. Traits were chosen to focus on phenotypic differences known to affect the foraging patterns of dipteran pollinators of *R. acris* (Totland 1994). Flowers were selected for measurement from plants at least 5 m apart to reduce the chances of sampling multiple flowers from single genotypes. Only plants with no visual signs of senescence or herbivory were chosen, and measurements of all flowers were taken on a single day. Inter-specific differences in measured traits were analysed in Matlab[®] (MathWorks 2007) using principal component analysis (PCA), which uses correlations between original variables to produce orthogonal components (Mot et al. 2010). Differences between species were then tested using analysis of variance (ANOVA) with first principal component (PC1) as the response variable (Luciano and Naes 2009). Because flower-stalk height in *R. acris* varies in different environmental conditions (Harper 1957), a two-way ANOVA was performed separately on height data to determine if any inter-specific differences were maintained between sites.

In order to assess if the flowers of *R. acris* and *R. repens* differed in colour, the spectral reflectance curves of petals from 20 flowers of each species were analysed. Flowers were sampled randomly from the study sites and their petals analysed using a USB4000 Plug-and-Play Miniature Fiber Optic Spectrometer (Ocean Optics, Duiven, Netherlands) with a PX-2 Pulsed Xenon Lamp as a light source (Ocean Optics). The spectrometer produced 2040 wavelength reflectance values in the range of 300–700 nm, which includes the visual range of most flower-visiting insects (Chittka et al. 1994) and was calibrated against a Diffuse Reflectance Standard (WS-1, Ocean Optics). Petals were placed beneath the aperture of a reflection probe holder (RPH-1, Ocean Optics) connected to the spectrometer and light source by a QR400-7-UV/BX Reflection Probe (Ocean Optics). The spectral reflectance of both the outer and inner areas of each petal was measured, as these were observed to differ from each other.

The spectral reflectance of sampled petals from each species was compared visually in Matlab[®] (MathWorks 2007), using the outputs of three models:

- 1) Spectra were subjected to PCA (Mot et al. 2010) and inter-specific differences between the spectra of outer petal areas and between the spectra of inner petal areas were tested for significance using ANOVA, with PC1 as the response variable (Luciano and Naes 2009). Reflectance was averaged across 5 nm bands in the measured range, giving 80 spectral variables.
- 2) Spectra were plotted on the bee colour-space hexagon (Chittka 1992), which weights colour differences according to the sensitivity and range of bee photoreceptors.
- 3) Spectra were also plotted using a fly colour-space model, which provides an estimate of the visual perceptions of dipteran floral visitors (Arnold et al. 2009b; Troje 1993). In this model, dipteran vision is interpreted as allowing discrimination between, but not within, four basic sectors of the colour space, represented visually by the four quarters of the square in which spectra are plotted.

Behaviour of social bees

During May 2009, observations of insect visitors to *R. acris* and *R. repens* flowers were undertaken at the three field sites described. In order to assess the fidelity of visitors, 30 individuals (10 per site) encountered while walking a straight line across each site were followed for a maximum of 2 minutes. In trials, dipteran and other floral visitors such as solitary bees were found to be too fast and erratic to follow, so it was only possible to observe social bees. All floral visits (when an insect landed on a flower) were recorded. Observations were undertaken between 10.30 h and 16.00 h on dry, sunny days to minimize variation in insect behaviour due to weather or time of day, and to ensure that flowers of *R. acris* and *R. repens* (which close at the end of each day) were fully open when surveys were undertaken.

Bateman's constancy Index (BI) (Bateman 1951) was used to determine the level of constancy shown by observed bees to the flowers of *R. acris* or *R. repens*. The index estimates constancy based on the number of inter- and intra-specific visits made to any pair of plant species, producing an output range from -1 (no intra-specific visits) to 1 (complete constancy). An output value of 0 represents random patterns of visitation (no constancy). The measure is insensitive to changes in absolute preference for one or other species. BI is calculated using Equation (1):

$$\text{Bateman's Index (BI)} = (AD)^{0.5} - (BC)^{0.5} \div (AD)^{0.5} + (BC)^{0.5} \quad (1)$$

where A and D represent the number of intra-specific movements between flowers of species one and two, respectively, and B and C represent the number of changes from species one to species two and from species two to species one, respectively (Bateman 1951).

There are two problems with the index when applied to field observations. First, if a foraging insect only visits one species of flower, the index returns a constancy value of 0. Similarly, if there is only one change in the species of flower visited, this positive value of B or C in the formula is multiplied by 0. To overcome this problem, zero values in the formula were replaced by 0.5. Second, the index assumes a 50/50 chance of moving to a flower of species one or two from any flower visited, but this assumption may be violated if flowers of the two study species are distributed patchily. Part of the observed constancy could then be a result of the distribution of flowers (Waser 1986).

In order to ascertain the extent to which the distribution of the flowers of *R. acris* and *R. repens* at the study sites was likely to account for any apparent constancy shown by foraging bees, a 'Behavioural BI' was calculated, for which the BI inputs A to D were weighted according to the real probability of intra- and inter-specific visits occurring. The weighting needed to fulfil certain criteria: (i) the values of A and D should decrease when the chances of intra-specific visits are greater than even (because the spatial distribution of flowers can explain more of the observed fidelity) and increase when the chances of intra-specific visits are less than even, and (ii) the weighting should be equally sensitive to positive or negative deviations from a probability of 0.5 for an inter- or intra-specific visit. To meet these criteria, each input (A to D) in the calculation of BI can be weighted by the probability (P) of the type of visits it represents occurring by chance, using the multiplier $2(1 - P)$. The resulting matrix represents an estimate of visitation figures if the values of P had been equal to 0.5 and allows calculation of the 'Behavioural BI'.

In order to estimate the actual probabilities of moves between flowers, the number and distribution of flowers along a 50 m transect was recorded at each site. Transects were divided into 10-cm squares, with the number of open flowers of each species recorded within each square. Flowers sharing a square were recorded as occupying the same position. The chances of moving from one flower to another of either species was calculated from these data, assuming that (i) insects foraging at random visit the closest flower to the one they occupy, and (ii) where multiple flowers are equidistant from the initial flower there is an equal chance of any being chosen. For each flower on the transect, the probability of an insect making an intra- or inter-specific visit was based on the proportion of same/other species flowers in the same transect square. If no other flowers were present within a square, the proportion of same/other species flowers in adjacent squares was used. The averages of the individual flower probabilities for each species were used to weight inputs A to D in the calculation of Behavioural BI for each bee observed, using Matlab® (MathWorks 2007).

The foraging constancy of bees was assessed using sign tests to determine if BI and Behavioural BI values differed significantly from 0 at the three field sites. In order to ascertain whether the spatial distribution of flowers could account for any constancy displayed, BI and Behavioural BI values were compared using Mann–Whitney tests. Mann–Whitney tests were also used to test for differences in the constancy shown by honey bees and bumblebees. Differences between sites in BI and Behavioural BI values were analysed using Kruskal–Wallis tests. Finally, to test if bees displaying high levels of constancy were more likely to favour *R. acris* or *R. repens* flowers, the number of bees making more than 70% of intra-specific visits to *R. acris* was compared to the number making more than 70% of intra-specific visits to *R. repens* using an exact binomial test.

Insect choices at artificial arrays

In summer 2008, the ability of insects to discriminate between flowers of *R. repens* and *R. acris* was tested by recording the number of visits made to flowers of both species arranged in artificial arrays. Flowers were collected at Site A using the same sampling method as for the phenotypic comparisons described above. Arrays consisted of seven flowers arranged to be equidistant from their neighbours, 10 cm apart and presented at a height of 5 cm.

Observations were made at Site A, according to the same restrictions of time and weather described above for the observation of bees. Mixed- and single-species arrays were used to determine whether insect choices were affected by the proximity of the two species. Mixed arrays consisted of four flowers of one type, and three of the other, with the majority type swapped between observation periods to equalize the chances of visits to each. All arrays were observed for 15 minutes at a time, with the order of observations randomized between array types to avoid confounding effects of weather and time of day (Kilkenny and Galloway 2008). Two arrays were observed at a time, placed 50 m apart to avoid interference. Flowers were replaced between observation periods to avoid the effects of bias from previous foraging.

For each array type, exact binomial tests were used to determine if there were significant differences in the number of initial visits made to flowers of each species (Goyret *et al.* 2008). For mixed arrays, visit numbers were weighted for analysis according to the relative number of flowers of each species presented ($P = 0.43$ and $P = 0.57$ for the majority and minority species, respectively). A chi-square test with Yates' correction was used to test for differences between the proportion of visits to flowers of the different species at single and mixed arrays.

At mixed arrays, the numbers of inter- and intra-specific visits between flowers in an array were recorded to test the fidelity of floral visitors. Visit numbers were weighted according to the probability of insects reaching same and other species flowers by chance, using the formula described for

weighting inputs to the Behavioural BI matrix. The values of P calculated were as follows: for majority flowers, $P = 0.29$ (intra-specific visits) and $P = 0.71$ (inter-specific visits); for minority flowers, $P = 0.44$ (intra-specific visits) and $P = 0.56$ (inter-specific visits). Significant differences in the number of inter- and intra-specific visits made were tested using exact binomial tests (Goyret *et al.* 2008).

RESULTS

Floral morphology and spectral reflectance

Measurement of the flower-stalk heights of 30 *R. acris* and 30 *R. repens* plants found that *R. acris* were significantly taller (mean height \pm standard error [SE]: 40 ± 1.61 cm) than *R. repens* (mean height \pm SE: 18.07 ± 1.02 cm; $F = 211.55$, $P < 0.001$). The interaction term of the two-way ANOVA (species \times site) was non-significant ($F = 1.11$, $P = 0.34$); although the heights of both species varied between sites ($F = 18.27$, $P < 0.001$), this did not significantly affect the inter-specific difference in height.

Analysis of the floral traits of 30 *R. acris* and 30 *R. repens* flowers using PCA (Fig. 1) and ANOVA revealed significant inter-specific differences between flowers along the PC1, which accounted for 51% of the variation between the species ($F = 19.19$, $P < 0.001$). PC1 was weighted mainly on petal width (means \pm SE: *R. acris*, 1.07 ± 0.03 cm; *R. repens*, 0.83 ± 0.02 cm), nectar guide length (means \pm SE: *R. acris*, 0.45 ± 0.02 cm; *R. repens*, 0.38 ± 0.01 cm) and petal length (means \pm SE: *R. acris*, 1.18 ± 0.05 cm; *R. repens*, 1.11 ± 0.02 cm).

No significant difference was found between the two species along PC2, which accounted for 20% of the variation between the species and was weighted mainly on floral depth (means \pm SE: *R. acris*, 0.71 ± 0.02 cm; *R. repens*, 0.74 ± 0.03 cm) and floral diameter (means \pm SE: *R. acris*, 2.02 ± 0.09 cm; *R. repens*, 1.93 ± 0.05 cm).

PCA of the spectral reflectance of *R. acris* and *R. repens* petals showed that the loci of the spectra of the two species overlapped (Fig. 2). However, ANOVA on PC1 (which accounted for 95% of variation between the samples) revealed significant inter-specific differences between the spectra of both outer ($F = 4.13$, $P < 0.05$) and inner petal areas ($F = 12.86$, $P < 0.01$). In the fly vision model, the spectra of *R. acris* and *R. repens* petals mostly plotted in the $p+ y-$ section of the colour space (Fig. 3), indicating that flies would be unable to differentiate between the majority of the sampled spectra. However, when the spectra of both species were plotted on the bee colour-space hexagon, a difference was visible between the loci of the spectra of inner petal areas of the two species (Fig. 4). The average distance in bee colour space between the inner petal area spectra of the two species was 0.15 units, with a maximum of distance of 0.45 units and a minimum distance of 0.01 units. The loci of outer petal spectra were highly overlapping with no clear inter-specific difference visible.

Behaviour of social bees in the field

During observations of foraging social bees, individuals of four species were recorded on the sites, with a total sample size of 88: the honeybee *Apis mellifera* L. (Hymenoptera: Apidae) (18

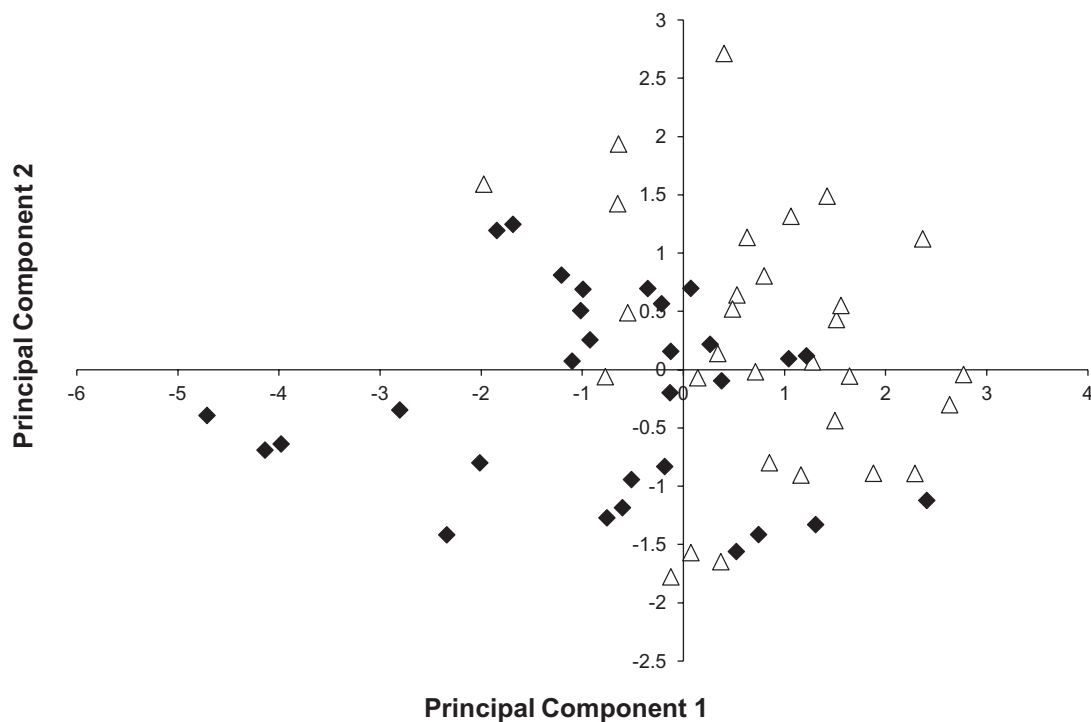


Figure 1: the first two principal components from a PCA of the floral traits of *R. acris* (◆) and *R. repens* (Δ).

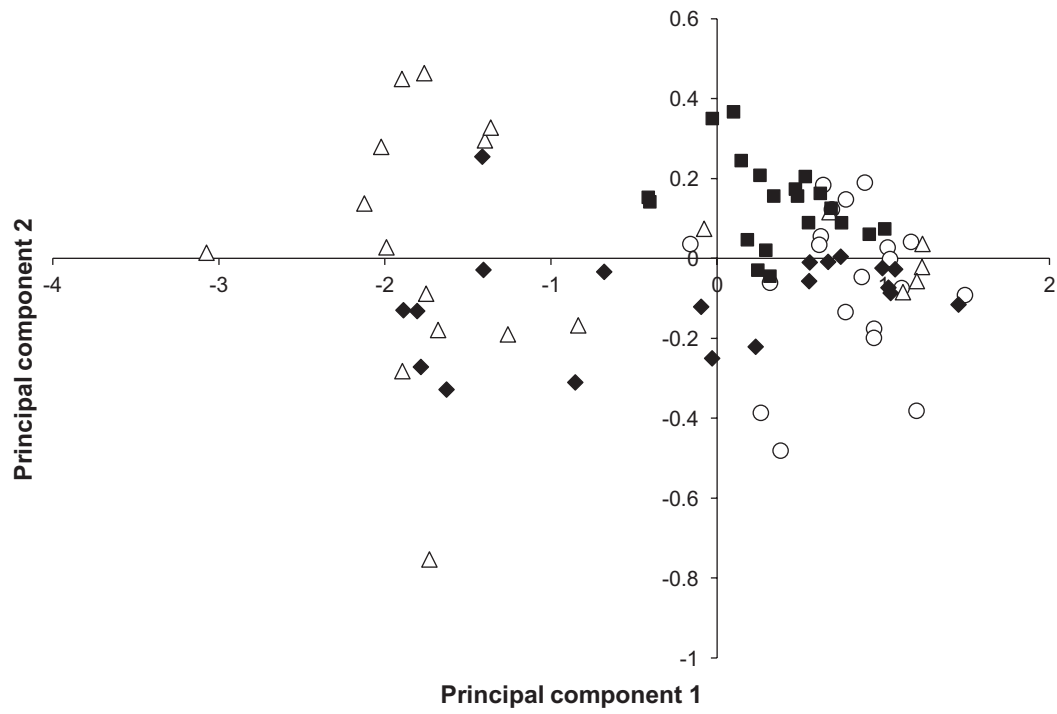


Figure 2: the first two principal components from a PCA of the floral spectral reflectance of *R. repens* and *R. acris*. *Ranunculus acris* inner petal area (■), outer petal area (◆); *R. repens* inner petal area (○), outer petal area (Δ).

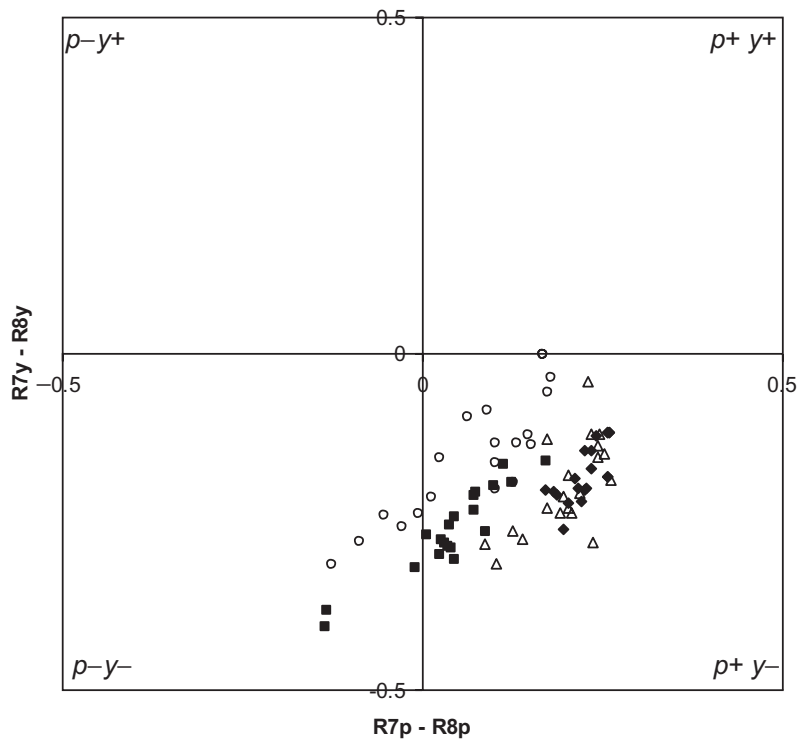


Figure 3: fly colour-space plots of *R. acris* and *R. repens* floral spectra. *Ranunculus acris* inner petal area (■), outer petal area (◆); *R. repens* inner petal area (○), outer petal area (Δ).

individuals) and three bumblebee species, *Bombus lapidarius* L. (Hymenoptera: Apidae) (45 individuals) *Bombus pratorum* L. (Hymenoptera: Apidae) (7 individuals) and *B. terrestris/lucorum* (18 individuals; exact species not identifiable in the field). Analysis showed that adjusting BI to account for the patchy distribution of *R. repens* and *R. acris* flowers significantly reduced BI values (Table 2). Sign tests on index values based on 1768 floral visits showed that BI values at three sites and Behavioural BI values at two sites differed significantly from 0 (Table 2).

Significantly more bees (53 vs. 17) displayed high levels of constancy to flowers of *R. repens* than displayed high levels of constancy to flowers of *R. acris* (Binomial Exact Test, $P < 0.001$). Eighteen bees showed little constancy to either species. The constancy levels of bumblebees and honeybees were the same using BI (0.73 for bumblebees, 0.74 for honeybees; $W = 685.5$) and using Behavioural BI (0.32 for bumblebees, 0.31 for honeybees; $W = 823.5$).

The behaviour of insects at floral arrays

Across the 93 arrays observed (51 mixed arrays and 21 single-species arrays for each species), all but seven recorded floral visits were made by dipterans, of which 24 were hoverflies (14 at mixed arrays and nine at single-species arrays). The remaining visits were made by social bees, but as the sample of this group was so small these visits were excluded from analyses. At mixed arrays, dipterans made significantly more initial visits to *R. acris* flowers than to *R. repens* flowers

(Table 3), but when flowers were displayed in single-species arrays there was no difference in the number of initial visits made to each species (Table 3). The proportion of visits made to *R. acris* was significantly higher at mixed arrays than at single-species arrays ($\chi^2_{d.f. 1} = 5.02$, $P < 0.05$). Twenty-two intra-specific and 56 inter-specific visits were observed for dipteran visitors moving from their initial flower to a second flower, representing significantly more inter-specific movements than expected by chance (exact binomial test, $P < 0.001$).

DISCUSSION

This study investigated the pollination systems of *R. acris* and *R. repens* in order to understand how these two co-flowering species with similar floral phenotypes are able to coexist within apparently overlapping and functionally generalist pollination niches. Evidence was found for all the factors that Levin and Anderson (1970) predicted would increase the chances of two co-flowering species being able to coexist: differences in floral phenotype, spatial patchiness in the distribution of flowers, discrimination between species by floral visitors and differences between pollinator guilds in behaviour when foraging at flowers of the two species. These findings are indicative of subtle differences in the pollination niches of *R. acris* and *R. repens*.

Table 2: results of observations of bee visits to *R. repens* and *R. acris* flowers at three sites, with the outcomes of Sign tests, Mann–Whitney tests (W) and Kruskal–Wallis tests (H)

| | BI | Behavioural BI | Difference (%) | W |
|--------|-------------------------|----------------|----------------|--------|
| Site A | 0.74^a | 0.33 | 56 | 1334** |
| Site B | 0.74 | 0.33 | 56 | 1197** |
| Site C | 0.71 | 0.12 | 83 | 1229** |
| H | 0.21 | 6.94* | | |

^aBold values indicate that index values differ significantly from 0: $P < 0.001$.

* $P < 0.05$.

** $P < 0.001$.

Table 3: analysis of the number of visits made by dipterans to *R. acris* and *R. repens* flowers in mixed and single-species floral arrays

| Array type | Species | Number of visits | P value, exact binomial test |
|--------------------|--------------------------|------------------|--------------------------------|
| Mixed ^a | <i>Ranunculus acris</i> | 209 | <0.01 |
| | <i>Ranunculus repens</i> | 147 | |
| Single Species | <i>Ranunculus acris</i> | 84 | NSD ^b |
| | <i>Ranunculus repens</i> | 91 | |

^aVisit numbers for mixed arrays are weighted according to the number of flowers of each species in observed arrays.

^bNSD = no significant difference at $P < 0.05$.

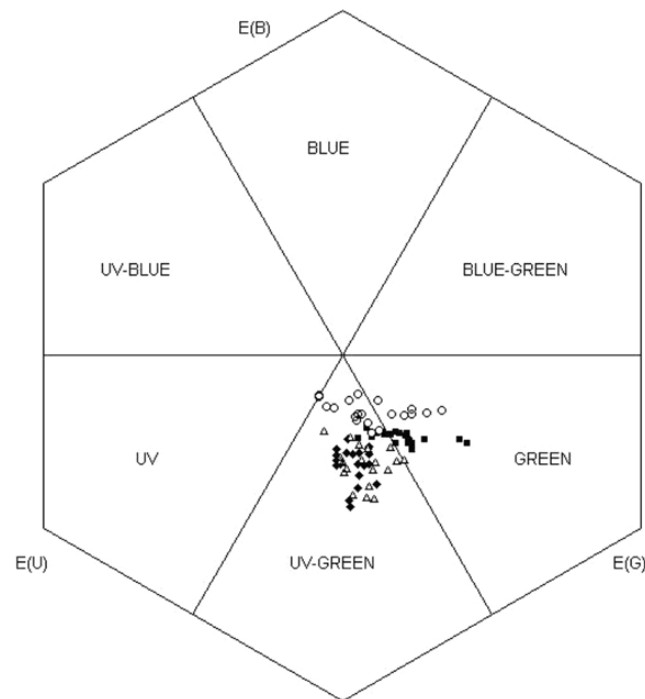


Figure 4: bee colour-space plots of *R. acris* and *R. repens* floral spectra. *Ranunculus acris* inner petal area (■) outer petal area (◆); *R. repens* inner petal area (○), outer petal area (△).

Floral phenotype and discrimination by pollinator guilds

Flowers of *R. acris* and *R. repens* differed from each other in floral morphology, with *R. repens* having narrower, shorter petals, with smaller nectar guides, than *R. acris*. Correlations between the traits revealed by their grouping in PCA suggest that they represent an overall difference in flower size, with flowers of *R. repens* being on average smaller than those of *R. acris*. Following PCA, ANOVA revealed inter-specific differences in spectral reflectance in both inner and outer petal areas based on PC1. There was also some visual indication of inter-specific differences between the spectra of the inner areas of petals in bee colour space, with little overlap between the loci of these spectra. The average colour-space distance between the inner petal area spectra of the two species was 0.15 units; Dyer and Chittka (2004) found that bees could differentiate well between colours separated by colour-space distances of this magnitude, although the minimum distance of 0.01 measured here is at the lower limit of the capability of bees to discriminate, and could be expected to be difficult for them to recognize in the field (Dyer and Chittka 2004). In contrast, there was a large overlap in the loci of outer petal area spectra in bee colour space (Fig. 4) in the loci of the spectra of both outer and inner petal areas using PCA and in fly colour space (Figs 2 and 3), indicating that the colour of individual flowers would not be a reliable guide to their species in these cases (despite the significant differences observed from ANOVA following PCA). Social bees have been shown to use both colour and shape to discriminate between similar targets (Dyer and Chittka 2004) and it may be that the observed differences in floral colour and morphology together provide a more reliable basis for discrimination than either of these factors would alone.

In addition to the spectral reflectance caused by floral pigments, Gaisterer *et al.* (1999) and Vignolini *et al.* (2012) describe the specular reflectance (glinting) produced by the glossy outer petal areas of *R. acris* and *R. repens*, which may act as a long distance attractant to floral visitors. Differences in floral morphology and colour described above may help to differentiate flowers of the two species from each other at close range, after insect have been attracted from distance by this generic specular reflectance. Consistent with this idea, there was no difference between the number of visitors to flowers of *R. acris* and *R. repens* in single-species arrays (Table 3), suggesting that the dipterans observed did not discriminate between the floral displays of the two species, whereas in mixed arrays more visits were made to flowers of *R. acris* indicating that there was discrimination at shorter distances. Plant–insect interactions of this type may provide facilitative advantages to *R. acris* and *R. repens*, with a larger, joint floral display likely to attract more floral visitors (Johnson *et al.* 2003), whereas close-range discrimination between species potentially allows the expression of floral fidelity once pollinator guilds have arrived at the flowers. Previous authors have suggested that facilitation may be more likely between related species, as a result of similarities in the types of pollinator they attract and require (Sargent and Ackerly 2008), and Sargent *et al.* (2011)

found a reduction in pollen limitation where a focal species flowered in the presence of close relatives. The findings described here may suggest similar facilitative interactions between the closely related *R. acris* and *R. repens*.

Hegland and Totland (2012) suggested that facilitation in pollination systems is more likely between species with similar floral phenotypes, showing that fruit set in *R. acris* was increased in the presence of the phenotypically similar flowers of *Potentilla erecta*; the patterns of discrimination shown by floral visitors here might provide a mechanism for this type of facilitation. Consistent with this idea, data from observations of social bees demonstrated that this group was able to exhibit constancy when foraging between *R. acris* and *R. repens*. There was also evidence that dipterans visiting more than one flower in mixed arrays discriminated between flowers of the two species, but in this case the number of inter-specific visits was higher than expected, suggesting that the ability to differentiate between species was used to switch between them, rather than to maintain fidelity. Although observation periods at floral arrays were kept short, it is possible that rewards in the cut flowers became depleted over time so that the dipterans observed were reacting to a lack of reward in a particular flower type by avoiding that type when choosing their next flower.

Without investigating the reaction of insects to manipulation of the floral traits studied, it is not possible to say whether the observed inter-specific differences in these traits were important to the discrimination displayed by floral visitors. However, Totland (2001) found that dipterans discriminated between flowers of *R. acris* on the basis of similar phenotypic differences, providing some evidence that such differences can be important to foraging insects. Further research would be required to quantify the effect of the observed inter-specific differences on the foraging behaviour of visitors to flowers of *R. acris* and *R. repens* in the communities studied, relative to other factors such as differences in floral scent.

It proved impossible to test the fidelity of dipteran floral visitors in the field, and bees were rare visitors to floral arrays so that a direct comparison of the proportion of visits to each plant species made by social bees and dipterans was not possible. However, dipteran visitors to mixed arrays made more initial visits to *R. acris* flowers than expected (Table 3) and social bees were more likely to display constancy to *R. repens* than to *R. acris* in the field. The greater number of bees displaying constancy to *R. repens* and the greater frequency of visits to *R. acris* by dipterans may indicate subtle functional specialization in the pollination niches of the two species. Harper (1957) observed how the close fit of the flowers of *R. repens* to foraging honeybees resulted in an extensive deposition of pollen on these insects while noting that the flowers of *R. repens* appear better adapted for insect pollination than those of *R. acris*, with more developed nectaries and nectar scales. These phenotypic factors might explain the greater number of social bees displaying constancy to flowers of *R. repens* than to those of *R. acris*.

Despite rarely visiting floral arrays, social bees were often observed in large numbers foraging at *R. acris* and *R. repens* in

the field. Temporal and spatial variation in the availability of different pollinator guilds may be expected to favour generalization in the reproductive strategy of plant species (Waser *et al.* 1996) and may represent one reason why there has not been further specialization of *R. repens* or *R. acris* flowers to either social bee or dipteran pollinator guilds. Although previous work suggests that both dipterans and social bees can act as pollinators of these two species (Harper 1957; Totland 1994; Whittington *et al.* 2004), studies of the effectiveness of the pollination service delivered by these guilds would be required to establish how observed differences in visit frequency affect plant reproductive fitness (Fenster *et al.* 2004) and to test whether indications of functional specialization accurately represent the pollination service received by the two species.

Floral constancy and the spatial distribution of flowers

Social bees observed foraging in the field showed high rates of fidelity to flowers of either *R. repens* or *R. acris* (Table 2) and patchiness in the floral distribution of the two species could account for between 55% and 80% of this observed constancy. This indicates that an insect foraging at random between *R. acris* and *R. repens* flowers might have been expected to show relatively high floral fidelity by chance.

Evidence of behavioural constancy at sites A and B (Table 2) indicates that social bees can discriminate between flowers of *R. acris* and *R. repens* and that they express constancy to one or other. The three field sites observed contained a low diversity of flowering insect-pollinated plants (Table 1), and social bees have shown to express greater floral constancy when foraging in species-poor relative to species-rich communities (Wilson and Stine 1996). As a result, patchiness in the distribution of *R. acris* and *R. repens* flowers may be of greater importance in reducing inter-specific pollen flow in more species-rich habitats where bees may show less constancy. The spatial differentiation of flowers of the two species is increased by the observed inter-specific differences in flower-stalk height, which could be expected to make randomly occurring intra-specific visits more likely.

Subtle differences between the environmental niches of different plant species are likely to be important in allowing them to coexist (Silvertown 2004), and it has long been recognized that *R. repens* occupies wetter locations than *R. acris* in grassland communities (Harper 1957). Differences between the environmental niches of these two species, as well as their ability to spread vegetatively (Fitter and Peat 1994; Harper 1957), are likely to increase the chances that flowers of the two species will exist in distinct patches. Here, it has been shown that such patchiness has the potential to increase the proportion of intra-specific visits made by pollinators, irrespective of their ability to discriminate between the flowers of the two species. This should in turn reduce the chances that where *R. acris* and *R. repens* occur sympatrically either will be excluded from a community through competition for pollinators (Levin and Anderson 1970).

CONCLUSION

The pollination systems of *R. acris* and *R. repens* appear to be characterized by subtle inter-specific differences, with floral visitors discriminating between the species and in some cases expressing fidelity, rather than by open relationships between indiscriminating pollinator guilds and flowers, which cannot be differentiated from each other. This study has not touched on other potential inter-specific differences, e.g. in floral scent and reward, and has not investigated the effectiveness of different floral visitors as pollinators of these species. Further research may therefore reveal still more complexity in these pollination systems. Subtle differences across a number of dimensions of their pollination niches may explain the coexistence of *R. acris* and *R. repens*. The exploration of apparently generalist pollination systems offers a fascinating insight into how diversity can be maintained in species-rich plant communities, without high levels of phenotypic specialization.

ACKNOWLEDGEMENTS

The authors would like to thank Mathieu Falcou, Rhiannon Watson, Céline Sagres and Julien Pannetier for their assistance with insect observations and Alan Gay for his help with spectral analyses.

Conflict of interest statement. None declared.

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