Bumble bees show an induced preference for flowers when primed with caffeinated nectar and a target floral odour

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Summary

Caffeine is a widely occurring plant defence chemical[1,2] that occurs in the nectar of some plants, e.g. *Coffea* or *Citrus* spp., where it may influence pollinator behaviour to enhance pollination[3,4]. Honey bees fed caffeine form longer-lasting olfactory memory associations[5], which could give plants with caffeinated nectar an adaptive advantage by inducing more visits to flowers. Caffeinated free-flying bees show enhanced learning performance[6] and are more likely to revisit a caffeinated target feeder or artificial flower[7-9], although it is not clear whether improved memory of the target cues, or the perception of caffeine as a reward is the cause. Here, we show that inexperienced bumble bees (*Bombus terrestris*) locate new food sources emitting a learned floral odour more consistently if they have been fed caffeine. In laboratory arena tests we fed bees a caffeinated food alongside a floral odour blend (priming), then used robotic experimental flowers[10] to disentangle the effects of caffeine improving memory for learned food-associated cues versus caffeine as a reward. Inexperienced bees primed with caffeine made more initial visits to target robotic flowers emitting the target odour, compared to control bees or those primed with odour alone. Caffeine-primed bees tended to improve their floral handling time faster. While the effects of caffeine were short-lived, we show that food locating behaviours in free-flying bumble bees can be enhanced by caffeine provided in the nest. Consequently, there is potential to redesign commercial colonies to enhance bees' forage-focus, or even bias bees to forage on a specific crop.

Keywords

alkaloids, associative learning, bumble bees, flower constancy, pollination

Results

Here we measure whether bees that can be trained to locate a food source using a learned odour do so more or less efficiently when also exposed to caffeine during the learning phase. We ‘primed’ inexperienced bumble bees by exposing them either to a sugar solution, a sugar solution and a target synthetic odour blend, or a caffeinated sugar solution and the odour blend. When their preference for robotic flowers carrying the odour blend or a distractor odour was subsequently tested in a laboratory arena, the initial preference of bumble bees primed with the target odour blend in combination with caffeinated sugar solution was significantly biased towards electronic artificial flowers bearing the target odour above chance (Figure 1C; M = 0.704, $CI_{95\%}$ = 0.519 – 0.852). This initial preference for the target odour decreased as the bees visited more flowers and gained experience (Figure 1C). In contrast, the bees receiving only plain sugar solution (Figure 1A; M = 0.448, $CI_{95\%}$ = 0.276 – 0.621), or plain sugar solution in combination with the target odour (Figure 1B; M = 0.600, $CI_{95\%}$ = 0.433 – 0.767) showed no clear preference for flowers with the target odour or distractor odour above chance at their first flower visit, nor along their visitation sequence. All groups eventually became indifferent in their preferences between the two robotic flower types overall.

Bees from the caffeine and odour-alone priming groups did not differ in their initial handling speeds on the artificial robotic flowers. As the bees made more visits over repeated foraging bouts, they increased their performance in handling the robotic artificial flowers. The mean time spent, per flower, to locate and consume the nectar decreased over repeated bouts (Figure 2A; visit duration $p < 0.001$), as in other studies[11]. However, this improvement in handling speed (slope of the “handling speed acquisition curve”) showed some trend to differ according to the priming treatment (visit duration x treatment interaction $p = 0.051$), such that bees receiving only plain sugar solution had faster flower handling initially, but also a less pronounced rate of improvement, compared to bees primed with sugar and caffeinated sugar solution in combination with the target odour.

The searching time between consecutive visits also decreased with increasing number of visits (Figure 2B, visit interval $p < 0.001$) irrespective of the priming treatment (visit interval x treatment interaction $p = 0.708$). The rate of learning of the bees was highest at the beginning and diminished with an increasing number of visits. We anticipated that the priming of bees to the target odour with caffeine would result in increased flower constancy, i.e. fidelity to flowers of one odour type. In fact, the constancy index (CI) (calculated as in[12,13]) did not differ between treatment groups (ANOVA, $F_{2,82}$ = 0.225, $p = 0.799$) (Figure S1).

In order to maximise the applicability of findings, the priming target odour was formulated to resemble commercial strawberry floral odour. Discrete varieties of commercial strawberries grown in the UK exhibit distinct floral odour profiles as demonstrated by headspace analysis (Figure 3). However, as the prototype synthetic floral odour broadly resembled commercial varieties such as Malling Centenary in terms of its multidimensional locus in “bee odour space”, it can be considered representative of a “typical” strawberry flower’s odour.

Discussion

Efficient use of flowers by polylectic pollinators involves a variety of behaviours dependent on learning and memory. Odour cues aid in more rapid and efficient detection of flowers[14] and can be memorised by bumble bees either at the flower, or alternatively in the nest when it is brought back in nectar by conspecifics[15]. Olfactory learning in the nest is similarly observed in honey bees[16,17]. We found that consumption of caffeinated food supplement by bumble bees in the nest, when combined with a floral odour blend (priming), induced a preference for target flowers emitting that same odour outside the nest, but not when bees were odour-primed without caffeine.

We demonstrate that caffeine enables worker bees to form stronger associations between floral odour and food – and, importantly, that they will continue to seek out this odour when foraging outside the nest. We demonstrate that this can occur even when no caffeine is present on the target and the target visiting behaviour is decoupled from the experience of receiving caffeine. This laboratory-based study provides the first evidence of caffeine-mediated memory enhancement in free-flying bees when target flowers were not baited with caffeine. This is an important difference from previous studies where either the target has contained caffeine as part of the reward[6,7], or the bees have been tethered and their memory tested by proboscis extension response[5]. It demonstrates that the preference can be induced via caffeine-aided learning in the nest without requiring the reward experience on the target to be enhanced by caffeine too. While the effect was not long-lived, this was unlikely to be due to caffeine being metabolised out of the bee body, as the caffeinated sugar solution was stored in the nest by bees so could be consumed *ad libitum*, and previous work[5] indicates the effects on memory retention can last several days. Bees will gradually become indifferent in their flower preferences when all types are equally rewarding[18], as in this setup. In an applied context such as on fruit farms, normally the colonies are located within the crop, with the target flower type also being the closest flowers in proximity. Consequently, the effect is predicted to be longer-lived in the field compared to a flight arena.

Bees receiving caffeine in the nest showed a trend to improve in their flower visit speeds more quickly than the control bees. While our study did not examine the motor skills explicitly or break down the components of flower handling that might be responsible for this change, it suggests the possibility that caffeine could enhance motor learning skills. These govern many aspects of foraging behaviours and effective pollen deposition, benefiting both the bee (by efficient resource collection) and the plant (by effective pollination), and this may be particularly relevant for complex flowers where handling requires learning to refine it, such as those requiring sonication or manipulation to access anthers[11,19-21]. Alternative explanations for this may be that bees change in their motivation to leave flowers or take flight with experience, or change the duration for which they continue to probe at an empty flower. These behaviours could also be influenced by caffeine and the memory of rewarding odour.

Caffeine priming did not significantly influence the bumble bees' visitation rate, contrary to expectations based on previous research showing that caffeine increases locomotor activity in Hymenoptera[22]. However, while they were spectrally inconspicuous, the electronic artificial flowers were relatively large, raised, targets in a small arena, on a simple background, meaning the search task was not cognitively demanding. A future question is whether caffeine can improve searching behaviours in a visually complex environment.

In our experiment, caffeine did not influence the floral constancy of the bees during the early foraging period; whether this also would change for bees in a complex foraging environment is uncertain. Flower constancy is affected by bumble bees' working memory[12] and processing of visual information. Further work could examine whether caffeine affects working memory and ability to deal with multiple different search images or flowers that vary across multiple modalities. However, other studies have suggested that caffeine affects retention of memories by bees more strongly than initial acquisition, so effects may mostly be observed over longer time periods (e.g. between days) rather than within foraging bouts where working memory is most important[5,6].

Alkaloids such as caffeine exhibit wide-ranging effects on the behaviour and decision-making of even relatively small-brained organisms such as bees. While caffeine is thought to have evolved as a natural pesticide and antifeedant[1], caffeine at the concentration used in this experiment and found naturally in nectars of *Coffea* and *Citrus* is not repellent to bees[23]. As more about the learning potential of caffeinated bees emerges, it appears the potential benefits to a plant of "drugging" in pollinators are considerable. Harnessing the behavioural responses to caffeine (initial primed flower preference and rapid acquisition of flower handling skills) could translate into value for farmers, particularly in partially-covered fruit crops such as strawberry and tomato where growers make significant investment purchasing commercial bumble bee colonies for pollination. Typically, only a limited cohort of the colony forage and many may become distracted with other flowering species[24]. Caffeine-odour priming as we demonstrate here may confer particular benefits for some popular commercial strawberry varieties (e.g. Malling Centenary) that show high dependence on pollination by commercial bees. Providing caffeine and crop-specific odour to captive commercial bumble bees could prime inexperienced foraging bumble bees to visit a target crop preferentially to other flowers in the surroundings, reducing competition with wild bees and providing enhanced value-for-money from the colony.

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Author contributions

Designed the experiments: SEJA/JHD/MTF/FLW/PCS; Developed and prepared odour dispensers: DRH/DIF; Collected data: JHD/KLJ; Analysed data: JHD/SEJA; Discussed the results: SEJA/JHD/MTF/FLW/PCS; Wrote the paper: SEJA/JHD; Commented critically on the manuscript: All authors.

Declaration of interests

FLW is an employee of Biobest NV. FLW was involved in the study design and interpretation but the funding bodies themselves were not involved in the design, data collection, analysis or decision to publish. At time of submission the authors hold no patents related to this work.

Main figure titles and legends

Figure 1 Preferences of bumble bees receiving the different priming treatments. Priming bumble bees with caffeinated sugar biased their initial preference (y-axis) towards the target odour at their first visit. The initial bias of the inexperienced bees at their first visit diminished along the visitation sequence (panel A, B and C). The effect of the priming treatment was mainly apparent during the bees' 1st forage bout (panel D). Circles represent the proportion of target odour choices across all tested bees at the given visit number for (A) control bees; (B) bees primed with sugar+odour; (C) bees primed with sugar+odour+caffeine. Circle areas are proportional to the sample size of the datum (number of bees). Error bars = 95% CI at first choice. Confidence intervals not overlapping the horizontal dashed line indicate choices significantly different from chance (0.5) at $p < 0.05$. The shaded area represents the density of the bootstrapped model parameter estimates. Model predictions and 95% CI are shown for all consecutive visits after the first visit. Dashed regression lines indicate where $n < 10$ bees. Points in panel D indicate the average choice of single bees for the respective forage bout and boxplots indicate the median, 25th/75th percentile and 1.5 x interquartile range. See also Figure S1 and Table S1.

Figure 2 Changes in visit duration over the foraging period of bumble bees in the different priming treatment groups. The visit duration (A) and the time intervals between consecutive visits (B) decreased with increasing number of flower visits. Circles represent the mean visit duration (A) and the mean interval between consecutive visits (B) across all tested bees at the given visit number. Circle areas are proportional to the sample size of the datum (number of bees). Model predictions and 95% confidence bands are shown for all consecutive visits (A) or for all consecutive visits after the first visit (B). Dashed regression lines indicate a sample size less than 10 bees. Note the different y-axis scaling above 40 s in (B).

Figure 3 Floral odour compositions for different strawberry varieties represented in an NMDS plot for comparison. Floral odour compositions from the three commercial strawberry varieties shown in the NMDS plot differed significantly (PERMANOVA, $p = 0.02$), with the synthetic blend created by us ("Odour blend") being non-identical but falling within the same point cloud and thus likely to resemble it perceptually.

STAR★Methods

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sarah Arnold (s.e.j.arnold@greenwich.ac.uk).

Materials availability

The odour blend generated in this study is available from the authors on request. Details of the formulation are provided within this manuscript.

Data and code availability

1. Raw data related to bee visits have been deposited at the Open Science Framework and are publicly available as of the date of publication. DOIs are listed in the key resources table. Raw data related to floral volatiles are available from the lead contact upon request.

2. R scripts used in analyses have been deposited at the Open Science Framework and are publicly available as of the date of publication. DOIs are listed in the key resources table.

3. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental model and subject details

We tested 86 *Bombus terrestris audax* workers from 12 commercial colonies (henceforth "hives"). The hives were obtained from Biobest NV (Westerlo, Belgium) as research hives, "2 weeks young" (relative to normal supply age), without cotton insulation. Hives were maintained in a windowless laboratory, at 25 ± 3°C and ambient humidity, and provided with sugar solution and pollen as detailed below. Hives were randomly assigned to a treatment group.

Method details

Location

Experiments took place indoors at the Natural Resources Institute, University of Greenwich, UK (51°23'48.9"N 0°32'19.3"E) in a windowless laboratory, at 25 ± 3°C and ambient humidity. Experiments were conducted between 09:00 and 17:00 each day during September 2016 to November 2017.

Priming treatment preparation and colony maintenance

Three priming treatments were prepared in which we exposed each entire bumble bee hive 24 h before the experiment to one of: 1) sucrose solution only (sugar treatment), 2) sucrose solution in combination with a target odour mix (sugar + odour treatment), and 3) caffeine laced sucrose solution in combination with the target odour mix (sugar + odour + caffeine treatment) (see Table).

Information on numbers of tested bees and how many visits were recorded for the choice trial. (S = sugar only; SO = sugar+odour; SOC = sugar+odour+caffeine). See also Table S1.

				Visits per bee recorded:		
Colony	Treatment	Round	Bees tested	Median	Min	Max
A	S	A	7	40	31	62
B	SO	A	9	50	8	107
C	SOC	A	6	76.5	17	103
D	S	B	5	52	29	61
E	SO	B	6	59	31	97
F	SOC	B	7	34	20	69
G	S	C	7	51	44	70
H	SO	C	7	55	23	68
I	SOC	C	8	44	6	62
J	S	D	8	45.5	10	72
K	SO	D	7	55	16	80
L	SOC	D	9	63	12	102

Immediately after arrival, we replaced the pre-installed feeding reservoirs (capacity 100 ml) in the hives with custom capillary feeders. The custom feeders contained either plain 1.5M sucrose solution (sugar treatment and sugar+odour treatment), or 1.5M sucrose solution with 0.1mM caffeine (sugar+odour+caffeine treatment). This is a typical concentration of caffeine shown to have biologically relevant effects on bees in other studies[5] and falls below the threshold at which bees show gustatory aversion[23]. Each custom feeder was additionally equipped on the outside with two odour lures containing either the target odour mix (sugar+odour treatment and sugar+odour+caffeine treatment) or plain paraffin oil as control (sugar treatment). The distance between the capillary feeder and the odour source was less than 20 mm. The bees were accustomed to a food source at this location in the hive due to the pre-installed feeders, and immediately accepted the custom feeders. Every three days the feeders were cleaned, and the feeding solutions and odour lures were replaced with fresh ones. Thus, the colonies were exposed to the priming treatments during the entire experiment. In addition to the sucrose solutions from the feeders, the colonies were provided with 5 g honey bee-collected multifloral pollen (Biobest NV, Westerlo, Belgium) every three days.

Odour sampling and priming blend preparation

Collections of volatiles from strawberry flowers were carried out on commercial strawberry varieties growing in polytunnels at the National Institute of Botany East Malling Research (NIAB EMR) field station, and in laboratories at the Natural Resources Institute. Data for blend creation were collected from Elsanta variety (N = 4) and those for data verification from Elsanta (N = 16), Malling Centenary (N = 11) and a proprietary commercial variety (N = 12).

Volatiles were collected from 1-3 strawberry flowers, *Fragaria* x *ananassa*, enclosed in polyethyleneterephthalate oven bags. Charcoal-filtered air was pumped into the bags (150 ml/min) and drawn out (100 ml/min) through stainless steel collection tubes (90mm X 6.35 mm; Markes International, Llantrisant, UK) containing Tenax TA (35-60 mesh, 200 mg) for 18-24 h. Adsorbed volatiles were desorbed in a Unity desorber (Markes International) into a gas chromatograph (HP6890; Agilent Technologies, Manchester, UK) coupled directly to a mass selective detector (MSD 5973; Agilent Technologies). The GC was fitted with a non-polar DB5 column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) with helium carrier gas (1 ml/min) and oven temperature programmed from 40°C for 2 min then at 10°C/min to 250°C. Compounds were identified by comparison of their mass spectra with those in the NIST05 library and retention indices relative to retention times of *n*-alkanes with those in the Pherobase[25]. Identifications were confirmed by comparison of mass spectra and retention indices with those of authentic standards where available.

A total of 40 compounds was detected and identified. In the initial collections from Elsanta variety, the eight major compounds were (*E*,*E*)-α-farnesene (28% of total volatiles), (*Z*)-3-hexenyl acetate (19%), (*E*)-β-ocimene (12%), nonanal (6%), methyl salicylate (5%) benzaldehyde (5%), decanal (4%) and 4-methoxybenzaldehyde (*p*-anisaldehyde; 2%). These compounds have all been found in volatiles from strawberry flowers previously[26-28] although the relative proportions reported vary widely (e.g. Figure 3).

As (*E*,*E*)-α-farnesene is not easily available in sufficient quantity, blends of the other compounds were prepared and formulated in dispensers consisting of a cellulose acetate cigarette filter in a polypropylene pipette tip (1 ml; Fisher Scientific) sealed at the large end with a Teflon-lined crimp seal; volatiles were released through the 0.2 mm aperture at the narrow end[29].

Release rates were measured from dispensers exposed in a laboratory wind tunnel at 8 km/h windspeed and 27 °C. Volatiles were collected on Porapak resin and analysed quantitatively by gas chromatography[29]. Numerous blends and co-solvents were tested to give a blend releasing the seven components at similar relative rates to those found in volatiles from strawberry flowers above. This contained (*Z*)-3-hexenyl acetate (1mg), ocimene (*E*:*Z* 2:1; 2mg), nonanal (1.2mg), benzaldehyde (3mg), decanal (1.8mg), methyl salicylate (2.1mg) p-anisaldehyde (14.9mg) in 100 µl paraffin oil. Linalool is a very common plant volatile, but was not detected in our analyses of volatiles from strawberry flowers and was not detected or detected only at very low levels in other reports[26-28]. This was formulated as "distractor odour" in pipette tip dispensers at 3 mg (±)-linalool in 100 µl paraffin oil giving a release rate of approx. 120 ng/h at 27°C. Previous work on honey bees indicates that learning of blends and single odour components occurs at a broadly similar rate[30].

Flight arenas and pre-trial acclimation

The bumble bee hives were connected to wooden flight arenas (1.1 x 0.7 x 0.3 m, L x D x H) having a transparent acrylic lid[31,32]. The arena floors were covered with a green polypropylene sheet to provide a consistent and easily cleaned background (Figure S2A). The hives were connected to the arena entrances via a clear acrylic plastic pipe (internal Ø 18mm) with several shutters, allowing control of the bee traffic (Figure S2D).

Each arena was equipped with two neutral feeders each containing plain 1.5M sucrose solution (Figure S2B) for the first two days after hive set up and in the time between the actual choice trials. During these periods, the bees could freely enter the arena to forage on the neutral feeders *ad libitum* to motivate them to explore and become accustomed to the setup. Bees who visited the neutral feeders were marked using coloured queen-marking paint (EH Thorne Ltd., Market Rasen, UK) to allow individual identification. The sucrose solution and the capillary wicks in the neutral feeders were replaced every three days.

Artificial flower system and choice trials

During the choice tests, the neutral feeders were removed, and each arena was equipped with eight artificial robotic flowers following previously published designs[10] (Fig S1C; see also supplementary material for details) of which four were equipped with lures containing the target odour mix and four with a lure containing a distractor odour. The lures for the artificial flowers contained either a 0.1x diluted version of the same volatile mixture used for the odour priming treatment or 3 mg of the distractor odour. The electronic artificial flowers detected the bee visits and refilled automatically after 12 s. The software included a "detection buffer" to account for erratic movements/probing by the bee; after the first probing was detected, if subsequent probing occurs the internal timer would reset until no new probing was detected for 10 s. The refill cycle then had a duration of 2 s.

Each artificial flower was loaded with 1 M plain sucrose solution and provided a reward of 1.7 µl (SD=0.24, N=20) sucrose solution at each visit. To aid bee orientation, white dots were attached to the otherwise green flower lids (Figure S2C).

For the choice trials, a single, previously marked bee entered the arena. At their first encounter, the bees were naïve to the electronic artificial flowers. A foraging bout (breakdown in Table S1) was determined to be complete either when the bee returned to the hive or if the bee remained inactive in the arena for at least 3 min. While we aimed to record at least 50 visits per bee, some bees completed fewer visits and did not reappear to forage the same day. Thus, we recorded the maximum possible number of consecutive visits in each trial. Bees that completed more than 100 visits were not further tested. After each forage bout, the arena was opened and ventilated to avoid an accumulation of volatiles. The artificial flowers were wiped with 70% EtOH to remove scent marks deposited by the foraging bee and their positions re-randomised.

Quantification and statistical analysis

The experiment was run in four consecutive rounds. In each round we allocated one colony to each of the three priming treatments. With each new round, the experimental treatments were rotated across three identical arenas. Flower visits were automatically detected and logged by the artificial flower system. In addition to the identity of the visited flower, the system also captured the start and end time of each visitation event.

To test for the effects of the priming treatments on the bees' odour preferences we modelled the bees' choices to a binomial distribution (1=target odour, 0=distractor odour) using a logit link function. We split the analysis into two models, one for the initial choice, where the bees were naïve to the artificial robotic flowers, and one for all consecutive choices. For the initial choice model, we included only the priming treatment as fixed effect. The model for all consecutive choices included the visit number (log-transformed), the priming treatment, and their interaction as fixed effects. We additionally included the identity of the prior choice as fixed effect to model potential autocorrelation of the visitation sequences (i.e. the effect of the former choice on the consecutive choice). Bee identity was included as random effect to account for the non-independence of choices made by an individual bee and potential inter-individual variation in the bees' odour preferences in both models. For the initial choice model, we calculated the 95% bootstrap confidence intervals of the fixed effect parameters to estimate if the initial choice differed from chance (i.e. 95% CI not including 0.5). For the model of all consecutive choices, we tested for the significance of fixed effect terms using likelihood-ratio tests comparing the nested models including and omitting the effect term of interest.

We further tested whether the priming treatments influenced the bees' performance in learning to handle the artificial flowers and foraging in the arenas. We modelled the visit duration (handling time), and the time interval between consecutive visits (search time) to a Gamma distribution using a log link function. Both models included the visit number (log-transformed), the priming treatment, and their interaction as fixed effects, and bee identity as random effect. We tested for the significance fixed effect terms using likelihood-ratio tests comparing the nested models including and omitting the effect term of interest.

All models were fitted using the *glmer()* function and bootstrapping was performed with 10,000 simulations using the *bootmer()* function (r-package lme4[33]) for parameter estimates and model predictions.

The floral constancy index (CI) was calculated using the approach proposed by Chittka et al.[13] and provided in equation 1, a modified version of Bateman's Index[34] that is more robust to bees showing complete constancy, including if they make constant visits on only one flower type/species[13]. This measures the proportion of "same type" sequential flower visits in a mixed display (made up of "species X" and "species Y"; "same type" = XX or YY) relative to "different type" visits (XY or YX).

$$\mathrm{CI} = 0.5\left[\frac{A-B}{A+B} + \frac{C-D}{C+D}\right] \quad (1)$$

In which A = the number of constant flights from species X to species X, B = inconstant flights from X to Y, C = constant Y to Y, and D = inconstant Y to X. This was calculated for each bee's first foraging bout (leaving the nest, visiting artificial robotic flowers until satiated and returning to the nest), and compared between treatments via a simple ANOVA.

The odour composition (components and ratios) for each flower sampled was input into an ordination plot (NMDS) using package "vegan" [35] and clouds of points for each strawberry variety were compared via a PERMANOVA in "vegan" with strawberry variety as a factor.

All analyses were done in R version 3.6.0[36] apart from the initial calculation of CI, which was performed in Microsoft Excel version 2104 for Microsoft 365.

Supplemental Information

Supplemental file is provided.

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