

A population comparison of the strength and persistence of innate colour preference and learning speed in the bumblebee *Bombus terrestris*

Thomas C. Ings · Nigel E. Raine · Lars Chittka

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Abstract Studies of innate colour preference and learning ability have focused on differences at the species level, rather than variation among populations of a single species. Initial *strength* and *persistence* of colour preferences are likely to affect colour choices of naïve flower visitors. We therefore study the influence of both the strength and persistence of innate colour preference (for blue) on an operant learning task (associating food reward with yellow flowers) in two populations of the bumblebee *Bombus terrestris*. We found that both strength and persistence of blue preference differed significantly between populations: *B. terrestris dalmatinus* had a weaker and less persistent blue preference than *B. terrestris audax*. These differences in preference also influenced learning performance. Considering only landing behaviours, one-trial learning occurred in the majority (73%) of bees, and was achieved sooner in *B. terrestris dalmatinus* because of its weaker blue preference. However, compared to landing behaviours the relative frequency of approach flights to rewarding and unrewarding flower types changed more slowly with task experience in both populations. When considering both approaches and landings, the rate of learning, following the first rewarded learning trial, was faster in *B. terrestris audax* than *B. terrestris dalmatinus*. However, the net effects of population differences in blue preference and learning dynamics result in similar final levels of task performance. Our results provide new evidence of behavioural differences among isolated populations within

a single species, and raise intriguing questions about the ecological significance and adaptive nature of colour preference.

Keywords Bumble bee · Cognitive ecology · Floral preference · Flower colour · Foraging behaviour · Learning speed · Sensory bias

Introduction

Many studies have examined the role played by colour preferences in the co-evolution of plants and their pollinators (Menzel 1967; Benschbach 1994; Giurfa et al. 1995; Kelber 1996; Lunau et al. 1996; Weiss 1997; Pohl et al. 2008). Recent work has also indicated that the strength of innate colour preferences can influence foraging performance in bumblebees under natural conditions (Raine and Chittka 2007), a finding consistent with the hypothesis that local variation in flower traits could drive selection for such innate sensory (colour) biases.

A common theme among studies of colour preference is that they only look at the influence of the initial strength of colour preference, and not its persistence over time (e.g. Giurfa et al. 1995; Lunau and Maier 1995; Hurlbert and Ling 2007; Spence and Smith 2008). Yet, persistence of preference could also be of great ecological importance to pollinators, particularly bees, that frequently have to learn to associate new colours with rewards as the most rewarding species of flower change through time and space (Heinrich 1979; Menzel 1985; Ollerton and Lack 1992; Willmer and Stone 2004).

Bumblebees are well known to be able to learn to discriminate between flowers offering different rewards using colour as a visual cue (Heinrich et al. 1977; Dukas

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T. C. Ings · N. E. Raine (✉) · L. Chittka
Research Centre for Psychology,
School of Biological and Chemical Sciences,
Queen Mary University of London,
Mile End Road,
London E1 4NS, UK
e-mail: n.e.raine@qmul.ac.uk

and Real 1991; Smithson and Macnair 1996; Gumbert 2000). Furthermore, the speed with which bumblebees learn to visit the most rewarding colour varies significantly among individual bees, colonies (Raine et al. 2006b; Raine and Chittka 2008) and possibly among populations (Chittka et al. 2004; Raine et al. 2006a). Previous studies have shown that geographically isolated populations (particularly island populations) of *Bombus terrestris* differ in a number of behavioural and sensory traits (Raine et al. 2006a; Skorupski et al. 2007). We therefore examine the strength and persistence of colour preferences, and the learning performance of two geographically isolated populations of *B. terrestris* (Coppée et al. 2008; Rasmont et al. 2008), known to differ in their foraging performance (Ings et al. 2006)—*Bombus terrestris dalmatinus* (Dalla Torre) from south-eastern Europe and *Bombus terrestris audax* (Harris) from the UK. We focus specifically on the comparison between commercially bred *B. terrestris dalmatinus* and wild *B. terrestris audax* because these populations have recently been brought into sympatry through the import of *B. terrestris dalmatinus* for commercial pollination in the UK; they might therefore compete for similar floral resources and potentially interbreed (Ings et al. 2005; Ings et al. 2006; Velthuis and van Doorn 2006).

Materials and methods

Fifteen colonies of British *B. terrestris audax* were reared from nest searching queens collected in and around Greater London during the spring of 2004. Collected queens were kept in a dark room (at 25–28°C and 60% relative humidity) inside bipartite wooden nest boxes (28×16×11 cm), with male pupae added to encourage them to initiate a colony (for further details see Raine et al. 2006b). A total of 21 colonies of *B. terrestris dalmatinus* were obtained from Koppert Biological Systems (Berkel en Rodenrijs, The Netherlands), nine in 2004 and 12 in 2005. These commercial stocks have been raised over multiple generations from queens caught in Greece and Turkey (Velthuis and van Doorn 2006). Rearing protocols for commercial bees remain confidential, but to the best of our knowledge are believed to be very similar to those we used for *B. terrestris audax*. The learning performance of *B. terrestris audax* was assessed during the summer of 2004 (17 June to 3 Sept) and that of *B. terrestris dalmatinus* during the winter of 2004/5 (29 Sept to 9 Feb) and the summer of 2005 (4 to 25 July).

Data collection

Individual colour preference and learning performance of foraging bees from each colony were measured using an

ecologically relevant associative learning task in which bees had to learn to use floral colour as a predictor of reward (Raine and Chittka 2008). The bees are presented with an operant, rather than classical, conditioning paradigm as such trial-and-error learning is more ecologically relevant here when considering how bees use colour cues to choose which flowers to visit when foraging. We presented bees with a foraging environment containing two different coloured flower “species” in a flight arena (120×100×35 cm) covered with a transparent Plexiglas® lid. Here, bees had to learn to associate yellow (bee green in bee colour space) artificial flowers with rewards (a 15- μ l droplet of 50% (w/w) sucrose solution) and to ignore blue (bee blue) flowers (unrewarding). Bees choosing a rewarding (yellow) flower were judged to be making a correct choice, whilst choosing an unrewarding (blue) flower was deemed to be an error. Test flower colours were selected so that bees had to overcome their unlearned preference for blue, before associating one of their innately less favoured colours (yellow) with reward (Chittka et al. 2004). This is a simple associative learning task that bees are able to learn, but the task is sufficiently difficult to reveal variation in learning performance between individuals and colonies (Raine et al. 2006b).

Naïve foragers (i.e. bees that had never encountered real or artificial flowers) were initially pre-trained to forage in the flight arena on 20 rewarded bicoloured flowers (24×24 mm—half blue (Perspex® Blue 727)/half yellow (Perspex® Yellow 260), with a 15- μ l droplet of 50% (w/w) sucrose solution placed in the centre). Pre-training allows bees to become used to visiting artificial flowers to collect nectar and it enables observers to determine which individuals are motivated foragers suitable for further training. The use of bicoloured flowers ensured that bees had an equal chance of associating both colours (blue and yellow) with rewards. Bees were allowed to forage on the pre-training flowers for at least five (typically 5–10) foraging bouts (i.e. they filled their honey crops and returned to the nest at least five times). Flower rewards were replenished shortly after they had been consumed and the bee had left the flower.

Once a nectar forager had been identified (by completing at least five consecutive foraging bouts) it was then subjected to the training phase. During training, only one bee at a time was ever allowed into the foraging arena where it was presented with 10 yellow, rewarding (containing a 15- μ l droplet of 50% (w/w) sucrose solution) and 10 blue, unrewarding (empty) flowers (24×24 mm), which were distributed at random positions in the arena (Raine et al. 2006b). Flowers of both colours each had a recessed well (depth=2 mm; diameter=4 mm) in the middle of their upper surface. Sucrose solution rewards were pipetted into the wells of yellow flowers. Nearest neighbour flowers in

the arena were 5–15 cm apart, and the maximum distance between any two flowers was 150 cm. These are typical distances between flowers that a bee would encounter in a mixed species flower patch under natural conditions. Bees were regarded as choosing a flower when they *approached* to within 2 cm of a flower with their head and antennae oriented towards the flower, or *landed* on a flower. We deemed landing to indicate that a bee had sampled a flower because it could detect the presence/absence of a sucrose reward by extending its proboscis to feed from the well of the flower (probing) or via chemosensors in its feet (Marshall 1935; de Brito Sanchez et al. 2008). All flower choices made by a bee were recorded from when it first entered the arena, until it made at least 100 choices starting with the first rewarded learning trial (i.e. the first time it sampled and consumed the sucrose reward on a yellow flower). Training spanned several foraging bouts, and between bouts, artificial flowers were replaced with clean ones (placed in re-randomized positions) to eliminate the potential effects of scent marking and spatial memory on task performance. Controlled illumination for all experiments was provided by high frequency fluorescent lighting (TMS 24F lamps with HF-B 236 TLD ballasts, Philips, The Netherlands, fitted with Activa daylight fluorescent tubes, Osram, Germany) which flicker at ca. 42 KHz—well above the flicker fusion frequency of bees (about 200 Hz, Srinivasan and Lehrer 1984).

Innate colour preference: strength and persistence

To differentiate colour preferences and learning, we subdivided the choices made during training into two consecutive stages. Stage 1 consisted of all the choices a bee made before its first rewarded learning trial (i.e. the first time a bee probed a rewarding flower). Stage 2, began with the first rewarded learning trial, and consisted of 100 flower choices. The initial strength of colour preference (for blue over yellow) was measured for all individual bees by calculating the proportion of blue flowers they *chose* (approached or landed on) during stage 1. This proportion represents a measure of their innate “blue preference” (over yellow) because bees had not yet received any positive reinforcement from yellow flowers during stage 1. This strength of preference was used as the starting level for the fitted learning curves (see below). To check that the strength of initial preference did not change appreciably during stage 1, we compared the proportion of blue flowers chosen during the first and last ten choices for all bees making at least 20 unrewarded flower choices before the first reinforced learning trial (138 individuals of *B. terrestris audax* and 115 of *B. terrestris dalmatinus*). We found that the proportion of blue flowers chosen changed very little, falling by only $4.0 \pm 1.3\%$ in *B. terrestris audax*

and $1.0 \pm 1.9\%$ in *B. terrestris dalmatinus*. Persistence of blue preference was taken as the total number of landings on blue flowers during stage 1.

Learning performance

The learning performance of 521 bees (8–15 per colony; Table 1) from 15 *B. terrestris audax* (n foragers = 213) and 21 *B. terrestris dalmatinus* (n foragers = 308) colonies across both stage 1 and 2 was assessed using several complimentary approaches:

1. Trial-based learning: landing behaviours

Firstly, we examined overall task performance (i.e. bees’ ability to associate yellow flowers with sucrose rewards) using a criterion-based approach. We calculated the number of learning trials required for bees to make 10 consecutive correct choices. We regarded every time a bee landed on either a non-rewarding blue, or rewarding yellow, flower to be a learning trial.

To investigate the learning dynamics from the first reinforced learning trial onwards (stage 2), we compared the number of positive and negative learning trials as a function of all flower choices divided into 10 consecutive bins of 10 choices (all 100 approaches and landing behaviours). For this analysis, landing on yellow flowers was a positive learning trial, while landing on blue flowers was a negative trial.

2. Effects of approach flights on learning

As all bees approached more flowers than they landed on, we also investigated changes in approaches to blue and yellow flowers after the first rewarding learning trial (stage 2). The number of approach flights were analysed as a function of all flower choices divided into 10 consecutive bins of 10 choices (all 100 approaches and landing behaviours).

3. Effect of all choices on learning

To examine the role of changes in the number of approaches and landings in the learning process, we considered both of these behaviours simultaneously. In this analysis, both approaches to, and landing on, yellow flowers were considered to be correct choices. Individual learning curves were fitted to the proportion of errors each bee made every 10 flower choices from the first rewarding trial (i.e. during stage 2 of training). The innate blue preference (see above) of individual bees was used as the starting point of the learning curves for bees that made five or more choices prior to the first yellow probe ($n = 423$; 81%). However, for bees that made fewer than five choices during stage 1 ($n = 98$; 19%), the mean innate blue preference for their colony was used as the starting point of

Table 1 Summary of flower choices made during stage 1 (prior to the first rewarded learning trial) and stage 2 (from the first rewarded learning trial onwards) of the training phase

Population	Colony	N	Stage 1				Stage 2			
			Approaches		Landings		Approaches		Landings	
			Blue	Yellow	Blue	Yellow	Blue	Yellow	Blue	Yellow
<i>B. terrestris audax</i>	A113	15	36.6±6.5	2.4±1.1	3.5±1.2	0.1±0.1	19.8±2.7	26.8±1.6	0.1±0.1	53.3±2.4
	A126	8	8.1±2.5	5.9±2.1	2.1±1.5	0.5±0.2	12.9±2.1	42.5±4.1	0.6±0.4	44.0±4.6
	A142	15	31.8±7.0	4.1±1.9	4.3±1.4	0.1±0.1	16.4±3.1	38.8±2.5	0.4±0.2	44.4±3.8
	A16	15	26.3±4.4	5.6±2.0	1.4±1.0	0.1±0.1	24.3±2.6	33.0±2.8	0.2±0.1	42.5±3.7
	A163	15	28.5±7.5	1.3±0.7	1.9±0.7	0.0±0.0	19.8±2.8	33.9±1.9	0.8±0.4	45.5±2.7
	A180	15	23.3±6.1	4.0±1.4	2.1±1.1	0.3±0.1	19.9±2.3	35.9±2.3	0.5±0.3	43.7±1.6
	A21	15	29.3±12.9	4.3±2.3	1.9±1.3	0.3±0.2	15.8±2.2	40.4±2.8	0.2±0.2	43.6±2.7
	A212	15	66.5±19.2	9.4±5.3	6.5±2.5	0.2±0.1	11.6±1.4	30.2±2.5	0.6±0.3	57.6±2.4
	A228	14	42.4±17.5	7.3±4.1	2.0±1.0	0.0±0.0	28.7±5.0	35.2±3.2	0.7±0.4	35.4±3.5
	A24	12	66.1±8.5	2.0±0.5	15.3±4.0	0.0±0.0	19.0±4.5	24.1±2.9	2.8±1.0	54.1±3.5
	A33	15	26.9±4.8	3.1±1.1	1.9±0.7	0.1±0.1	15.5±2.7	30.0±2.1	0.3±0.2	54.2±2.5
	A42	15	36.5±9.9	1.3±0.4	4.9±2.5	0.1±0.1	21.5±1.8	28.1±1.8	0.9±0.4	49.5±1.9
	A62	15	26.6±4.8	5.6±1.6	3.9±1.2	0.1±0.1	23.8±2.5	44.4±2.9	3.5±0.8	28.3±2.4
	A65	15	48.3±9.4	2.3±1.0	10.7±3.2	0.1±0.1	16.0±2.3	34.5±2.4	1.5±0.6	48.0±2.6
	A99	14	26.1±5.7	0.9±0.3	6.2±1.6	0.1±0.1	16.2±2.4	33.4±3.3	2.4±0.9	48.0±3.6
	<i>B. terrestris dalmatinus</i>	D1	15	15.7±3.1	11.5±5.1	0.3±0.1	0.5±0.3	21.3±2.9	48.3±2.4	0.1±0.1
D10		15	15.2±4.4	8.8±2.7	0.6±0.2	0.5±0.2	21.9±2.4	46.3±2.7	0.9±0.7	30.9±2.7
D11		14	15.8±5.2	5.7±1.8	1.0±0.6	0.1±0.1	23.3±2.5	40.7±2.3	1.1±0.4	34.9±1.4
D12		15	10.7±2.3	4.8±1.3	0.5±0.4	0.3±0.2	20.2±2.4	35.9±2.6	0.6±0.2	43.3±3.4
D13		15	8.7±3.0	6.1±3.3	0.3±0.3	0.0±0.0	13.1±1.9	40.2±1.7	0.5±0.4	46.1±2.1
D14		14	12.9±3.5	1.1±0.4	1.4±0.7	0.5±0.2	28.6±3.5	37.1±1.9	0.6±0.4	33.6±2.7
D15		14	14.9±4.8	5.9±1.6	0.3±0.2	0.6±0.3	24.0±3.9	45.4±1.9	0.3±0.1	30.4±3.4
D16		12	5.6±2.9	3.8±1.6	0.0±0.0	0.2±0.1	23.0±3.3	38.8±3.1	0.8±0.4	37.4±3.0
D17		15	22.9±4.8	5.7±1.8	0.9±0.6	0.3±0.1	31.6±3.6	38.2±2.2	0.3±0.3	29.9±2.8
D18		15	43.1±20.6	6.8±3.5	2.4±1.5	0.1±0.1	27.0±1.4	39.2±2.0	1.0±0.4	32.8±2.3
D19		15	11.5±3.5	1.3±0.6	0.3±0.2	0.0±0.0	18.3±1.8	47.1±2.2	0.6±0.2	34.1±2.0
D2		15	12.8±3.5	14.9±3.8	0.9±0.7	0.1±0.1	14.2±2.5	55.1±3.2	0.4±0.2	30.3±2.4
D20		15	10.5±3.2	1.7±0.9	0.1±0.1	0.0±0.0	34.1±1.9	34.7±2.5	0.7±0.4	30.5±1.8
D21		15	15.0±4.0	1.9±0.7	0.5±0.3	0.6±0.3	27.9±3.1	42.9±2.0	0.0±0.0	29.2±3.1
D22		14	10.5±2.8	4.2±2.2	0.2±0.2	0.1±0.1	16.9±2.4	45.7±1.7	0.1±0.1	37.2±3.1
D3		15	18.7±3.1	18.7±4.1	0.1±0.1	0.1±0.1	9.5±1.2	60.6±2.1	0.0±0.0	29.9±2.0
D4	15	5.2±1.2	5.0±2.2	0.2±0.1	0.1±0.1	9.0±1.6	53.5±2.9	0.1±0.1	37.4±2.8	
D5	15	12.5±3.7	15.4±4.0	0.3±0.3	0.5±0.2	15.1±2.6	54.7±1.7	0.8±0.4	29.4±2.6	
D6	15	13.2±3.3	9.4±4.5	0.0±0.0	0.3±0.2	25.9±2.6	48.7±2.2	0.1±0.1	25.3±2.2	
D8	15	17.5±4.3	9.4±3.3	0.9±0.7	0.2±0.1	20.7±2.8	47.8±2.4	0.1±0.1	31.4±2.2	
D9	15	8.1±2.0	3.7±1.5	0.5±0.2	0.7±0.3	19.5±1.8	44.3±2.3	0.2±0.1	36.1±2.5	
<i>B. terrestris audax</i>			35.3±2.7	3.9±0.6	4.5±0.5	0.1±0.0	18.9±0.8	33.9±0.7	1.0±0.1	46.1±0.9
<i>B. terrestris dalmatinus</i>			14.4±1.3	7.0±0.7	0.6±0.1	0.3±0.0	21.1±0.7	45.1±0.6	0.4±0.1	33.3±0.6

Colony means (\pm SE) are given for the number of blue or yellow flowers approached and landed on. Population means are given in italics at the end of the table. Colony order follows that of the figures

their individual learning curve. This was necessary because stochastic processes reduce the accuracy with which assessment of individual colour preferences can be made when a bee visited fewer than five flowers. These bees were

assigned a colony, rather than population, mean value for strength of preference as previous studies have shown this can vary significantly among colonies within the same population (Raine and Chittka 2007). Analyses were

repeated omitting individuals for which mean values for strength of preference were assigned to ensure this procedure did not introduce artefacts (see details below). Learning curves were fitted to the 11 points (starting level and 10 choice bins) for all individual bees, using a first order exponential decay function ($y = y_0 + Ae^{-x/t}$) in Microcal Origin® (Raine et al. 2006b). Overall differences between the learning performance of *B. terrestris dalmatinus* and *B. terrestris audax* were assessed by comparing two curve derived parameters. First, the slope coefficient t , is inversely proportional to learning speed, i.e. fast learning bees have a low t value. Second, the y asymptote, y_0 , denotes the saturation performance level of learning in this task.

Statistical analysis

All colour preference measures for stage 1 were analysed using nested ANOVAs. The dynamics of learning during stage 2 were analysed using both repeated measures ANOVA for numbers of lands and approaches (based on colony average performance during each consecutive bin of 10 choices), and nested ANOVA for individual learning curve parameters (the population–colony interaction was used as the error term for the population effect). Strength of blue preference data were arcsine-square root transformed to normalize residuals, and persistence of blue preference and learning speed (t) were natural log transformed. The Greenhouse–Geisser correction was used for within-subject effects where the assumption of sphericity was not met. The relationship between the initial strength and persistence of blue preference over time was examined by calculating the Spearman rank correlation for each population, because at least one variable in each population had a non-normal distribution that could not be corrected by transformation. All tests including the strength of blue preference were run excluding bees that made fewer than five choices during stage 1, i.e. before probing their first rewarding (yellow) flower, because the strength of their colour preference had to be estimated from the colony average preference.

Twelve individual bees showed no appreciable learning during the task, and the software generated “learning curves” that were essentially horizontal lines for these individuals. The t values of these bees were either very high (>400) or negative and could thus have a strong influence on colony means. Therefore, bees with t values above 400 (1 of 213 *B. terrestris audax* and 5 of 308 *B. terrestris dalmatinus*), or negative values (one *B. terrestris audax* and five *B. terrestris dalmatinus*) were excluded from analyses. However, this had no effect on the significance of any tests. In three *B. terrestris dalmatinus* colonies, the starting level (strength of blue preference) for learning curves had to be estimated for a large proportion of bees ($n = 7/15$, $9/15$ and $8/12$). Therefore, analyses involving learning curve derived

parameters were run both including and excluding these three colonies. However, removal of these colonies had no impact on the observed differences, so only results from analyses including all colonies are presented here.

Results

In total, we trained 570 bees (241 *B. terrestris audax* from 15 colonies; 329 *B. terrestris dalmatinus* from 21 colonies), of which 28 *B. terrestris audax* and 21 *B. terrestris dalmatinus* did not complete the learning task (19 *B. terrestris audax* and 5 *B. terrestris dalmatinus* never probed a yellow flower, and 9 *B. terrestris audax* and 16 *B. terrestris dalmatinus* ceased foraging before completing the task: i.e. did not make 100 flower choices including the first time they probed a yellow flower). Learning performance (number of trials to reach criterion, and learning speed) was assessed for all 521 bees that completed the learning task (213 *B. terrestris audax* and 308 *B. terrestris dalmatinus*).

Initial strength of blue preference

Most bees tested (i.e. 85% of those making ≥ 5 pre-yellow probe choices: $n = 423$) chose more blue flowers than yellow before probing their first rewarding yellow flower. However, the initial strength of blue preference was significantly ($F_{1,34} = 26.35$, $P < 0.0001$) weaker in *B. terrestris dalmatinus* (mean \pm SE, $69.8 \pm 1.6\%$) than in *B. terrestris audax* ($88.6 \pm 1.1\%$), despite significant variation among colonies within populations (colony–population interaction— $F_{34,387} = 3.91$, $P < 0.0001$; Fig. 1).

Persistence of blue preference over time

Considerable variation in the number of choices made before probing a rewarding flower (i.e. during stage 1) was seen among individual bees within many colonies, irrespective of population (Fig. 2). For example, in one *B. terrestris audax* colony (A212), one bee made 373 flower choices before probing the first yellow flower, whilst another probed a yellow as its first flower choice. On average, *B. terrestris audax* inspected and sampled twice as many flowers (mean \pm SE, 43.90 ± 3.25) as *B. terrestris dalmatinus* (22.3 ± 1.76) during stage 1 ($F_{1,34} = 29.19$, $P < 0.0001$; Fig. 2). The persistence of the blue preference, as measured by the number of landings on blue flowers, was also variable among colonies within populations (colony–population interaction— $F_{34,485} = 2.92$, $P < 0.0001$) but differed significantly between populations ($F_{1,34} = 46.27$, $P < 0.0001$). On average, *B. terrestris audax* landed on a larger number of blue flowers (mean \pm SE, 4.49 ± 0.53) than *B. terrestris dalmatinus* (0.57 ± 0.11) before probing a

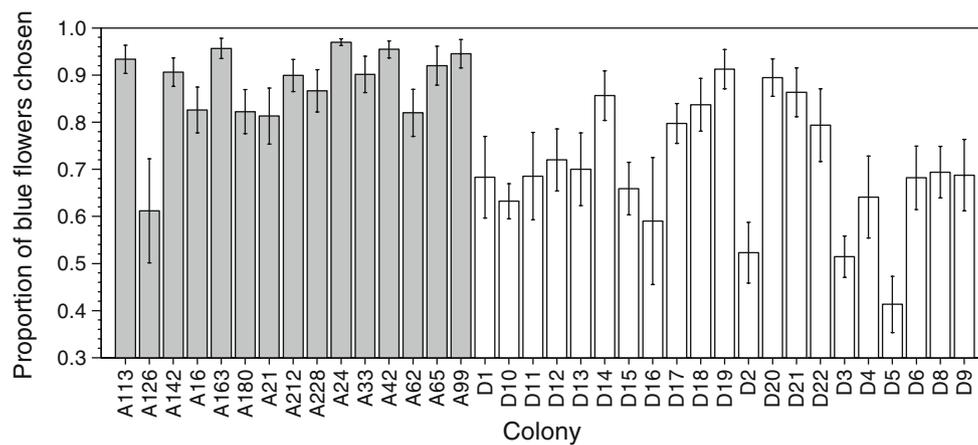


Fig. 1 The mean (\pm SE) strength of blue preference for 15 colonies of *B. terrestris audax* (grey bars) and 21 colonies of *B. terrestris dalmatinus* (white bars). Blue preference is the mean proportion of

blue flowers chosen during bins of 10 flower choices before the first rewarding yellow flower was probed. Colonies are ordered alphanumerically and the same order is used in all figures

rewarding flower for the first time. Furthermore, a greater proportion of *B. terrestris audax* foragers (7.9%) failed to probe a yellow flower compared to *B. terrestris dalmatinus* (1.5%).

Relationship between strength and persistence of blue preference

Strong blue preferences were more persistent (as measured by the number of landings on blue flowers in stage 1) than weak preferences in both populations (*B. terrestris audax*— $r_s = 0.209$, $n = 193$, $P = 0.004$; *B. terrestris dalmatinus*— $r_s = 0.306$, $n = 230$, $P < 0.001$). Similarly, bees with strong blue preferences also approached more blue flowers than bees with weak preferences (*B. terrestris audax*— $r_s = 0.570$, $n = 193$, $P < 0.001$; *B. terrestris dalmatinus*— $r_s = 0.369$, $n = 230$, $P < 0.001$). However, for *B. terrestris dalmatinus*, bees with stronger blue preferences tended to make fewer choices overall (both approaches and landings) during stage 1 prior to feeding from a yellow flower ($r_s = -0.274$, $n = 230$, $P < 0.001$). No such pattern was evident for *B. terrestris audax*.

Learning performance

1. Trial-based learning: landing behaviours

Considering each landing behaviour as a learning trial (where landing on yellow is positive and blue, negative reinforcement) across the entire task, we found that the British population, *B. terrestris audax*, required a significantly ($F_{1,34} = 25.86$, $P < 0.0001$) greater number of learning trials (16.49 ± 0.70) than the Mediterranean population, *B. terrestris dalmatinus* (11.38 ± 0.19), before reaching the criterion of 10 consecutive yellow landings. Although most

(85%) bees tested started out with a preference for blue, the majority (72.8%) never landed on a blue flower again (Table 1; Fig. 3a,b) after feeding from the first yellow flower (stage 2, *B. terrestris audax*— $n = 144/213$; *B. terrestris dalmatinus*— $n = 234/308$). On average, the remaining (27.2%) bees landed on fewer than three (2.52 ± 0.19) blue flowers (*B. terrestris audax* = 3.19 ± 0.31 ; *B. terrestris dalmatinus* = 1.89 ± 0.89 ; Fig. 3a,b). Hence, when looking at flower landings only, the majority of bees effectively exhibited one-trial learning: i.e. they required only one rewarding exposure to associate yellow with reward.

To investigate the learning dynamics from the first reinforced learning trial onwards (stage 2) in more detail, we compared the number of positive learning trials (landing on yellow flowers) per bin of 10 choices (all approaches and landings). Both populations showed distinct learning during stage 2: i.e. on average, they increased the number of yellow flowers on which they landed per bin of 10 choices (Fig. 3a,b). However, the dynamics of this improvement differed between populations: the number of yellow landings increased more rapidly with experience for *B. terrestris audax* than *B. terrestris dalmatinus* (population-bin interaction— $F_{8,3,4283.3} = 8.86$, $P < 0.001$; Fig. 3a,b).

2. Effects of approach flights on learning

When looking exclusively at flower landings (the most “determinate” of flower choices), there is risk of overlooking a much more gradual learning process. The learning procedure requires both the association of yellow with reward, and the suppression of the innate attraction to blue flowers. While the number of landings on blue flowers dropped rapidly after the first rewarding learning trial (Fig. 3a,b), the frequency of approach flights to blue flowers declined much more slowly during stage 2

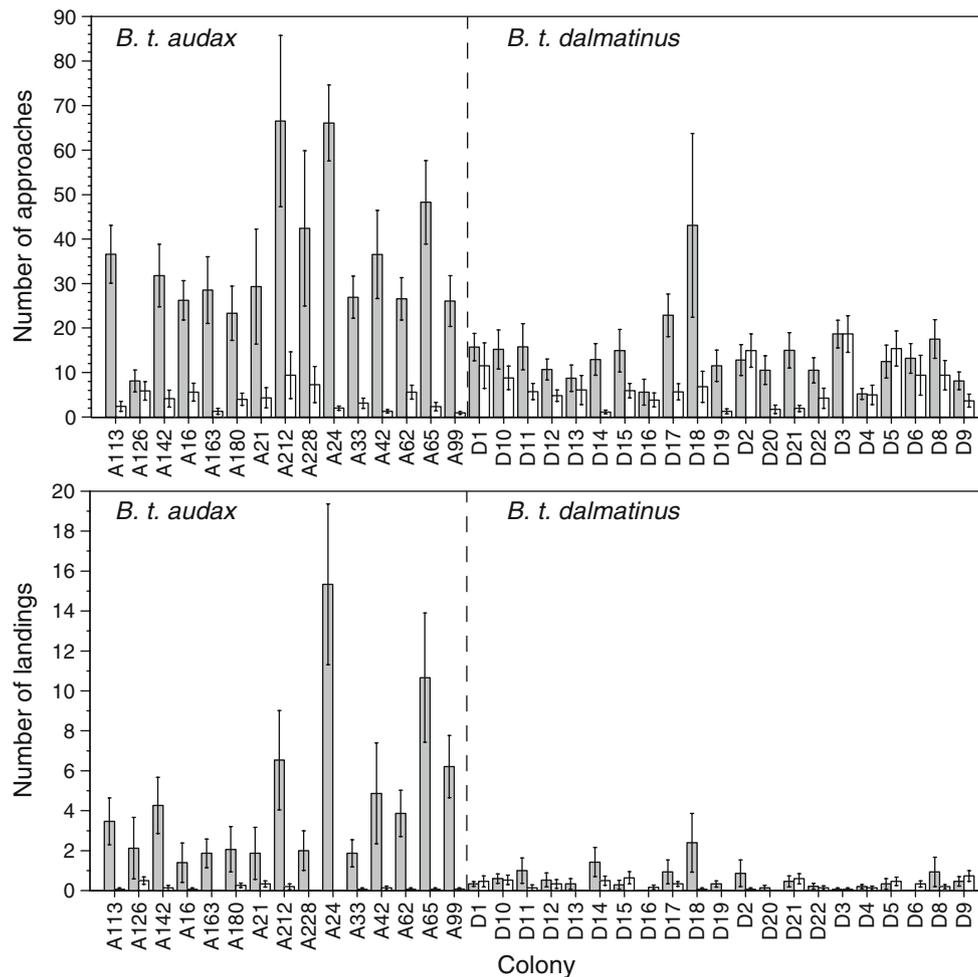


Fig. 2 Mean (\pm SE) number of flower approaches (*top panel*) and landings (*bottom panel*) made by bees prior to their first positive learning trial (i.e. during stage 1). Blue flowers are shown by grey

bars and yellow flowers are shown by white bars. In each panel, data from each population are segregated by a dashed vertical line

(Fig. 3c,d). Both populations showed a strong reduction in the number of blue flowers approached, but the rate of change was greater for *B. terrestris audax* (population–bin interaction— $F_{7.2,4009.9} = 7.02$, $P < 0.001$). The number of yellow flowers approached during stage 2 showed a slight increase in both populations (bin— $F_{8.8,4640.6} = 55.31$, $P < 0.001$), but overall, *B. terrestris dalmatinus* approached significantly more yellow flowers than *B. terrestris audax* (population— $F_{1,519} = 131.32$, $P < 0.001$). These changes in behaviour, towards both rewarding and unrewarding flowers, clearly show that an approach flight represents a choice for a particular flower (even if the approach does not lead to the bee landing on that flower), and is therefore indicative of the bee’s preference (innate at first, then learnt).

3. Effect of all choices on learning

As learning is demonstrated by changes in both the number of landings (Fig. 3a,b) and approaches (Fig. 3c,d),

both of these behavioural categories need to be considered simultaneously. Hence, for this analysis, both approaching and landing on yellow flowers were considered correct choices. Learning speed (t) varied significantly among colonies *within* populations (Fig. 4a; colony–population interaction— $F_{34,473} = 1.70$, $P = 0.010$). However, there was no significant difference in learning speed *between* populations ($F_{1,34} = 0.86$, $P = 0.361$), although *B. terrestris dalmatinus* were on average slightly slower learners (mean \pm SE, $t = 28.9 \pm 2.0$) than *B. terrestris audax* ($t = 23.34 \pm 1.7$). Both populations achieved a high level of accuracy by the end of stage 2 (Fig. 4b) and were close to the saturation levels of learning (y_0). These predicted saturation levels of learning did not differ significantly ($F_{1,34} = 2.31$, $P = 0.1379$), with both populations making less than 1 error (by approaching or landing on a blue flower) in every 10 flower choices (y_0 —*B. terrestris audax* = 0.76 ± 0.07 ; *B. terrestris dalmatinus* = 0.98 ± 0.08).

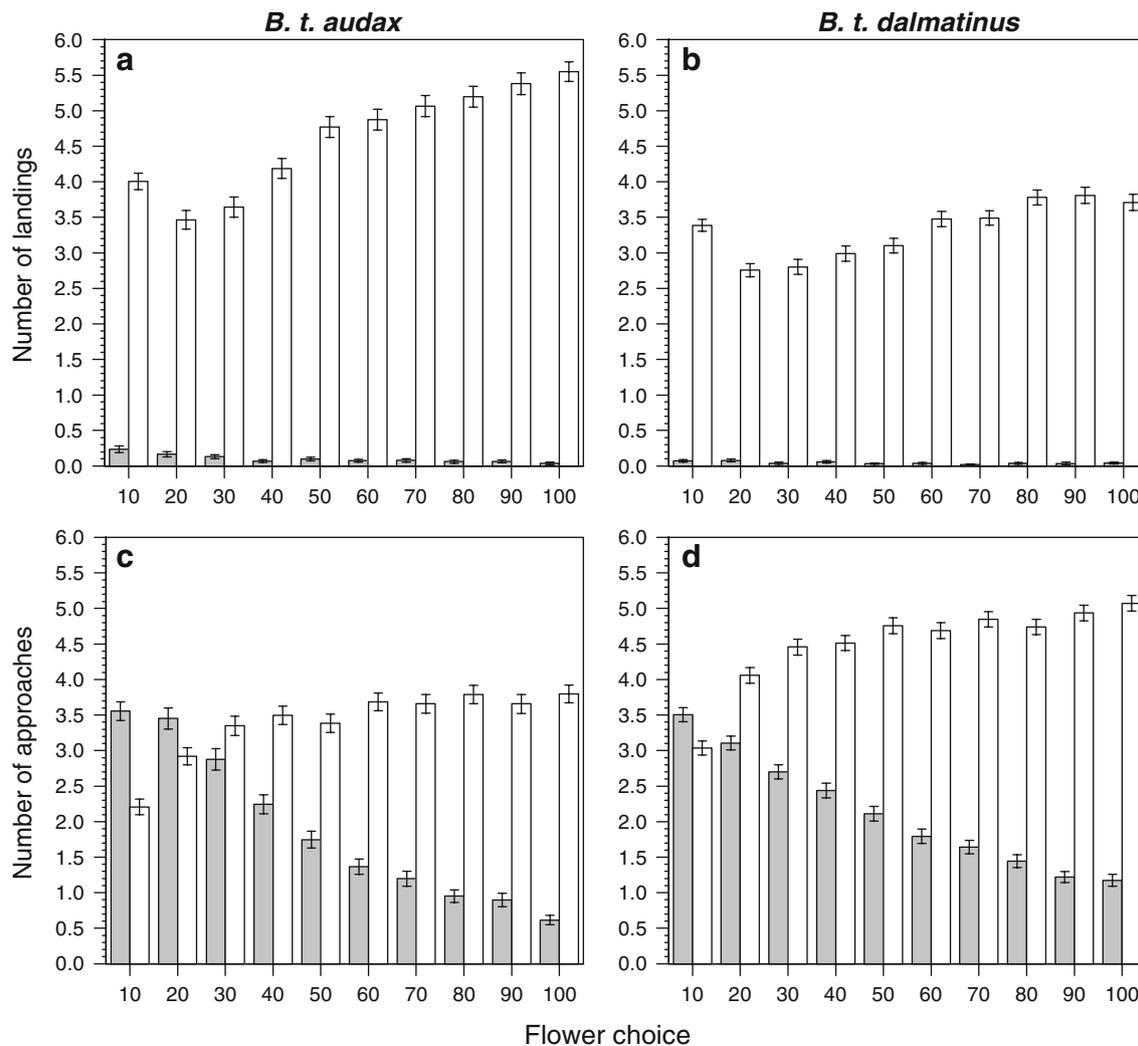


Fig. 3 Summary of all the flower choices made by *B. terrestris audax* (left panels—**a**, **c**) and *B. terrestris dalmatinus* (right panels—**b**, **d**) during stage 2 of training (i.e. during the 100 flower choices starting with the first rewarded learning trial). Choices are broken down into

the mean (\pm SE) number of blue (grey bars) and yellow (white bars) landings (top panels—**a**, **b**) and approaches (bottom panels—**c**, **d**) made during consecutive bins of 10 flower choices

Influence of blue preference on learning

Initial blue preference had a strong influence on overall learning performance (learning curves based on analysis including both approaches and landing on yellow flowers as correct choices) in both populations (Fig. 4b). The average learning curve for *B. terrestris audax* started from a much stronger initial blue preference which also persisted for a considerably greater number of flower choices compared to the curve for *B. terrestris dalmatinus*. On average, *B. terrestris dalmatinus* made fewer errors, i.e. they chose rewarding yellow flowers more frequently, than *B. terrestris audax* until 100 flower choices had been made, at which point the population average learning curves cross. After this point, both population learning curves have

almost reached saturation performance (y_0), with *B. terrestris dalmatinus* making a greater number of errors.

Discussion

We demonstrate that the strength and persistence of colour preference in *B. terrestris* varies among individuals, colonies and populations. The British population, *B. terrestris audax*, had a significantly stronger blue preference than the south-eastern European population, *B. terrestris dalmatinus* (Fig. 1). Furthermore, this blue preference was much more persistent in *B. terrestris audax*, even though blue flowers were completely unrewarding (Fig. 2). These differences in colour preference influenced

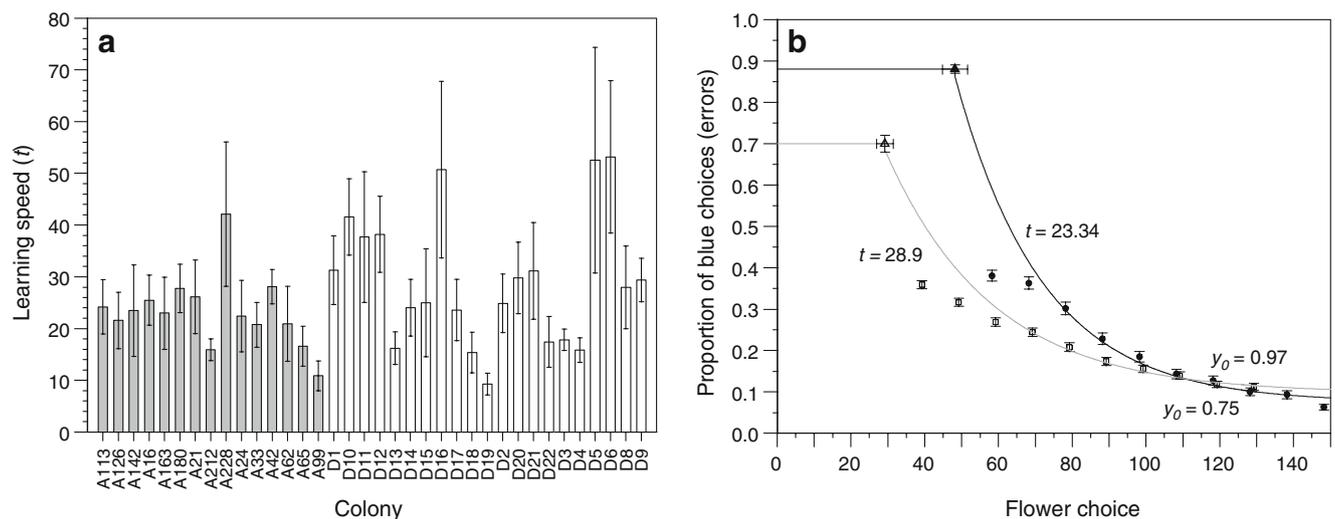


Fig. 4 Summary of learning performance for which all approaches to, and landings on, yellow flowers were deemed correct choices. **a** Mean (\pm SE) learning speed of 15 *B. terrestris audax* colonies (grey bars) and 21 *B. terrestris dalmatinus* colonies (white bars). Learning speed (t) is the decay constant from the learning curves with low t values indicating fast learning bees. **b** Average population learning curves fitted to mean proportions of errors made by bees within each population. The average curve of *B. terrestris audax* is shown by the

black line and the curve for *B. terrestris dalmatinus* is shown by the grey line. Filled circles are the mean (\pm SE) number of errors per 10 choices for *B. terrestris audax* and open squares are mean errors for *B. terrestris dalmatinus*. For each population, the first data point is indicated by a triangle that represents the mean strength of initial blue preference on the y axis, and the mean number of choices made before probing the first yellow, rewarding flower on the x axis

the overall shape of the population learning curves such that *B. terrestris audax* made a greater number of errors (i.e. chose unrewarding blue flowers more frequently) than *B. terrestris dalmatinus* during the learning process. However, the average performance of *B. terrestris audax* in this learning task was equal to that of *B. terrestris dalmatinus* after 100 flower choices (Fig. 4b).

The observation that *B. terrestris audax* had a significantly stronger innate blue preference than *B. terrestris dalmatinus* is surprising given the relatively consistent strength of blue preference seen in all other *B. terrestris* populations (including *B. terrestris dalmatinus*) tested to date (Chittka et al. 2004; Raine et al. 2006a). Our paradigm involved a choice between just two colours compared to six in previous studies (violet (bee UV-blue), blue (bee blue), white (bee blue-green), yellow, orange and red (all bee green)), which might affect the relative preference for blue over yellow.

We also found that the initial blue preference of *B. terrestris audax* was more persistent in comparison to that of *B. terrestris dalmatinus*, even though no rewards were present in blue flowers. Although there were significant differences between populations in both strength and persistence of blue preference, the same positive correlation between strength and persistence of blue preference (over yellow) exists within both populations. This means that an individual (*B. terrestris audax* or *B. terrestris dalmatinus*) bee with a stronger blue preference landed on, and thus sampled, a greater number of blue flowers before landing

on and feeding from a yellow flower (than individuals with weaker initial blue preferences). Furthermore, bees from both populations with stronger blue preferences approached more blue flowers suggesting that approach flights (which give bees no direct feedback on the reward status of a flower) are important in the learning process. We also found that in *B. terrestris dalmatinus* (but not in *B. terrestris audax*), bees with the strongest preference for blue make the fewest overall flower choices during stage 1. Since these bees (with stronger blue preferences) inspect a greater number of blue flowers, we can conclude that they must approach fewer yellow flowers.

Intuitively, this observation for *B. terrestris dalmatinus* seems at odds with what we might expect, i.e. that bees with a strong blue preference would make more flower choices overall before probing a yellow flower. However, in both populations, bees with a strong preference for blue landed on a higher proportion of the blue flowers they approached, thus giving them the opportunity to learn that they contained no rewards. Bees with a strong blue preference are therefore likely to receive greater negative reinforcement from blue flowers, potentially leading them to sample yellow after fewer flower choices (Rescorla and Wagner 1972). The fact that this only occurred in *B. terrestris dalmatinus* potentially indicates a population level difference in bees' sensitivity to negative feedback from non-rewarding, but preferred colours.

Although we see strong variation in the number of flower choices (either all choices or only landings) made

before their first rewarding learning trial (during stage 1), almost all subsequent landings (during stage 2) were on yellow flowers (Fig. 3a,b): i.e. bees effectively exhibit one-trial learning if approaches are not taken into account. However, while bees receive direct positive feedback from imbibing sucrose rewards when they sample a yellow flower, it also seems that they are learning something about the flowers by performing inspection flights, and that this behaviour changes as learning progresses (Fig. 3c,d). Whilst bees could be visually assessing the presence (or absence) of a droplet of odourless sucrose solution, this is unlikely as rewards (presented in a recessed well in the upper surface of the flower) were concealed from the bee's line of sight as it approached from the side, typically level with (or just below) the level of the flower. Approach flights to yellow could also serve to familiarise the bee with these flowers, as a kind of preparation for landing: for example by assessing the safety of the flower by scanning for potential predators (Gonçalves-Souza et al. 2008; Ings and Chittka 2008; Yokoi and Fujisaki 2009). The frequency of inspection flights is informative as the relative frequencies of approaches to yellow and blue flowers change over time with increasing individual experience of the task. Notably, shortly after a bee probes a yellow flower for the first time, the proportion of all approaches made to blue starts to fall (Fig. 3c,d).

These results differ somewhat from those of earlier studies, of both pattern learning in honeybees (Giurfa et al. 1999) and scent learning in bumblebees (Molet et al. 2009), in which choices were assessed using both approach flights and touches/landings on targets. In unrewarded extinction trials, Giurfa et al. (1999) found that honeybees touched the trained pattern with their antennae significantly more often than the alternative, but that approach flights were uninformative indicators of choice. Similarly, observations of bumblebees discriminating between visually identical (unrewarded) artificial flowers based on scent cues, showed that approach flights were a much less informative choice measure than landings (Molet et al. 2009). In both previous studies, the frequency of approach flights to the trained target did not differ significantly from chance performance. In contrast, our results showed the frequency of both inspection (approach) flights and landings on each flower colour changed over time with experience of the task, suggesting that both these behaviours are meaningful indicators of choice. However, choosing a flower is a hierarchical procedure and landing should be interpreted as a stronger choice indicator than an inspection flight (which did not progress into a landing).

Results of comparisons of learning performance between populations depend to some extent on which behaviours are considered a 'correct' choice. If we consider only positive and negative learning trials as important, i.e. landings on

yellow and blue flowers respectively, we see that *B. terrestris dalmatinus* reached the performance criterion after significantly fewer learning trials than *B. terrestris audax* (mean \pm SE = 11.38 ± 0.19 vs. 16.49 ± 0.70). However, focusing on the learning dynamics after the first rewarding trial (during stage 2), we see a somewhat different picture as task performance changes more rapidly in *B. terrestris audax* than *B. terrestris dalmatinus*. *B. terrestris audax* showed a more rapid increase in the number of yellow landings (Fig. 3a,b) and a more rapid decrease in number of blue flowers approached (Fig. 3c,d) than *B. terrestris dalmatinus* with experience of the task. Finally, when all choices of yellow flowers (both approaches and landings) were considered as correct, there was no significant difference in learning speed between populations, although the average learning speed of *B. terrestris audax* was slightly faster (average *t* value was lower) than for *B. terrestris dalmatinus*. On average, *B. terrestris dalmatinus* individuals are approaching a greater number of yellow flowers than *B. terrestris audax* after the first rewarding trial (*B. terrestris dalmatinus* = 45.11 ± 0.63 ; *B. terrestris audax* = 33.94 ± 0.74 ; Table 1; Fig. 3c,d). Whilst inspecting takes less time than sampling flowers, it seems that *B. terrestris dalmatinus* bees are still wasting time inspecting (but not sampling) potentially rewarding flowers towards the end of the task (stage 2). One possibility is that *B. terrestris dalmatinus* bees could be approaching flowers which they have already sampled (and emptied of rewards) more frequently than *B. terrestris audax*. Although this could indicate potential differences in spatial learning and/or memory between populations, future experiments would be needed to examine this further.

Our results provide additional evidence of significant behavioural differences among isolated populations within a single species. Comparative studies of bees typically focus on behavioural differences between species (Heinrich et al. 1977; Dukas and Real 1991; Dyer and Seeley 1991; Raine and Chittka 2005); hence, variation among populations (or subspecies) is often overlooked. Interesting behavioural differences in hygienic behaviour (Kamel et al. 2003), timing of the onset of foraging (Brillet et al. 2002), likelihood of collecting and processing pollen (Fewell and Bertram 2002) and learning performance (Lauer and Lindauer 1973; Menzel et al. 1973) have been shown among populations (or subspecies) of *Apis mellifera* (L.). Similarly, geographically isolated populations of *B. terrestris* differ in a number of sensory and behavioural traits, including ability to detect flowers (Spaethe et al. 2001; Chittka et al. 2004; Skorupski et al. 2007), foraging performance (Ings et al. 2006) and learning ability (Chittka et al. 2004). Such population (or subspecies) level behavioural differences are likely to be ecologically relevant and deserve further attention.

The results of this study raise a number of questions about the potential adaptive significance of colour preferences. In particular, it would be very interesting to quantify the ecological significance of the persistence of colour preference by correlating it with foraging success and fitness in natural habitats, and by quantifying the volatility of the floral market (i.e. how frequently the most rewarding flower species change over time) in the respective habitats to which these *B. terrestris* populations should be locally adapted. It is also possible that the behaviour of commercially reared *B. terrestris dalmatinus* is no longer representative of its natural counterparts, and that selection and/or chance processes in the breeding programme have caused the commercially available stock to diverge from their ancestors. However, even if we assume a conservative scenario in which the behaviour of the two natural populations (*B. terrestris audax* and *B. terrestris dalmatinus*) are indistinguishable, then the observation that multiple and strong differences in behaviour could be generated in only 20 years since the start of commercial bumblebee rearing (Velthuis and van Doorn 2006) is in itself interesting, and indicative of high evolvability of the traits under investigation here. Finally, because *B. terrestris dalmatinus* is now used for commercial pollination purposes in the UK (Ings et al. 2005; Ings et al. 2006; Velthuis and van Doorn 2006), the commercial stock are interacting with native *B. terrestris audax* and their relative success in the wild might be directly determined by the extent to which their floral colour preferences and learning behaviour are adaptive in the local pollination market.

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