

The innate responses of bumble bees to flower patterns: separating the nectar guide from the nectary changes bee movements and search time

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Abstract Nectar guides can enhance pollinator efficiency and plant fitness by allowing pollinators to more rapidly find and remember the location of floral nectar. We tested if a radiating nectar guide around a nectary would enhance the ability of naïve bumble bee foragers to find nectar. Most experiments that test nectar guide efficacy, specifically radiating linear guides, have used guides positioned around the center of a radially symmetric flower, where nectaries are often found. However, the flower center may be intrinsically attractive. We therefore used an off-center guide and nectary and compared “conjunct” feeders with a nectar guide surrounding the nectary to “disjunct” feeders with a nectar guide separated from the nectary. We focused on the innate response of novice bee foragers that had never previously visited such feeders. We hypothesized that a disjunct nectar guide would conflict with the visual information provided by the nectary and negatively affect foraging. Approximately, equal numbers of bumble bees (*Bombus impatiens*) found nectar on both feeder types. On disjunct feeders, however, unsuccessful foragers spent significantly more time (on average 1.6-fold longer) searching for nectar than any other forager group. Successful foragers on disjunct feeders approached these feeders from random

directions unlike successful foragers on conjunct feeders, which preferentially approached the combined nectary and nectar guide. Thus, the nectary and a surrounding nectar guide can be considered a combination of two signals that attract naïve foragers even when not in the floral center.

Keywords Nectar guides · Bee foraging · Orientation · Navigation · Pollination · Floral constancy

Introduction

Sprengel (1793) first suggested that contrasting floral patterns, such as dots or radiating lines surrounding the nectary, hereafter referred to as a “nectar guide”, assist pollinators in finding floral nectar. Multiple studies have found evidence for this hypothesis (reviewed in Dafni and Giurfa 1999). Nectar guides can benefit plant and pollinator, increasing plant fitness (Waser and Price 1983) and the processing speed and potential energetic gain of pollinators (Lunau 1991; Lunau et al. 2006; Leonard and Papaj 2011; Leonard et al. 2013).

Recent work has identified complex multi-component, visual and olfactory signaling in flower patterns (Dötterl and Jürgens 2005; Leonard et al. 2011). Even within a single modality, such as vision, attractive floral visual signals can have multiple components (Leonard et al. 2011). For example, the nectar guide often surrounds the nectary in radially symmetric flowers (Biesmeijer et al. 2005; Leonard and Papaj 2011), and both nectar guide and nectary can facilitate visual orientation. In addition, the center of a flower can be attractive to bees, particularly if the center is darker (Biesmeijer et al. 2005). This attraction has likely evolved because nectaries are usually in the center of radially symmetric (actinomorphic) flowers, a common morphology (83 and 72 % of dicot and monocot families, respectively, are actinomorphic; Neal et al. 1998).

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Can the attraction of nectary and nectar guide be tested separately from the potential attraction of the floral center? Previous experiments focusing on linear nectar guides have not investigated this question because the nectar guide and nectaries (or dots representing the nectaries) were presented together, the nectar guide surrounded the floral center, or both conditions were present (Manning 1956; Free 1970; Dinkel and Lunau 2001; Leonard and Papaj 2011; Leonard et al. 2013).

We therefore tested if a radiating nectar guide around a nectary allows foragers to find the nectary more easily, even when these elements are not near the floral center. We made an artificial flower (feeder) in which these two visual elements competed for a forager's attention. We created "disjunct" feeders in which the nectar guide was separated from the nectar and "conjunct" feeders in which the radiating lines of the nectar guide surrounded the nectary (Fig. 1), as they often do occur in nature. To eliminate the potentially attractive effect of the flower center, the nectary and nectar guide were off-center on the conjunct feeder. On the disjunct feeder, these two elements were separated and off-center.

Methods

We tested the behavior of foragers from three successive *Bombus impatiens* colonies towards a 10-cm square feeder placed horizontally on the bottom of a foraging arena. On top, we attached a printed label: a blue circle, representing the flower, on a green background. The feeder was either conjunct or disjunct (Fig. 1), and the nectar guide, based upon Leonard and Papaj (2011), consisted of four white lines (each 10×2 mm wide). The nectary was a 6-mm diameter well drilled into the plastic feeder and appeared dark against the blue flower because of shadows cast by the well wall (see Supplement). Into the nectary, we placed 1.5-M unscented analytical-grade sucrose solution, which bumble bees cannot smell (Kunze and Gumbert 2001). To eliminate social copying, we only used a naïve bee's first visit if it was made in the absence of other bees. After each visit, we cleaned the feeder to remove potential odor marks. We recorded the amount of time a naïve bee spent on the blue flower during its first floral visit and the direction from which it first crossed into the blue flower. Directly approaching the nectar guide was defined as 0° . We tested if the feeder treatment (conjunct or disjunct) influenced bee ability to find the nectary with a χ^2 test. We used circular statistics (Rayleigh's Z test and a V test for a unimodal circular distribution) to see if treatment or success influenced bee approach angle. Finally, we analyzed if treatment or success influenced time spent on the blue flower with a generalized linear model (GLM). Detailed methods are in the Supplement.

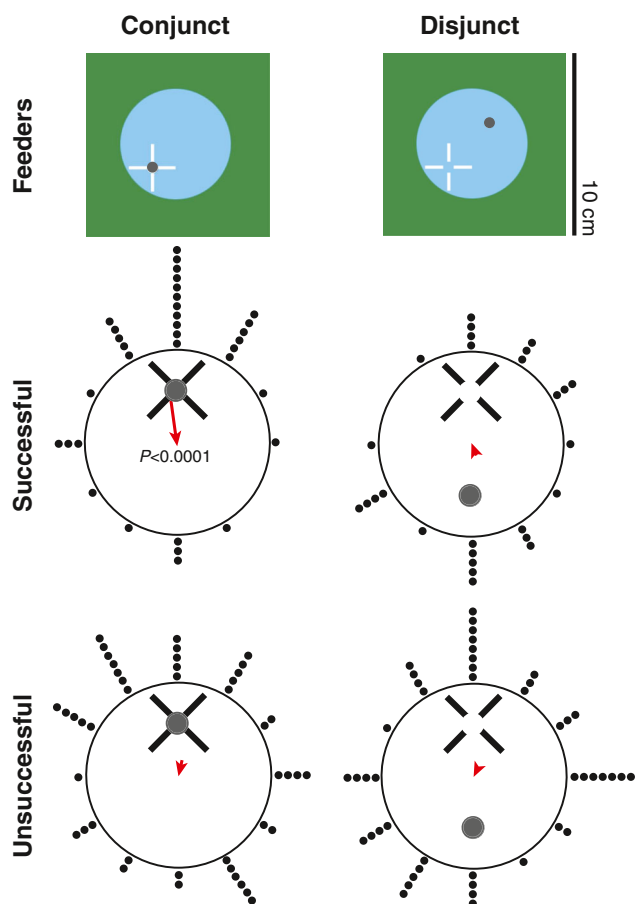


Fig. 1 The approach orientations of successful and unsuccessful bumble bee (*B. impatiens*) foragers towards conjunct and disjunct feeders. White lines on the feeders show the nectar guide, and the gray circle (see Supplement) is the nectary. Each black dot represents a different bee. The nectar guide was defined as the 0° position on conjunct and disjunct feeders. The arrows show mean vector magnitude (length relative to circle radius) and approach direction (Table S1). Only successful foragers approaching the conjunct feeders showed a distribution significantly different from random

Results

Bees took a variety of paths, but were often attracted to the nectar guide, circling and inspecting it closely (see Fig. S1). Most bees walked onto the feeder like bumble bees conducting walking nectar searches between large natural inflorescences (Pyke 1980; Thomson and Plowright 1980; Thomson 1986). On disjunct feeders, 96 % of bees approached the nectary or the nectar guide (crossed over the circular outer boundary defined by the nectar guide or an equally sized circle surrounding the nectary). Of these approaching bees ($n=74$), exactly 50 % approached the nectar guide first, demonstrating that both elements were equally attractive. Foraging success, however, was not affected by the spatial configuration of nectar guide and nectary. On the conjunct feeders, 43.8 % of foragers ($n=80$) successfully found nectar, while on the disjunct feeders, 35.1 % of foragers

($n=77$) were successful (not a significant difference, $\chi_1^2=1.03$, $P=0.31$).

However, the spatial configuration of nectar guide and nectary affected bee approaches (Fig. 1). On the conjunct feeders, successful foragers crossed the blue floral circle at a mean angle of 352° . This approach distribution is non-uniform ($P<0.0001$, Table S1) and is significantly similar to 0° ($V=3.95$, 34 d.f., $P<0.0001$), the closest approach to nectary and nectar guide on the conjunct feeders. Unsuccessful foragers approached the conjunct feeders from random directions ($P=0.41$). On the disjunct feeders, successful and unsuccessful foragers approached the flower from random directions ($P=0.20$). Separating nectary and nectar guide evidently disrupted forager approaches.

We hypothesized that search times on a disjunct feeder would increase because the nectar guide should compete with the nectary for the bees' attention. Indeed, there is a significant interaction of feeder type \times foraging success (GLM, $\chi_1^2=4.50$, $P=0.03$) on blue flower search times (feeder treatment has no overall effect, $\chi_1^2=1.93$, $P=0.16$, although foraging success does, $\chi_1^2=8.11$, $P=0.004$). There is no significant colony effect ($\chi_2^2=4.35$, $P=0.11$). Essentially, bees that failed to find nectar on the disjunct feeder spent significantly more time searching on the flower as compared to all other groups (Fig. 2, contrast test, L-R $\chi_1^2=14.53$, $P=0.0001$). On the conjunct feeders, there is no significant difference between the search times of successful vs. unsuccessful foragers (contrast test, L-R $\chi_1^2=0.34$, $P=0.56$).

Discussion

Other studies have demonstrated the attraction of bees to a nectar guide consisting of radiating lines around the center of a flower relative to a flower without such lines (Manning 1956; Free 1970; Dinkel and Lunau 2001; Leonard and Papaj 2011; Leonard et al. 2013). Our study has three distinguishing features: (1) testing an off-center nectar guide, (2) separating the attraction of nectary and nectar guide by creating a disjunct feeder which provides information conflict, and (3) using bees that were not pre-trained and whose responses should thus indicate innate preferences (see Supplement). We demonstrate that such radiating lines can attract the attention of naïve bees even when not centered in the flower (an inherently attractive position; Biesmeijer et al. 2005) and not connected to a visible nectary (see Supplement about the visibility of the nectary in this and past research).

Our results show that the separation of nectar guide and nectary on the disjunct feeders confused bees in two ways. Successful bees did not directly approach the nectary (unlike their behavior on conjunct flowers), and unsuccessful bees spent significantly more time on the disjunct feeder than bees in any other situation. On the conjunct feeder, successful

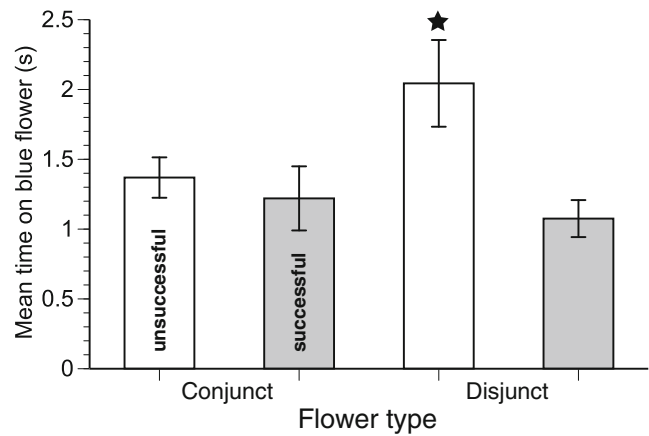


Fig. 2 The effect of feeder type on the amount of time that successful (gray bars) and unsuccessful bumble bee (*B. impatiens*) foragers (white bars) spent on conjunct and disjunct feeders. The star shows the group that is significantly different from all other groups. Each group consists of a different set of bees and the choice of each bee was tested only once. Standard error bars are shown

foragers surprisingly did not find the nectary faster than unsuccessful foragers. Overall success was also not affected by the spatial configuration of nectar guide and nectary. The stronger result of Leonard and Papaj (2011), who showed a significant increase in foraging success, may arise from the combination of a nectary and surrounding radial nectar guide in the floral center and the use of pre-trained foragers (see Supplement). Our results may also differ because, in our experiment, bees primarily walked onto the feeder. However, multiple bumble bee species are known to search by walking between large natural inflorescences, particularly when inflorescences are sufficiently close together (Pyke 1980; Thomson and Plowright 1980; Thomson 1986; see Supplement).

Considering nectary and the nectar guide as separately attractive floral elements may be useful because it expands our current understanding of multi-component floral signals and how they evolve. Usually, we think of such components as being in different sensory modalities (Hebets and Papaj 2005), i.e., the visual and olfactory components of nectar guides (Dötterl and Jürgens 2005; Leonard et al. 2011). Understanding multiple components of signals is important because combined information can influence the speed, accuracy, or both of pollinator decisions (Kulahci et al. 2008). Also, manipulating the elements of an intricate visual signal and testing the innate preferences of naïve bee may be useful for learning more about the complex phenomenon of floral nectar guides.

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References

- Biesmeijer JC, Giurfa M, Koedam D, Potts SG, Joel DM, Dafni A (2005) Convergent evolution: floral guides, stingless bee nest entrances, and insectivorous pitchers. *Naturwissenschaften* 92:444–450
- Dafni A, Giurfa M (1999) The functional ecology of nectar guides in relation to insect behaviour and vision. In: Wasser S (ed) *Evolutionary theory and processes—modern perspectives*. Kluwer Academic, Dordrecht, pp 363–383
- Dinkel T, Lunau K (2001) How drone flies (*Eristalis tenx* L., Syrphidae, Diptera) use floral guides to locate food resources. *J Insect Physiol* 47:1111–1118
- Dötterl S, Jürgens A (2005) Spatial fragrance patterns in flowers of *Silene latifolia*: lilac compounds as olfactory nectar guides? *Plant Syst Evol* 255:99–109
- Free JB (1970) Effect of flower shapes and nectar guides on the behaviour of foraging honeybees. *Behaviour* 37:269–285
- Hebets EA, Papaj DR (2005) Complex signal function: developing a framework of testable hypotheses. *Behav Ecol Sociobiol* 57:197–214
- Kulahci IG, Dornhaus A, Papaj DR (2008) Multimodal signals enhance decision making in foraging bumble-bees. *Proc R Soc Lond B* 275:797–804
- Kunze J, Gumbert A (2001) The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. *Behav Ecol* 12:447–456
- Leonard AS, Papaj DR (2011) 'X' marks the spot: the possible benefits of nectar guides to bees and plants. *Funct Ecol* 25:1293–1301
- Leonard AS, Dornhaus A, Papaj DR (2011) Flowers help bees cope with uncertainty: signal detection and the function of floral complexity. *J Exp Biol* 214:113–121
- Leonard AS, Brent J, Papaj DR, Dornhaus A (2013) Floral nectar guide patterns discourage nectar robbing by bumble bees. *PLoS ONE* 8:e55914
- Lunau K (1991) Innate flower recognition in bumblebees (*Bombus terrestris*, *B. lucorum*; Apidae): optical signals from stamens as landing reaction releasers. *Ethology* 88:203–214
- Lunau K, Fieselmann G, Heuschen B, van de Loo A (2006) Visual targeting of components of floral colour patterns in flower-naive bumblebees (*Bombus terrestris*; Apidae). *Naturwissenschaften* 93:325–328
- Manning A (1956) The effect of honey-guides. *Behaviour* 9:114–139
- Neal PR, Dafni A, Giurfa M (1998) Floral symmetry and its role in plant-pollinator systems: terminology, distribution, and hypotheses. *Annu Rev Ecol Syst* 29:345–373
- Pyke GH (1980) Optimal foraging in bumblebees: calculation of net rate of energy intake and optimal patch choice. *Theor Popul Biol* 17:232–246
- Sprengel CK (1793) *Das entdeckte Geheimniss dem Natur im Bau und in der Befruchtung der Blumen*. Friedrich Vieweg der Ältere, Berlin
- Thomson JD (1986) Pollen transport and deposition by bumble bees in *Erythronium*: influences of floral nectar and bee grooming. *J Ecol* 74:329–341
- Thomson JD, Plowright RC (1980) Pollen carryover, nectar rewards, and pollinator behavior with special reference to *Diervilla lonicera*. *Oecologia* 46:68–74
- Waser NM, Price MV (1983) Pollinator behaviour and natural selection for flower colour in *Delphinium nelsonii*. *Nature* 302:422–424

Supplement

Materials and Methods

We conducted our experiment at the University of California San Diego in La Jolla, California, USA (32°52.690'N and 117°14.464'W), from April 2012 through January 2013. We sequentially used three *B. impatiens* colonies purchased from Biobest Biological Systems (Ontario, Canada). This species occurs in Canada and the eastern United States and ranges from Maine and Ontario in the north to Florida in the south and as far west as Michigan, Illinois, Kansas and Mississippi (Heinrich 1979). Each colony contained approximately 100 adult bees at any given time and had an average lifetime production of approximately 280 bees (Hagbery and Nieh 2012). Each adult bee was uniquely marked with a numbered and colored bee tag (Queen Marking Kit, The Bee Works, Orillia, Ontario, Canada) attached to its thorax with cyanoacrylate adhesive (Hagbery and Nieh 2012). All bees were marked immediately after we received the colony. We marked newly emerged bees each morning.

Colonies were exposed to a 12-h light cycle (6:00am-6:00pm) illuminated with three 20W halogen bulbs positioned around the foraging arena and placed in a temperature-controlled room (~21°C). We housed the colony in a wood nest box (33 x 28 x 15 cm) with a clear lid that was normally occluded to maintain darkness. The nest box was connected in series to a foraging antechamber that was connected to the foraging arena, each connection consisting of a clear plastic tube (10 cm long, 3.5 cm diameter) with a slit into which a plastic gate could be inserted to control access. The antechamber (31 x 19 x 10 cm) allowed us to better limit the number of bees entering the foraging arena. The foraging arena consisted of a clear plastic box (32 x 54 x 27 cm) and lid with two mesh panels on one side for ventilation.

To maintain the colony, we fed it daily *ad libitum* for two hours either in the morning (10:00 am) or afternoon (2:00 pm) in the foraging chamber. If a trial was conducted in the morning, we fed the colony in the afternoon. If a trial would be conducted in the afternoon, we fed the colony in the morning. We randomized feeding times (afternoon or morning) to avoid training bees to expect food a specific time. We fed the colony 1.5 M unscented sucrose solution in a clear rectangular glass dish (7.5 x 6 cm, 31 ml

volume) and pollen (collected from honey bees, fresh frozen, and thawed by grinding prior to feeding) in two 4 cm diameter clear plastic petri dishes. We chose these feeding vessels because they were markedly different in appearance from the test feeders and we wished to avoid having bees associate nectar with the appearance of the experimental feeders before their first choice.

We used two experimental feeders: a conjunct feeder in which nectar guide surrounds the nectary, and a disjunct feeder in which nectar guide is separated from the nectary (Fig. 1). The experimental feeders consisted of 10 x 10 x 1.25 cm (L x W x H) white plastic blocks covered with sheet of photo paper upon which we color laser-printed a green background with a central 6 cm diameter light blue circle to represent the flower (Fig 1). We used a zygomorphic (bilaterally symmetric) design, placing the nectary to one side of the feeder, rather than the center. We chose these colors to be similar to the test flowers created by Leonard and Papaj (2011) and used colors from the standardized HKS-N-series, following Worden et al. (2005): HKS58n for dark green, HKS 46n for light blue. Like Leonard and Papaj (2011), we presented our flowers horizontally. This is a normal position for many flowers (Giurfa et al. 1999), and is used in other studies of bumble bee floral orientation (e.g., Spaethe et al. 2001). Makino (2008) also demonstrated that bumble bees prefer foraging on a horizontal as compared to a sloping array and had decreased foraging performance on a vertical as compared to a horizontal array.

Nectar guide

The nectar guide consisted of four white lines (each 10 mm long and 2 mm wide) arranged in a cross (Fig. 1). Both visual elements are visible to bumble bees, which can discriminate a floral nectar guide that is ≥ 0.5 mm in diameter at close distances, such as when they walk on the flower (Lunau et al. 2009). On the disjunct feeders, the nectary was displaced 37 mm away, to the opposite side of the feeder (Fig. 1). This nectar guide was highly reflective and contrasted strongly with the blue flower background.

Nectary

Each nectary consisted of a circular well (6 mm diameter and 7 mm deep) drilled into the underlying white plastic block. We always placed the nectary with its center 12 mm away from the nearest point on the circumference of the blue circle in conjunct and disjunct feeders (Fig. 1). Thus, a bee randomly approaching this point would have an equal chance of finding the nectary in conjunct and disjunct feeders if nectar guides provide no orientation information. The well appeared as a darkened hole because only $24\pm 5\%$ of incident photons (for wavelengths from 410 to 655 nm) were available for reflection out of the well. Bumble bee (*B. impatiens*) color photoreceptors have spectral peaks within 347-539 nm (Skorupski and Chittka, 2010).

To measure light levels, we used a quantum meter (Apogee model MQ-200, Logan, Utah, USA, spectral range of 410-655 nm) underneath the base of the well using the same lighting and feeder positions as in our experiment. To make these measurements, we took a feeder and drilled a hole into the back to accommodate the sensor, which was therefore placed at the bottom of a 6 mm diameter and 7 mm deep well. In our experiment, the center of the feeder was always in the same position, but the location of the off-center well (see Fig. 1) was randomly rotated among four different positions relative to the nest entrance to avoid bias. For our light measurements, we therefore placed the nectar well at the same height and in the same four positions used in our experiment. To measure how much light reached the surface of a nectary, we separately positioned the sensor underneath a 6 mm diameter hole in the printed photo paper (0.33 mm thick) that we used to cover our feeder and repeated our measurements ($n=16$). In all four positions, the nectar well was equally illuminated ($F_{1,14}=0.36$, $P=0.56$). On average 13.4 ± 0.8 $\mu\text{mol photons m}^2 \text{ s}^{-1}$ reached the surface of the well, but only 3.2 ± 0.5 $\mu\text{mol m}^2 \text{ s}^{-1}$ reached the bottom of the well.

Running trials

We ran trials either in the morning or afternoon (randomly chosen), conducting an equal number of disjunct and conjunct feeder trials. Each trial lasted 10 min. We conducted a maximum of two trials per

day. An hour before the beginning of a trial, we closed off the foraging chamber and antechamber gates and used aspirators to remove all bees, placing them back inside the colony. We then thoroughly cleaned these chambers with deionized water and 100% ethanol and waited for them to fully dry. We placed a clean feeder (either conjunct or disjunct, randomly determined such that half of the trials were conjunct) and pipetted 150 μL of 1.5 M unscented analytical-grade sucrose solution into the nectar well (which held 198 μL), making sure that all the solution was completely inside the nectary and did not spill out. We placed the feeder in the center of the foraging arena and rotated it so that the actual location of the nectary was randomized relative to the arena entrance. We suspended a video camera above the feeder and recorded bee choices with a digital video recorder (model#: QSDF8204, Q-See, Anaheim, California, USA).

Before the start of a trial, we opened a gate to allow approximately 10 bees to enter the antechamber. Usually, only about one-third of the bees during any given trial would show interest in the feeder. We therefore began a 10 min trial by allowing three bees to enter the foraging arena. We allowed this number of bees into the foraging arena to increase the chances of at least one bee finding the feeder. However, once a bee found the feeder, we did not analyze the responses of the other bees in the foraging arena. We also permanently excluded these bees to rule out the possibility that a bee could have learned about the feeder through social copying (Dawson et al. 2013). In some trials, no bees were attracted to the feeder. In total, we ran 190 trials with three colonies and 157 bees made usable choices. The majority of the bees walked to the feeder, with only 14 of 157 bees flying to the feeder. There was no significant effect of how bees arrived at the feeder (flew or walked) on the amount of time that they spent on the feeder ($\chi_1^2=2.80$, $P=0.09$). On natural flowers, particularly large ones, bumble bees can walk while searching for nectar and pollen (Heinrich 1979). On multiple plant species, bumble bees can walk from one inflorescence to the next while searching for nectar (Pyke 1980; Laverty 1980; Thomson and Plowright 1980; Thomson 1986).

At the end of each bee's visit, we turned off the white lights, illuminated the foraging arena with a 630 nm red LED light that darkened the arena for bees (which see red light poorly) and therefore reduced their motions to facilitate capture with an aspirator. This capture technique did not elicit alarm behavior in other bees. We noted the unique tag color and number of each bee. We retained captured bees in aspirators until the end of all trials on a given day and then returned them to the nest. We did not kill foragers because they are necessary to keep the colony in good condition. We focused on the choices of foragers and thus did not test the choices of males (identified by their head and antennae) and queen-like individuals (identified by their size and appearance late in the colony life). We removed males and queen-like individuals as soon as they entered the antechamber.

After each trial, we cleaned the plastic feeder block thoroughly with low-residue laboratory detergent after each choice, rinsing with deionized water, and ending with a 100% ethanol rinse. We then allowed the feeder to thoroughly dry. We replaced the photo paper after each trial.

Behavioral measurements

We measured the time that bees spent on the blue circle (flower time), the green background (green background time), and summed these to obtain the time spent on the entire feeder (total feeder time). We calculated the total feeder time because it is possible that bees treat the entire feeder as a single unit and do not distinguish between the blue flower and the green background. In some cases, a bee could cross over the blue flower multiple times during a single foraging attempt (Fig. S1). As long as the bee did not leave the green square surrounding the blue flower, we added up the times spent on the blue flower to obtain the total flower time. A floral visit could be successful (bee's mouthparts contact the nectary) or unsuccessful (bee leaves the feeder without finding nectar).

We also measured the approach direction of the bee to the feeder, scoring this direction to the nearest 30°. For example, a bee landing or walking onto a point immediately adjacent to the nectar guide would be scored as approaching from 0°. On conjunct flowers, the nectary and nectar guide are both located at 0°. On disjunct flowers, the nectary was located opposite the nectar guide, and thus a bee

landing or walking onto a point immediately adjacent to the disjunct nectary would be scored as approaching from 180° (Fig. 1). We separately scored approaches to the green background and to the blue flower. For example, a bee could cross over the green background at 30° and then walk on the green background before crossing over the blue flower at 0°. Bees that landed in the center of the feeder did not have an approach angle. Therefore, the approach data (Table S1) has a slightly smaller sample size than the overall data.

For disjunct feeders, we wished to determine if bees approached the nectary or nectar guide first. In our video analysis, we therefore defined an imaginary circle 2.5 cm in diameter around the nectar guide (corresponding to the edge to edge limit of the nectar guide) and defined a circle of equal diameter centered on the nectary. We scored a bee as first approaching the nectary if its head first crossed over the circle around the nectary. Alternatively, it would be scored as first approaching the nectar guide if its head first crossed over this circle around the nectar guide.

We viewed videos using Windows Media Player software and manually measured total time on the flower and total time on the nectar guides time to the nearest 0.03 s (based upon 30 video frames per second). All video analysts underwent extensive training, and all time measurements were double-checked at the end of the experiment by a different student. We used the center of the bee's thorax to define when it entered or exited a specific zone. To illustrate the paths taken by bees on the flowers, we analyzed a subset of the bee choices in detail, using Tracker v4.751 software and digitized the center of the forager's thorax at 30 frames per second (Fig. S1).

Statistics

We used a χ^2 test to compare the number of bees that successfully found nectar on disjunct as compared to conjunct feeders. We used a Generalized Linear Model (GLM, Poisson distribution, Maximum Likelihood Estimation, Identity Link) to analyze time spent on the feeder, with colony as a fixed effect. We ran planned contrast tests (L-R χ^2 tests) to test for significant differences between groups. To analyze

bee approach angles, we used a circular statistics package (StatistiXL v1.8, running on Excel 2007) to calculate the mean vector (a dimensionless value between 0 and 1), the mean angle, and the angular standard deviation. We use Rayleigh's Z -test to determine if the circular data are uniformly distributed. For data that exhibit a significant directional vector, we then used the V -test for a unimodal circular distribution to determine if mean vector is the same as a hypothesized value. Unlike non-circular mean tendency tests, the V -test yields a significant P -value ($P < 0.05$) if the mean direction of a circular distribution is the same as a hypothesized direction (Batschelet 1965).

Results

In the main text, we provided results on how bees oriented towards the blue flower. However, is it possible that measuring bee behavior towards the entire feeder (blue and green region) yields a different result? It does not. Measuring the total feeder time (sum of time spent on the green background and the blue flower) yields a similar result to measuring time spent on the blue flower only. On the disjunct feeders, bees that were not successful spent more time searching than all other bees (contrast test, L-R $\chi_1^2 = 11.89$, $P = 0.0006$). This is the same result shown in Fig. 2, which considers time spent only on the blue flower. In the overall model, looking at total time spent on the feeder, there was no significant effect of feeder type ($\chi_1^2 = 1.75$, $P = 0.19$) and no significant interaction of feeder type*foraging success ($\chi_1^2 = 0.17$, $P = 0.67$) on search times. However, there was a significant effect of foraging success ($\chi_1^2 = 15.32$, $P < 0.0001$) on total feeder time, just as there was for time spent on the blue flower (see main Results).

Likewise, measuring the approach angle using the first approach to the green background, rather than to the blue flower, also gives qualitatively similar results. Although none of the approach distributions to the green background were significantly different from random, the mean angle for successful foragers on the conjunct feeder was 337° (Rayleigh $Z = 2.00$, $P = 0.14$), similar to the mean blue flower approach angle of 352° (Table S1).

Discussion

Innate bee preferences

Earlier studies testing bee responses to nectar guides composed of radiating lines have all used pre-trained bees with some experience of artificial flowers (Manning 1956; Free 1970; Leonard and Papaj 2011; Leonard et al. 2013). In contrast, we only considered the first choice a bee made in its lifetime, and we permanently excluded any bees that moved onto the feeder when another bee was at or near the feeder, because these bees could have learned from observation. We subsequently disregarded any choices that such bees made throughout their lifetimes. In addition, we regularly fed the colony from clear rectangular glass feeders with a markedly different appearance from the experimental feeders to ensure that bees did not associate nectar with the appearance of the experimental feeders (Fig. 1) before their first choice. Whenever we placed the glass feeders in the arena, many bees recognized and rapidly visited them. Thus, the choices bees made at conjunct and disjunct feeders represent the innate preferences of bees that were likely seeking out new food sources.

On conjunct feeders, we were puzzled that successful foragers did not find the nectary faster than unsuccessful foragers. The nectar guide should have made the nectary more visually conspicuous. However, naïve bumble bee foragers can take some time to determine how to find nectar initially, even in a natural flower with natural nectar guides (Heinrich 1979). Thus, the combined visual stimuli provided by the conjunct nectary and nectar guide were evidently not enough to decrease the nectar discovery time of naïve foragers when nectary and nectar guides are not centered in the flower.

Why did successful and unsuccessful bees take roughly equal amounts of time to find the nectary? The slow and somewhat convoluted paths taken by naïve bees searching on the feeders (Fig. S1) may also explain why bees could closely approach the nectary without immediately finding it. In this figure, there are two examples of successful bees (bees 1 and 3), both of which approach close to the nectary but also move around the nectar guide. In fact, bee 3 walks right past the nectary while searching, follows the line of a nectar guide, and then returns along the guide before finding the nectar (Fig. S1).

Walking vs. flying during floral searches

Nearly all foragers in our study walked onto our artificial flowers. Such walking searches have been reported during natural foraging on floral resources by multiple bumble bee species (Pyke 1980). When bees can walk between inflorescences, walking may be a preferred search method (Thomson and Plowright 1980). For example, bumble bees preferred walking to flying between the large, distinct inflorescences of *Erythronium americanum* and *Clintonia borealis* (Thomson and Plowright 1980). *Bombus bifarius* and *B. occidentalis* often stepped from one inflorescence to the next on *E. grandiflorum* (Thomson 1986). Such walking may be beneficial to the plant because predicted pollen carryover (pollination) was greater for walking than for short flights between flowers (Thomson 1986).

Moreover, as shown in our results (Fig. 1 & S1), bees can focus on visual floral patterns during these walking searches. Lavery (1980) observed a bumble bee forager (*B. flavifrons* or *B. sylvicola*) searching for pollen by walking among the flowers of *Pedicularis groenlandica*, and showed that foragers focus their searches in central flower areas delimited by color patterns. Nectar guides can reflect UV light (Leonard et al. 2011) and Daumer (1956) reported that honey bee foragers show innate proboscis extension responses when passing over a UV-reflecting part of a flower.

Visibility of nectar guide and nectary

Based upon the results of other studies, our nectar guide and nectary were visible to bees. Bumble bees can approach and discriminate artificial flowers with circular nectar guides at least 2 mm in diameter over artificial flowers without nectar guides (Lunau et al. 2009). However, as they approach a flower, bumble bees can detect even smaller nectar guides and will antennate artificial flowers with circular nectar guides as small as 0.5 mm (Lunau et al. 2009). Leonard et al. (2013) used a white artificial flower with a central dark nectary that was 2.5 mm in diameter. This nectary should therefore be clearly visible to bumble bees at a distance. Leonard et al. (2011) similarly used a dark central circular nectary (1.5 mm diameter) that contrasted with a lighter background and which should have been visible once the bees were close to the flower. We used a darkened nectary well that is 6 mm in diameter and a nectar guide with a span of 25

mm (edge to edge). Both should therefore have been clearly visible to bees (based upon Lunau et al. 2009).

References cited in this supplement:

- Batschelet E (1965) Statistical methods for the analysis of problems in animal orientation and certain biological rhythms. American Institute of Biological Sciences. 57 page monograph.
- Daumer K (1956) Reizmetrische Untersuchung des Farbensehens der Bienen. Z Vergl Phys 38:413–478.
- Dawson EH, Avarguès-Weber A, Chittka L, Leadbeater E (2013) Learning by observation emerges from simple associations in an insect model. Curr Biol 20:727-730
- Giurfa M, Dafni A, Neal PR (1999) Floral symmetry and its role in plant-pollinator systems. Int J Plant Sci 160: S41-S50
- Hagbery J, Nieh JC (2012) Individual lifetime pollen and nectar foraging preferences in bumble bees. Naturwissenschaften 99:821-832
- Heinrich B (1979) Bumblebee Economics. Belknap Press of Harvard University Press, Cambridge, Massachusetts
- Laverty TM (1980) The flower-visiting behaviour of bumble bees: floral complexity and learning. Can J Zool 58:1324–1335
- Leonard AS, Papaj DR (2011) ‘X’ marks the spot: The possible benefits of nectar guides to bees and plants. Funct Ecol 1293-1301
- Lunau K, Unseld K, Wolter F (2009) Visual detection of diminutive floral guides in the bumblebee *Bombus terrestris* and in the honeybee *Apis mellifera*. J Comp Physiol A 195:1121-1130
- Makino, TT (2008) Bumble bee preferences for flowers arranged on a horizontal plane versus inclined planes. Funct Ecol 22:1027-1032
- Pyke GH (1980) Optimal foraging in bumblebees: Calculation of net rate of energy intake and optimal patch choice. Theor Popul Biol 17:232–246.

Skorupski P, Chittka L (2010) Photoreceptor spectral sensitivity in the bumblebee, *Bombus impatiens* (Hymenoptera: Apidae). PLoS ONE 5: e12049.

Spaethe J, Tautz J, Chittka L (2001) Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. P Natl Acad Sci USA 98:3898-3903.

Worden BD, Skemp AK, Papaj DR (2005) Learning in two contexts: the effects of interference and body size in bumblebees. J Exp Biol 208:2045-2053

Supplemental Table S1. The mean approach direction of successful and unsuccessful bees to the conjunct and disjunct feeders. A direction of 0° corresponds to directly approaching the nectar guide and nectary on the conjunct feeder. A direction of 180° corresponds to directly approaching the nectar guide on the disjunct feeder. We measured approaches to the green background and to the blue flower separately (see Supplemental Methods above).

Approach to:	mean vector magnitude	mean angle (°)	angular standard deviation (°)	N	Rayleigh's Z	P-value
<i>Green background</i>						
Unsuccessful forager at conjunct flower	0.15	32.2	74.9	42	0.90	0.41
Successful forager at conjunct flower	0.25	336.6	70.0	31	2.00	0.14
Unsuccessful forager at disjunct flower	0.08	53.8	77.8	47	0.29	0.20
Successful forager at disjunct flower	0.07	111.7	78.4	25	0.10	0.20
<i>Blue flower</i>						
Unsuccessful forager at conjunct flower	0.17	4.1	73.8	44	1.27	0.28
Successful forager at conjunct flower	0.48	351.7	58.2	34	7.97	<0.0001*
Unsuccessful forager at disjunct flower	0.09	21.2	77.3	47	0.37	0.20
Successful forager at disjunct flower	0.11	135.0	76.7	27	0.30	0.20

*Mean vector is the same as 0°

Figure S1

