

# Breeding system, pollinator choice and variation in pollen quality in British herbaceous plants

M. E. Hanley<sup>\*1</sup>, M. Franco<sup>1</sup>, S. Pichon<sup>2</sup>, B. Darvill<sup>3</sup> and D. Goulson<sup>3</sup>

<sup>1</sup>School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth. PL4 8AA. UK; <sup>2</sup>Ecology, Evolution, Symbiosis, UMR CNRS 6556, University of Poitiers, Poitiers Cedex. France; and <sup>3</sup>School of Biological and Environmental Sciences, University of Stirling, Stirling. FK9 4LA. UK

## Summary

1. Although it is well established that different plant species vary considerably in the quality of pollinator rewards they offer, it is unclear how plant reproductive systems, in particular an obligate dependence on insects for pollination, might influence the evolution of pollinator rewards. Moreover, unlike the interaction between nectar reward and pollinator visitation, we have a limited understanding of the way in which pollen quality influences pollinator foraging behaviour.

2. We quantified the pollen protein and amino acid content for 23 NW European plant species. Pollen quality was compared with breeding system (facultative- vs. obligate insect-pollinated). A subset of 18 plants was sampled from a single habitat. For these we compared the proportion of pollen collection visits made by bumblebees with the quality of pollen offered.

3. We found a significant association between pollen quality and reproductive system; pollen of obligate insect-pollinated species contained higher protein content. We also found a significant relationship with pollinator use; plants most frequently visited by pollen-collecting bumblebees produced the highest-quality pollen.

4. We discuss how the close relationship between pollen quality and bumblebee attraction may have important benefits for plant reproductive success. However, we also show how the disruption of this mutualism can have detrimental consequences for plant and pollinator alike.

**Key-words:** bumblebee, foraging behaviour, plant reproduction, pollination, pollination syndrome, pollinator reward

## Introduction

Pollen quality varies greatly between different plant species (Roulston, Cane & Buchmann 2000; Somerville & Nicol 2006), but the causes and consequences of this variation remain unclear. One of the longest standing hypotheses is that pollen quality (usually measured as protein content) is associated with animal (zoophilous) or wind (anemophilous) pollination (Lidforss 1899). Anemophilous plants in general do seem to offer relatively low-quality pollen, probably by virtue of not having to attract pollinators to their flowers by offering a food reward (Roulston *et al.* 2000). However, within zoophilous plants there is considerable variation in the quality of pollen offered (Roulston *et al.* 2000). Given the taxonomic diversity within such a large group of plants, this is perhaps unsurprising. Nevertheless, there are also important differences in the pollination systems exhibited by zoophilous

plants, and the behaviour of the pollinators that visit them, which may account for variation in pollen quality.

A large number of zoophilous plant species are also capable of self- or wind-pollination, a strategy which provides reproductive assurance when pollinator populations are low or absent (Darwin 1877; Moeller 2006). Although the relationship between breeding system and pollen quality remains poorly explored, some obligate zoophilous species, such as those which rely on the vibrations produced by a visiting bee to stimulate pollen release (so-called ‘buzz-pollination’), do seem to possess particularly protein-rich pollen (Roulston *et al.* 2000). Pollinator fidelity may also be important. A number of plant species, particularly members of the Fabaceae, are pollinated exclusively by large bees from the Apidae (honeybees and bumblebees). Flower morphology may be one explanation for this close relationship; large, zygomorphic flowers are often only accessible to large bees. However, pollinator reward may also play a part in the mutualism. Specialisation on plants offering high-quality

\*Correspondence author. E-mail: mehanley@plymouth.ac.uk

pollen would be of clear benefit to bees which, unlike nectar feeding Lepidoptera and Diptera, are entirely dependent on pollen for all of their protein requirements (Goulson 2003; Smeets & Duchateau 2003). However, beyond the study by Roulston *et al.* (2000), there have been no attempts to examine the relationship between pollen reward and pollinator floral preferences. Thus, it is unclear whether there is any general trend for obligate zoophilous plants to offer higher quality pollen than plants capable of self- or wind-pollination, or whether bees preferentially visit plant species which offer higher-quality pollen.

In this study, we examined variation in pollen quality (approximate relative protein and essential amino acid [EAA] content) in 23 herbaceous species drawn from nine plant families native to southern England. Within this group of plant species 10 were facultative insect-pollinated (i.e. capable of self- or in the case of *Calluna vulgaris*, wind-pollination), with the remainder being entirely dependent on insects for pollen transfer. Thus, we were able to test the hypothesis that an absolute dependency on insects for pollination is associated with the quality of pollen offered by flowers. Our final goal was to determine whether variation in pollen quality is linked to the number of pollen collection visits made by bumblebees. More specifically, we tested the hypothesis that bumblebees selectively visit flowers which offer high-quality pollen, irrespective of their relative abundance in the plant community. Although it is well known that many pollinators, including honeybees and bumblebees, exhibit fidelity towards certain flowers (reviewed in Goulson 2003; Cane & Sipes 2006), we understand very little about the role of pollen quality in this relationship. Pollen from all but five of our plant species was collected from plants growing in the chalk grassland and scrub ecosystems of Salisbury Plain. We were able to compare pollen protein and EAA content with bumblebee visitation rates to these plant species to determine whether bumblebees favour plants offering the highest-quality pollen.

## Methods

### PLANT SPECIES AND STUDY SITES

Salisbury Plain in Wiltshire, southern England is the largest area of unimproved chalk grassland in NW Europe. During August 2003 and July/August 2004, we collected and analysed the relative EAA and pollen protein content of 18 plant species common throughout Salisbury Plain (Table 1). We also collected pollen from two heathland species (*C. vulgaris* and *Potentilla erecta*) from the New Forest National Park (50°51' N, 1°31' W), two plants from early successional woodland habitats (*Chamerion angustifolium* and *Digitalis purpurea*) from Southampton (50°58' N, 1°25' W) and from *Symphytum officinale*, a riparian species located at Ringwood (50°50' N, 1°47' W). Although this group of plants varies in their dependency on insects for pollination, each species is frequently visited by bumblebees for pollen or nectar, and together the 23 plant species included in this study attract a majority of all flower visits by bumblebees in southern England (Goulson *et al.* 2005; M.E. Hanley personal observation).

Using information gathered from the literature (Knuth 1909; Clapham, Tutin & Moore 1987; Grime, Hodgson & Hunt 2007), the

reproductive system of each species was coded as either obligate insect-pollinated (self-incompatible), or facultative insect-pollinated (self-compatible or wind-pollinated). Data on pollen grain diameter was also sourced from the literature (Andrew 1984; Peat & Fitter 1994).

### POLLEN COLLECTION AND ANALYSIS

For three species (*Echium vulgare*, *Papaver rhoeas* and *Senecio jacobaeae*) it was possible to collect pollen samples directly from flowers. For most species, however, small flower size or low pollen volume meant that direct sampling of pollen from inflorescences was not practical. For these plants we took pollen from the legs of foraging bees, having first observed the individual for 5 min to ensure that it was foraging on the target plant species only. Following removal of the pollen from the bee's legs, samples of pollen were examined under a microscope to ensure that the loads consisted of only the target plant species. Pollen collected in this way was washed in 0.1 M H<sub>2</sub>SO<sub>4</sub> prior to analysis in order to remove sugars used to cement the pollen to the bee's legs. Samples of pollen collected from *E. vulgare* using each of these methods were initially compared using the Bradford (1976) method to ensure that pollen collection method did not influence the results. Following freeze drying, pollen protein and EAA content was determined by ion-exchange analysis of hydrolysed proteins (Spackman, Stein & Moore 1958; Fountoulakis & Lahm 1998), using at least four replicate samples for each species. We were unable to quantify tryptophan or cystine, or separate asparagine from aspartic acid, or glutamine from glutamic acid using this method. 'Essential' amino acids for bumblebees were considered to be those identified as essential for honeybees (arginine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine), omitting histidine as it can be metabolically replaced by the  $\alpha$ -keto acid carnosine (De Groot 1953). Although consumed by young queens, pollen represents the major protein source for larvae and as such is vital to bumblebee nest success (Goulson 2003).

### BUMBLEBEE OBSERVATIONS

Observations of bumblebee foraging behaviour were made in areas of c. 100 m radius searched for 1-man-hour in conditions favourable to bumblebee activity, precisely following the methods of Goulson & Darvill (2004). This approach has been widely used for studies of bumblebee forage use (Goulson & Darvill 2004; Goulson & Hanley 2004; Goulson *et al.* 2005). Observations were conducted between 09:00 and 16:00 h throughout the main worker forage period of June–August, thus reducing the possibility of any temporal bias in flower visitation by bumblebees. Bees were only recorded as collecting pollen if they were observed actively gathering pollen and placing it in their corbiculae. A subset of the Goulson *et al.* (2005) data for bumblebee pollen-collecting visits on Salisbury Plain was combined with further observations undertaken during June–August 2004/2005, such that a total of 65 separate 1-man-hour records of bumblebee foraging were made at different locations on Salisbury Plain (see Table S1 in Supplementary Material).

### PLANT PHYLOGENY AND COMPARATIVE ANALYSIS

The relationships between visitation rate, pollen quality (protein and EAA content) and plant reproductive characteristics were tested by means of comparative analyses by independent contrasts as implemented by the CAIC software (Purvis & Rambaut 1995). This

**Table 1.** The relative protein content (% by dry weight) of pollen collected from 23 plant species native to southern England. The proportion of protein comprised of essential amino acids, and the relative proportion of observed bumblebee visits to each species to collect pollen (Goulson *et al.* 2005) are also shown. Nomenclature follows Clapham *et al.* (1987)

Family	Species	Protein content mean % ( $\pm$ SE)	Essential amino acids (%)	Maximum pollen diameter ( $\mu$ m)	Proportion of pollen visits (%)	Pollination mechanism
Asteraceae	<i>Centaurea nigra</i>	27.5 (1.5)	31.1	33	0.8	Insect
	<i>Centaurea scabiosa</i>	24.2 (1.3)	32	75	2	Insect
	<i>Cirsium arvense</i>	21.9 (0.3)	31.9	43	0.2	Insect/Self
	<i>Cirsium vulgare</i>	22.1 (1.4)	30.2	56	0.3	Insect/Self
	<i>Senecio jacobaea</i>	17.2 (1.5)	34.9	22	0	Insect
Boraginaceae	<i>Echium vulgare</i>	44.1 (2.2)	39.9	15	5.4	Insect
	<i>Symphytum officinale</i>	17.5 (1.3)	35.1	33	N/A	Insect/Self
Dipsacaceae	<i>Scabiosa columbaria</i>	24.8 (1.7)	34.2	80	0	Insect
Ericaceae	<i>Calluna vulgaris</i>	13.9 (0.2)	35.2	36	N/A	Insect/Wind
Fabaceae	<i>Genista tinctoria</i>	22.8 (0.5)	34.5	30	0.2	Insect/Self
	<i>Lotus corniculatus</i>	35.8 (1.1)	40.6	18	8.1	Bee
	<i>Melilotus altissima</i>	39.2 (1.1)	40.5	30	15.6	Bee
	<i>Onobrychis vicifolia</i>	37.5 (1.8)	40.2	41	20.2	Bee
	<i>Trifolium pratense</i>	40.8 (3.6)	40.6	45	16.7	Bee
	<i>Trifolium repens</i>	35.2 (0.8)	41.6	27	3.2	Bee
Onagraceae	<i>Chamerion angustifolium</i>	16.2 (1.8)	36.1	91	N/A	Insect/Self
Papaveraceae	<i>Papaver rhoeas</i>	19.1 (0.5)	36.6	30	6.9	Insect
Rosaceae	<i>Potentilla erecta</i>	16.3 (0.3)	35	28	N/A	Insect
	<i>Rosa canina</i>	14.5 (0.4)	32.8	30	0	Insect/Self
	<i>Rubus fruticosus</i>	15.1 (1.4)	34.5	24	1.5	Insect/Apomict
Lamiales	<i>Digitalis purpurea</i>	20.9 (0.4)	36.2	23	N/A	Bee/Self
	<i>Rhinanthus minor</i>	20.8 (0.9)	32.1	33	0.8	Bee/Self
	<i>Odontites verna</i>	36.6 (0.6)	40.8	20	8.6	Bee

required the construction of a reliable phylogeny for the 23 species in the data set. This phylogeny was obtained with PHYLOMATIC (<http://www.phylodiversity.net/phyloomatic/>, see Webb, Ackerly & Kembel 2007), employing the maximally resolved seed-plant tree with branch length interpolation (BLADJ function) and Wikstrom, Savolainen & Chase's (2001) aged megatree. As specified in the CAIC manual, when variables were continuous, option 'crunch', which allows quantification of contrasts for each variable at each node of the phylogeny, was employed. On the other hand, when dichotomous, categorical variables were involved (reproductive system, parity), option 'brunch', which sets all the contrasts in these categorical predictor variables to +1, is required. This is because there is only one comparison to be made, for example, obligate insect pollination (which was coded as 1) vs. facultative insect pollination (which was coded as 2). In this case, predominance of positive contrasts (in the continuous variable whose relationship with the categorical variable is being investigated) means that higher values in the continuous variable are found in cases where the categorical variable is coded with the higher value (in our example, facultative insect pollination). On the other hand, predominance of negative values in the continuous variable would indicate that this variable is higher when the categorical variable has the value with the lower code (in our example, obligate insect pollination). Continuous variables where proportions were involved (e.g. protein and EAA content, pollinator visitation rate) were arcsine-transformed prior to analysis. Those involving absolute measures (pollen size) were log-transformed. As is standard in these analyses, the relationships between continuous variables were investigated by linear regression through the origin (i.e. no change in one variable is expected to correspond to no change in the other). Those involving dichotomous variables were evaluated with a Wilcoxon sign test and a randomisation test. The results from these two tests were qualitatively identical, and we only report those from the Wilcoxon tests.

## Results

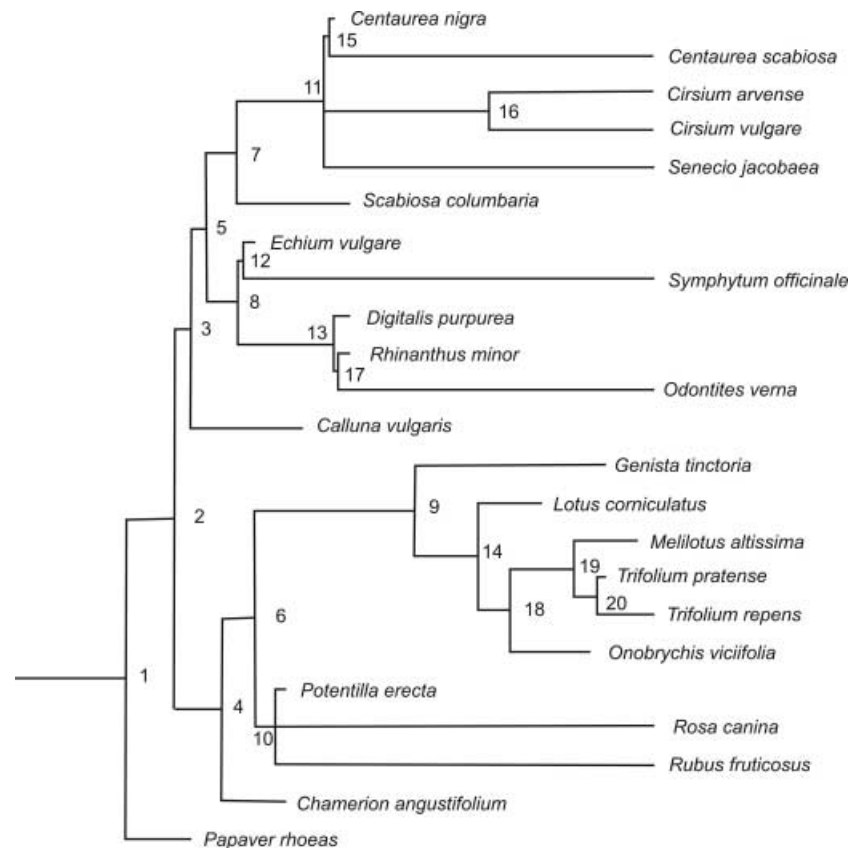
### VARIATION IN POLLEN QUALITY BETWEEN PLANT SPECIES

Despite the fact that plants belonging to the Fabaceae in general produced higher-quality pollen (Table 1), it was clear that there was much variation in pollen quality between and within plant family. For example, *Genista tinctoria* pollen contained relatively little protein, and a smaller proportion of this protein was comprised of EAAs compared with other members of the Fabaceae. Similarly within the Lamiales, *Odontites verna* pollen contained considerably more protein, and had a higher relative EAA content than the other two members of this clade (*Rhinanthus minor* and *D. purpurea*). Although there was marked variation in pollen quality within the Asteraceae, in no instance did protein or EAA content approach the levels noted within the Fabaceae. Members of the Rosaceae were consistent in their production of low-quality pollen.

### COMPARATIVE ANALYSES BY INDEPENDENT CONTRASTS

Reproductive system (facultative insect-pollinated vs. obligate insect-pollinated) provided seven possible contrasts within the 23 species data set (nodes 2, 5, 9, 10, 11, 12 and 17 in Fig. 1). This is because, when dealing with categorical variables, contrasts can only be used once. Although this results in a

**Fig. 1.** Phylogeny employed in the comparative analysis by independent contrasts used to examine the relationship between pollen quality and pollination syndrome, bumblebee visitation rate, style length and pollen size for 23 NW European herbaceous plant species. To allow reference to individual contrasts, nodes have been numbered in reverse order of CAIC's output (branch order). Branches are scaled to their estimated lengths (see text).



relatively low number of contrasts, it did allow us to investigate the direction of possible relationships. When we considered pollen protein content, pollen quality was higher for obligate- compared to facultative insect-pollinated species ( $z = -2.028$ , two-tailed asymptotic significance  $P < 0.05$ ). However, EAA content ( $z = -1.352$ , two-tailed asymptotic significance  $P = 0.176$ ) did not vary significantly between the two pollination groups.

When we compared the pollen protein content offered by the 18 different plant species collected from Salisbury Plain with bumblebee pollen collection visits to those species, we again found a significant positive correlation; that is, visits to plants offering higher-quality pollen were more frequent (Fig. 2a). However, the relative proportion of EAAs contained within pollen was not significantly associated ( $P = 0.06$ ) with bumblebee visits (Fig. 2b). While bumblebees appear to concentrate their pollen foraging activities on plants offering the highest pollen protein content, this preference may simply reflect differences in floral resource abundance. To test the possible relationship between visitation rate and inflorescence abundance, we compared average inflorescence density recorded along a  $5 \times 1000$  m grassland transect at Weather Hill ( $51^{\circ}16' N$ ,  $1^{\circ}42' W$ ), Salisbury Plain during June/August 2005 (M.E. Hanley, unpublished data), with the relative proportion of pollen collection visits to plants at the same site throughout the same period (a small subset of the visitation data analysed here). Using linear regression, we found a remarkably weak correlation ( $r^2 = 0.004$ ), suggesting that bumblebees did not simply utilize flowers in proportion to

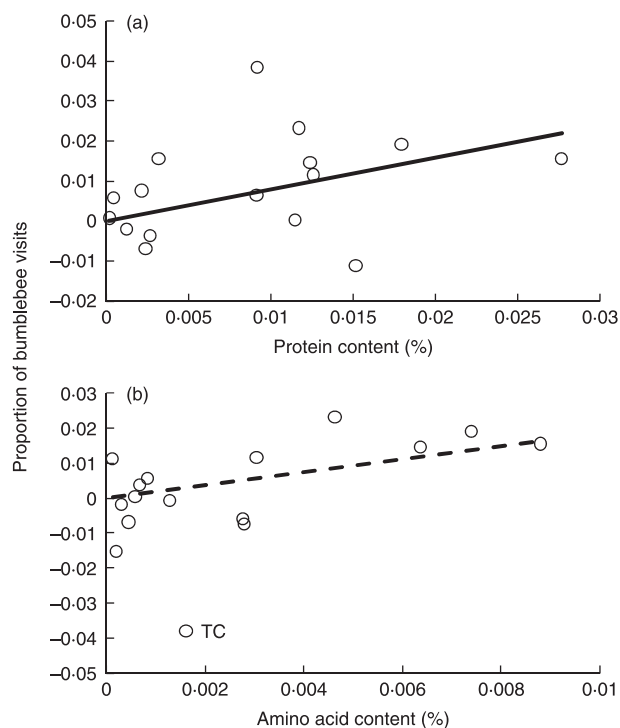
their relative abundance. Thus, we conclude that bumblebees preferentially collect pollen from plants offering higher pollen protein content.

The results of the independent contrasts showed that pollen protein content ( $r^2 = -0.05$ ;  $F_{1,18} = 0.04$   $P > 0.84$ ), EAA content ( $r^2 = -0.05$ ;  $F_{1,18} = 0.15$   $P > 0.70$ ) and pollen collection visits ( $r^2 = -0.06$ ;  $F_{1,14} = 0.02$   $P > 0.87$ ) by bumblebees on Salisbury Plain were not correlated with pollen grain size (diameter).

## Discussion

An absolute necessity to attract pollinators would seem to be a compelling reason why plants might offer high-quality pollen rewards. On average, pollen from the facultative insect-pollinated species we examined contained much less protein (18.6%) than that from obligate insect-pollinated species (30.6%). In fact, the nine most protein-rich pollens were recorded in obligate zoophilous species. Although there is clearly a bias towards the Fabaceae in our data set, this in itself is not surprising given the close association between members of this plant family and their bumblebee pollinators. However, even after accounting for phylogeny, we found a significant association between plant breeding system and pollen quality, with a general trend towards high protein content in obligate insect-pollinated species.

We also found a clear relationship between pollen protein content and pollinator attraction; bumblebees appear to



**Fig. 2.** The relationships between (a) the contrasts of the proportion of bumblebee visits in Salisbury Plain (arcsine-transformed) and the contrasts of protein content (arcsine-transformed), and (b) the contrasts of the proportion of visits (arcsine-transformed) and the contrasts of amino acid content (arcsine-transformed). Regressions are  $y = 0.79x$  ( $r^2 = 0.37$ ;  $F_{1,15} = 8.83$   $P < 0.01$ ) and  $y = 1.84x$  ( $r^2 = 0.22$ ;  $F_{1,15} = 4.16$   $P < 0.06$ ), respectively. Excluding the outlier contrast between the two *Trifolium* species (point labelled TC; node 20 in Fig. 1) in the latter relationship would yield  $y = 2.14x$  ( $r^2 = 0.50$ ;  $F_{1,14} = 13.86$   $P < 0.01$ ).

fine-tune their foraging behaviour to select plants offering the most rewarding pollen. Moreover, while the relationship between EAA content and bumblebee visitation was not significant at the  $P = 0.05$  level, a close relationship was evident, and this interaction may be worthy of further investigation. Pollen quality, including amino acid content, is known to be important for larval growth in bees (De Groot 1953; Roulston & Cane 2002), and for social bee species with relatively short brood cycles, larval provision of high-quality pollen may be essential in ensuring nest success (Goulson *et al.* 2005). There is some debate as to whether pollinators can actively discriminate between flowers on the basis of pollen quality (Roulston *et al.* 2000; Minckley & Roulston 2006). Nevertheless, there is evidence to suggest that bees are able to detect and select protein-rich pollen (Rasheed & Harder 1997; Robertson *et al.* 1999), possibly through the release of volatile compounds (Dobson 1987; Dobson, Groth & Bergstrom 1996). In any event, the evolution of pollen specialisation could arise without active selection, simply because bees which favour plant species producing nutrient-rich pollen are more likely to thrive, whatever the cue used to select the plant. Thus, preferential selection of pollen

may be one of many ways in which insect pollinators influence evolutionary change in floral traits (Fenster *et al.* 2004; Bronstein, Alarcon & Geber 2006).

It is possible that pollen characteristics other than nutritional quality may influence pollen collection by bumblebees. However, unlike Roulston *et al.* (2000) who found a negative correlation between pollen grain size and pollen quality, we found no relationship between pollen size and pollen protein or EAA content, or pollinator attraction. It is also conceivable that pollen longevity may affect pollinator selection (Beardsell *et al.* 1993), although it is unlikely to be a factor guiding the mid-season pollen collection visits reported here, since pollen is not stored for long periods in bumblebee nests (Alford 1975).

For the plant an obligate relationship with a relatively small group of pollinators such as bumblebees is of great benefit since it ensures efficient pollen transfer. This is especially important for species which occur at low densities, since generalist pollinators cannot be guaranteed to transfer pollen from one individual to another when rival forage sources are easier to locate (Kunin 1997). Protein-rich pollen is one way in which plants can promote the fidelity and reliability of bumblebees. It has long been known that bumblebees rapidly respond to highly rewarding flowers (in terms of nectar sugar content) by developing learned associations between the reward and flower scent, colour or shape so that they preferentially search out rewarding flowers (Goulson 1999; Stout & Goulson 2002). As a result, individual bees tend to exhibit flower constancy; repeated visits to a single species of flower, which experience has taught them, provides reward. This behaviour is clearly advantageous to the plant. It is intriguing to speculate that, if bees are able to assess pollen protein content (in addition to nectar quality), then high floral protein could be an adaptive response to promote pollinator constancy and hence ensure an efficient pollination service.

However, a close relationship between mutualists has many potential pitfalls. The loss of dedicated, obligate pollinators may disrupt pollen transfer with all the concomitant problems this has for gene flow and reproduction within a plant population (Bloch, Werdenberg & Erhardt 2006; Kwak & Bekker 2006). The fact that Fabaceae-specialist bumblebees were introduced to New Zealand in order to improve seed set in red clover (*Trifolium pratense*) underscores the obligate association between plant and pollinator (Goulson & Hanley 2004). Similarly, the recent rapid decline of Fabaceae-specialist bumblebees seems to be associated with the loss of species-rich, calcareous grasslands and hay meadows throughout Northern Europe (Goulson *et al.* 2005, 2006; Carvell *et al.* 2006). Those bumblebees which have remained abundant have been able to adapt their diets to encompass arable crops and garden flowers (Fussell & Corbett 1992; Goulson *et al.* 2005, 2006).

While it is impossible at present to determine whether pollinator decline results in reduced reproductive potential for obligate insect-pollinated plants, or *vice versa*, it is clear that both specialist pollinators and obligate zoophilous plants have declined markedly in diversity and abundance over recent decades (Goulson *et al.* 2005; Biesmeijer *et al.* 2006; Carvell *et al.* 2006; Goulson, Lye & Darvill 2008). The

realisation that decreasing pollinator abundance may have significant detrimental consequences for plant fitness (Kearns, Inouye & Waser 1998; Biesmeijer *et al.* 2006; Bloch *et al.* 2006) highlights a pressing need to elucidate how plant–pollinator interactions have evolved and are maintained. A host of factors, including inflorescence morphology, nectar reward, floral display size and floral density (Benitez-Vieyra *et al.* 2006; Ishii & Harder 2006; Liu *et al.* 2007; Makino, Ohashi & Sakai 2007) all play a role in pollinator preference and foraging behaviour. However, the link between pollination syndrome, pollen protein content and pollinator attraction established here highlights another important interaction that should be considered in our attempts to understand how the loss of preferred forage plants impacts on pollinator species.

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### Supplementary material

The following supplemental material is available for this article:

**Table S1.** Pollen collection preferences for eight *Bombus* species native to southern England. Preferences determined for 665 individuals surveyed between 2002 and 2005 at 65 sites on Salisbury Plain. Records for *Bombus terrestris* include possible observations of *B. lucorum* due to difficulties in distinguishing between workers of both species in the field

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2435.2008.01415.x>  
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