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RESEARCH ARTICLE

How to know which food is good for you: bumblebees use taste to discriminate between different concentrations of food differing in nutrient content

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ABSTRACT

In view of the ongoing pollinator decline, the role of nutrition in bee health has received increasing attention. Bees obtain fat, carbohydrates and protein from pollen and nectar. As both excessive and deficient amounts of these macronutrients are detrimental, bees would benefit from assessing food quality to guarantee an optimal nutrient supply. While bees can detect sucrose and use it to assess nectar quality, it is unknown whether they can assess the macronutrient content of pollen. Previous studies have shown that bees preferentially collect pollen of higher protein content, suggesting that differences in pollen quality can be detected either by individual bees or via feedback from larvae. In this study, we examined whether and, if so, how individuals of the buff-tailed bumblebee (*Bombus terrestris*) discriminate between different concentrations of pollen and casein mixtures and thus nutrients. Bumblebees were trained using absolute and differential conditioning of the proboscis extension response (PER). As cues related to nutrient concentration could theoretically be perceived by either smell or taste, bees were tested on both olfactory and, for the first time, chemotactile perception. Using olfactory cues, bumblebees learned and discriminated between different pollen types and casein, but were unable to discriminate between different concentrations of these substances. However, when they touched the substances with their antennae, using chemotactile cues, they could also discriminate between different concentrations. Bumblebees are therefore able to discriminate between foods of different concentrations using contact chemosensory perception (taste). This ability may enable them to individually regulate the nutrient intake of their colonies.

KEY WORDS: Social insects, Apidae, Pollination, Diet, Perception, Foraging

INTRODUCTION

Like all other animals, bees need the right amount and composition of resources to raise their brood (Keller et al., 2005; Khoury et al., 2013) and to maintain colony health (Brodschneider and Crailsheim, 2010). Proteins and carbohydrates, and to a lesser extent lipids, represent important macronutrients for all herbivorous and pollinivorous insects, including bees (Friend, 1958). Carbohydrates provide the basic energy needed for all metabolic processes, while proteins provide amino acids that are used for the animal's own protein biosynthesis (Campbell, 1997). Bees obtain all these nutrients from pollen and nectar gathered from various plant species. Nectar primarily contains carbohydrates, while most other macronutrients are provided by pollen (Roulston and Cane, 2000). Because bees have a generally high metabolic activity and comparatively low energy stores, they are particularly prone to suffering from an imbalanced nutrient intake if nutrients are provided in excess or deficient amounts (Raubenheimer and Simpson, 1993). For instance, too much protein (DeGroot, 1953; Standifer et al., 1960; Herbert et al., 1977; Pirk et al., 2010) and specific amino acids (Simpson and Raubenheimer, 2009) can be detrimental, and honeybees have shorter life spans when fed a diet high in proteins (Pirk et al., 2010; Archer et al., 2014). In contrast, pollen of comparatively higher protein content was found to benefit larval growth and development (Herbert et al., 1977; Herbert, 1992; Tasei and Aupinel, 2008) and, up to a certain amount, to increase adult survival and immune functioning (Génissel et al., 2002; Brunner et al., 2014). Likewise, adult bees and larvae performed better on higher sugar concentrations (Kaftanoglu et al., 2011). Bees would therefore strongly benefit from assessing the nutrient content and composition of their food and adjusting their foraging behavior accordingly. For instance, as they can detect the sugar content of nectar via gustatory receptors on their antennae, mouthparts and tarsi (de Brito Sanchez, 2011), they preferentially collect nectar of high sugar concentrations (Hagler, 1990). Several studies have shown that bees also preferentially collect pollen that is rich in protein (Regali and Rasmont, 1995; Cook et al., 2005; Kitaoka and Nieh, 2009; Leonhardt and Blüthgen, 2012; Konzmann and Lunau, 2014; but see Pernal and Currie, 2002), suggesting that bees are further able to assess pollen quality (e.g. protein content). This would enable them to better regulate nutrient intake, which would be highly beneficial for their colonies, as well-nourished individuals can better withstand stressors like parasites, infections, insecticides and drought periods (Szymas and Jedruszuk, 2003; Brodschneider and Crailsheim, 2010; Archer et al., 2014; Kay et al., 2014).

Regulation of nutrient intake has already been observed in several ant species, e.g. *Solenopsis invicta* (Sorensen et al., 1985), *Iridomyrmex humilis* (Markin, 1970) and *Rhytidoponera metallica* (Dussutour and Simpson, 2009), as well as in honeybees (*Apis mellifera*) (Schmickl and Crailsheim, 2004). Thus, social insect colonies are generally able to satisfy the nutritional needs of queen, larvae and workers (Altaye et al., 2010). However, the precise mechanisms behind the regulation of nutrient intake are unclear. It can take place at either the colony or individual level. Regulation at the colony level does not necessarily require the sensory ability of adults to detect nutrient quality, but could be achieved through feedback from larvae, i.e. their rate of food consumption, and subsequently through varying food uptake rates of different food sources (Cassill and Tschinkel, 1999; Behmer, 2009). This is to say, if pollen rich in

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protein was readily consumed by larvae and hence taken over by nurses, foragers would be more inclined to re-visit the same protein-rich food source. While this mechanism would work for honeybees, it is unlikely in bumblebee colonies where foragers do not interact with nurses (Goulson, 2003) or with each other as neither trophallaxis nor waggle dances are performed to communicate food source locations. Here, regulation at the individual level through direct quality assessment is more likely. Individual bees may assess pollen quality by using either cues unrelated to pollen nutrient content (e.g. odor, color) or the concentration of specific nutrients (e.g. protein, lipids, amino acids, etc.).

While some studies suggest that, unlike honeybees (Pernal and Currie, 2002), bumblebee foragers are able to differentiate between food of different protein concentrations (Hanley et al., 2008; Leonhardt and Blüthgen, 2012; Vanderplanck et al., 2014), others claim that bumblebees can distinguish between different concentrations of sugar, but not other nutrients (e.g. Alaux et al., 2010; Konzmann and Lunau, 2014). Detailed studies on the bees' ability to assess the nutrient concentration of food are, however, still missing.

In this study, we investigated whether buff-tailed bumblebees, *Bombus terrestris* (Linnaeus 1758) (Hymenoptera: Apidae), can discriminate between different concentrations of food mixtures. We consider this ability one potential mechanism behind the individual assessment of food quality, which would enable regulation of nutrient intake. We assumed that, if individual bees were able to differentiate between food mixtures of different concentrations (i.e. pollen/casein:cellulose mixtures), they would use either smell (i.e. olfactory cues) or taste (i.e. chemotactile cues), or both. Note that the definition of taste follows de Brito Sanchez et al. (2014) and refers to a specific form of contact chemoreception that activates certain antennal receptors and can therefore be perceived by the animal. We tested whether bumblebees can distinguish between pollen/casein:cellulose mixtures of different concentrations when provided with either olfactory cues alone or with olfactory and chemotactile cues.

To address this question, we used absolute and differential conditioning of the proboscis extension response (PER), which was used to test associative learning (Takeda, 1961; Vareschi, 1971) and relies on the bees' behavior to extend their proboscis when their antennae, tarsi or parts of the mouth are touched with a sugar solution (de Brito Sanchez et al., 2007). The sugar solution represents the unconditioned stimulus (US) (Bitterman et al., 1983). The conditioned stimulus (CS) can be visual (Hori et al., 2006), tactile (Erber et al., 1998), thermal (Hammer et al., 2009) or olfactory (Hammer and Menzel, 1995; Menzel, 1999; Giurfa, 2007; Hannaford et al., 2013). In any case, during the presentation of the CS, the US is presented to establish an association between the two stimuli. After learning the CS–US association, an animal will extend its proboscis at the mere presentation of the CS (Bitterman et al., 1983; Matsumoto et al., 2012). Honeybees have primarily been used as a model organism for PER conditioning (Bitterman et al., 1983), but it has also been successfully applied to bumblebees (Laloi et al., 1999; Hannaford et al., 2013; Sommerlandt et al., 2014).

Using PER, it was shown that honeybees can smell (Arenas and Farina, 2012), taste (Grüter et al., 2008) and differentiate between different pollen types (Cook et al., 2005). They can also discriminate between different concentrations of simple (one molecule) odors (Bhagavan and Smith, 1997). Here, we tested whether bumblebees can also differentiate between different pollen types as well as between different food mixtures in olfactory and, for the first time, chemotactile conditioning. We predicted that bumblebees would differentiate between different pollen types. We further expected that bumblebees, unlike honeybees (Pernal and Currie, 2002), should be able to differentiate different concentrations of food mixtures.

RESULTS

Absolute conditioning (olfactory)

Bumblebees learned all substances tested in the absolute conditioning, with an average of 65±3% of individuals responding to the CS after 10 trials (Fig. 1). The number of responses in the paired groups differed from that in the control groups (control and unpaired) for 1-nonanol (H=73.50, P<0.001) as well as for the different pollen types (washed pollen: H=26.37, P<0.001; unwashed pollen: H=25.43, P<0.001) and casein (H=21.17, P<0.001) (Fig. 1), while we found no significant differences among the different substances (H=2.48, P=0.478), demonstrating that all substances could be learned equally well.

Differential conditioning (olfactory)

Bumblebees learned to differentiate between pollen and casein as well as between apple and almond pollen using olfactory cues, but did not learn to discriminate between different concentrations (Table 1, Figs 2, 3). The bees' learning performance for casein versus pollen and almond versus apple pollen was 61±2% and thus approximately as high as the learning performance in the absolute conditioning (Figs 1, 2).

Differential conditioning (chemotactile)

When the bumblebees were allowed to touch the substances with their antennae, they learned to discriminate between mixtures of 1:10 versus 10:1 and 1:5 versus 5:1, but failed to differentiate between the 1:2 versus 2:1 concentrations (Table 1, Fig. 4). The learning effect set in later (after approximately five rewarded trials), but a higher learning performance was reached (85±4%) after 10 trials in comparison to the absolute and differential olfactory conditioning.

Bees were, however, unable to discriminate pollen:cellulose mixtures differing only in water content (supplementary material Fig. S2 and Table S4). A visual inspection of the surface texture of the fresh and dried stimuli revealed no obvious differences in texture (supplementary material Fig. S3), even though the stimuli lost about 62% of water within 2 h of drying (62±7%, N=5).

Bumblebees successfully discriminated casein:cellulose mixtures of 1:10 and 10:1 under red light (supplementary material Fig. S4 and Table S5).

DISCUSSION

A balanced diet is highly important for the survival, immune functioning and reproduction of (social) bees (Herbert et al., 1977; Herbert, 1992; Regali and Rasmont, 1995; Génissel et al., 2002; Tasei and Aupinel, 2008; Brunner et al., 2014), rendering the regulation of nutrient intake a highly beneficial ability. The potential mechanisms behind such a regulation in individual bees and entire colonies have, however, been little investigated. Using absolute and differential PER conditioning we showed that bumblebees can differentiate between different food sources and pollen types using olfactory cues. However, they were unable to differentiate between pollen/casein:cellulose mixtures of different concentrations based on smell alone, which agrees with findings for honeybees that failed to discriminate between different concentrations of pollen odors (Wright et al., 2005). In our study,

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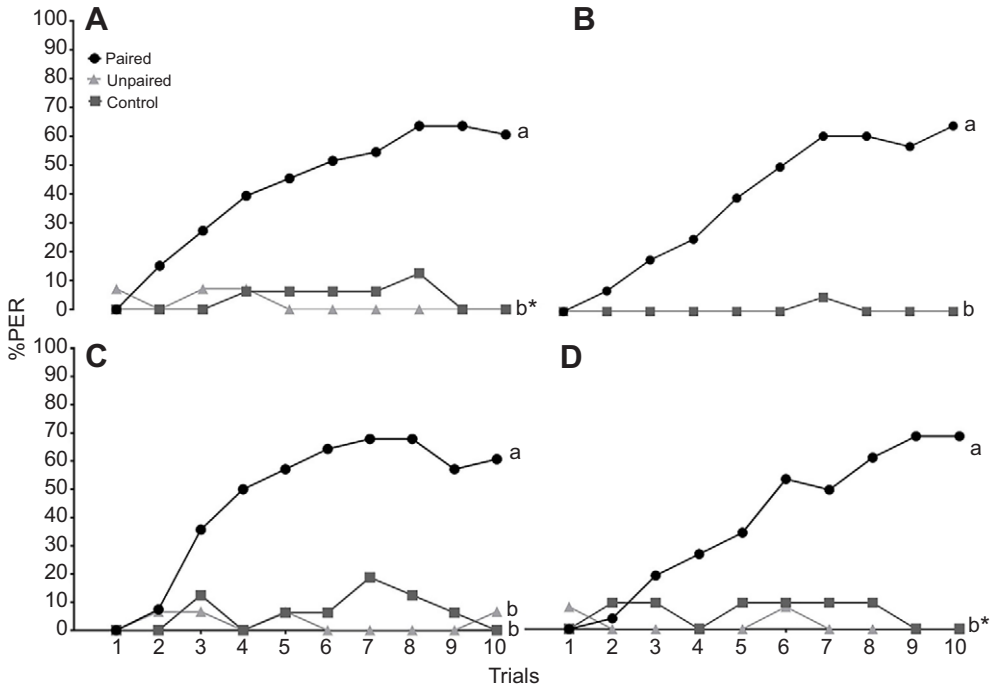

Fig. 1. The proportion of *Bombus terrestris* individuals showing a proboscis extension response (PER) over 10 trials following odor conditioning. Bees were conditioned on the odor of (A) unwashed bee pollen, (B) 1-nonanol, (C) washed bee pollen and (D) casein. Bumblebees were divided into three groups: individuals of the 'paired' group received both the conditioned stimulus and the reward in the same trial, individuals of the 'unpaired' group were presented with the conditioned stimulus and the reward in separate trials and individuals of the 'control' group received only an air stream per trial. The number of tested individuals for unwashed pollen was N_p=33 in the paired, N_{up}=14 in the unpaired and N_c=16 in the control group; for 1-nonanol, N_p=28 and N_c=20; for washed pollen, N_p=28, N_{up}=15 and N_c=16; and for casein, N_p=27, N_{up}=13 and N_c=11. Different letters to the right of the learning curves indicate significant differences in learning performance between groups; letters with an asterisk indicate that the two (overlapping) curves had the same letter.

differences between different concentrations could only be perceived and thus learned when the animals were allowed to (additionally) use their sense of taste (i.e. chemotactile cues).

The learning performance in our study was at least 50% after six trials, with an average of more than 60% after 10 trials, which was as high as in the study of Sommerlandt et al. (2014) and higher than in other studies with *B. terrestris*, where learning performances of only 20–30% were reported (Laloi et al., 1999; Laloi and Pham-Delegue, 2004). The performance differences between studies may be attributed to different experimental setups (e.g. different periods of starvation or differences in the mounting procedure, see discussion in Sommerlandt et al., 2014). Learning performance did not differ between absolute and differential conditioning, indicating that bumblebees are able to learn the comparatively difficult task of concentration differences as well as they can associate an odor with a reward. The high learning performance of almost 90% reached for the chemotactile conditioning was even higher than any performance reached in olfactory conditioning experiments and also higher than performances reported for honeybees tested in tactile conditioning, where bees discriminated different symbols engraved onto a plate (ca. 80%; Scheiner et al., 1999), suggesting that it is easier for bumblebees to learn substances

Table 1. Statistical results (*U*- and *P*-values) of Mann–Whitney *U*-tests analyzing differences between the rewarded (CS+) and unrewarded stimulus (CS−) in differential olfactory and chemotactile conditioning

				PER individuals			
Substance	Concentration	Cue	*N*	CS+	CS−	*U*	*P*
Casein vs pollen		Olfactory	56	41	8	513.5	<0.001
Apple vs almond			46	38	10	241.0	<0.001
Pollen:cellulose	1:5 vs 5:1		45	34	33	922.0	0.463
Casein:cellulose			48	41	42	979.5	0.204
Pollen:cellulose	1:10 vs 10:1		48	34	36	1026.0	0.350
Casein:cellulose			47	29	35	874.5	0.076
Pollen:cellulose	1:2 vs 2:1	Chemotactile	32	29	29	396.0	0.117
Casein:cellulose			32	28	28	419.0	0.211
Pollen:cellulose	1:5 vs 5:1		32	30	29	187.0	<0.001
Casein:cellulose			30	28	25	245.5	0.002
Pollen:cellulose	1:10 vs 10:1		31	29	29	268.5	0.002
Casein:cellulose			29	27	25	180.0	<0.001

The table shows the concentrations and substances tested, the cues presented, the number of individuals used (*N*) and the number of individuals that performed a PER at least once after 10 trials (PER individuals).

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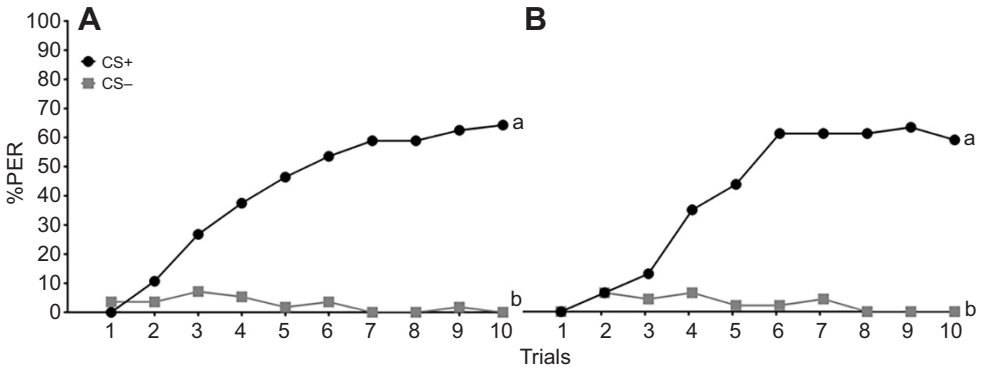

Fig. 2. The proportion of *B. terrestris* individuals showing a PER over 10 trials when differentially conditioned on the odor of casein versus pollen and apple versus almond pollen. (A) Casein versus pollen (*N*=56); (B) apple versus almond pollen (*N*=46). CS+ represents the rewarded conditioned stimulus, CS– the unrewarded conditioned stimulus. Both substances were used as CS+ and CS– with no significant differences between groups (see supplementary material Table S3). Different letters to the right of the learning curves indicate significant differences in learning performance between groups.

based on chemotactile than olfactory cues and that bees learn chemotactile cues even better than other tactile cues.

In our olfactory conditioning, the PER was clearly associated with food odor, because neither the air stream of the syringe ('control' group) nor the odor presentation alone induced learning. Moreover, bumblebees did not differ in their learning performance between different substances, suggesting that all substances can be perceived equally well and that it does not matter whether an odor is familiar (like pollen odors) or completely novel (like 1-nonanol and casein). Although bumblebees most likely prefer to collect pollen over casein or any other pollen surrogate (Pernal and Currie, 2002), this preference does not influence their learning performance.

As we only removed sugar from bee-collected pollen, besides protein it contained additional substances, such as inorganic components (Togasawa et al., 1967b), vitamins (Togasawa et al., 1967a) and lipids (Katsumata et al., 1975; Almeida-Muradian et al., 2005). However, we do not know which compounds are actually used for discrimination. Insects are generally able to perceive tastes similar to humans and have receptors for bitter and sweet substances as well as for several amino acids (Zhang et al., 2010; Toshima and Tanimura, 2012; for bees, see Linander et al., 2012), glycerol and water (as reviewed for *Drosophila* by Liman et al., 2014). Bees are even able to sense very small amounts of sugar (de Brito Sanchez, 2011). However, the protein content of casein was about 60% (supplementary material Table S1), with the rest consisting mainly of lipids (according to the manufacturer), which renders amino acids or lipids likely cues for discrimination. Bees can detect comparatively large concentrations of the amino acids tyrosine, cysteine, tryptophan, asparagine and proline using olfactory cues (Linander et al., 2012), but it is not clear whether they can also discriminate among these amino acids. In general, the concentration of water-soluble amino acids is positively correlated with the total amount of all amino acids and thus the protein content in pollen (r=0.40, P<0.001; data obtained from Weiner et al., 2010). Thus, if bumblebees have appropriate taste receptors, they may perceive water-soluble amino acids and use them to infer overall pollen protein content, which represents one potential mechanism for assessing pollen quality.

Alternatively, they could also have used cellulose as a chemotactile cue to differentiate between concentrations in our setup, but, to the best of our knowledge, it is as yet unknown whether bees can taste cellulose. Moreover, cellulose is inert and most likely too large as a molecule to be perceived by any taste receptors. It is also unlikely that water content, the concentration of

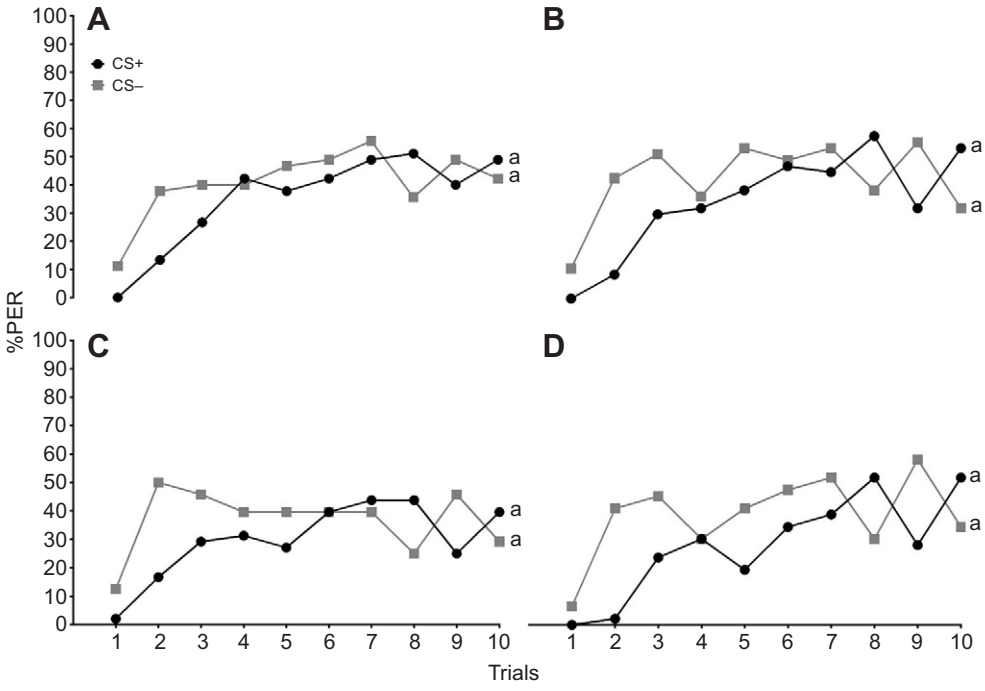

Fig. 3. The proportion of *B. terrestris* individuals showing a PER over 10 trials when differentially conditioned on the odor of pollen:cellulose or casein:cellulose. (A) Pollen:cellulose (*N*=45) and (B) casein:cellulose (*N*=48), both in concentrations of 5:1 versus 1:5; (C) pollen:cellulose (*N*=48) and (D) casein:cellulose (*N*=47), both in concentrations of 10:1 versus 1:10. CS+ represents the rewarded conditioned stimulus, CS– the unrewarded conditioned stimulus. Both substances were used as CS+ and CS– with no significant differences between groups (see supplementary material Table S3). The same letters to the right of the learning curves indicate no significant differences in learning performance between groups.

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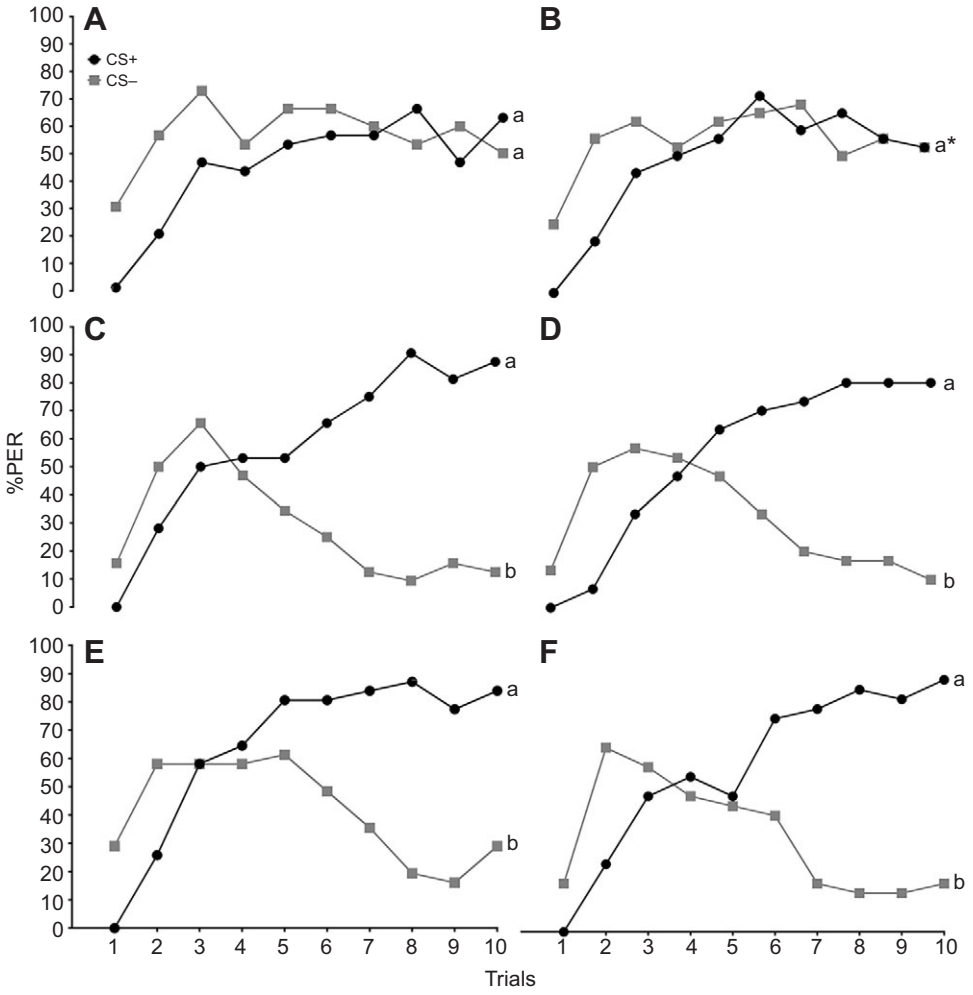

Fig. 4. The proportion of *B. terrestris* individuals showing a PER over 10 trials when differentially conditioned on the taste of pollen:cellulose and casein:cellulose. (A) Pollen:cellulose (*N*=32) and (B) casein:cellulose (*N*=32), both in concentrations of 2:1 versus 1:2; (C) pollen:cellulose (*N*=32) and (D) casein:cellulose (*N*=30) both in concentrations 5:1 versus 1:5; (E) pollen:cellulose (*N*=31) and (F) casein:cellulose (*N*=29) both in concentrations of 10:1 versus 1:10. CS+ represents the rewarded conditioned stimulus, CS– the unrewarded conditioned stimulus. Both substances were used as CS+ and CS– with no significant differences between groups (see supplementary material Table S4). Different letters to the right of the learning curves indicate significant differences in learning performance between groups; letters with an asterisk indicate that the two (overlapping) curves had the same letter.

water-soluble compounds (e.g. amino acids) or stimulus texture was used for discrimination, because neither the moisture content of food mixtures nor food texture affected discrimination by bumblebees (see supplementary material Figs S2, S3). The use of visual cues can also be ruled out as the bumblebees learned the difference between 1:10 versus 10:1 concentrations even when conditioning was performed in the dark (see supplementary material Fig. S4).

Our findings can help to explain the results of previous studies that have investigated pollen foraging in bumblebees. For example, Kitaoka and Nieh (2009) showed that *Bombus impatiens* prefers pure over diluted pollen, and several authors found a preference of bumblebees for pollen with high protein content (Robertson et al., 1999; Leonhardt and Blüthgen, 2012). These studies indicate that bumblebees assess (directly or indirectly) pollen quality. Our own study shows that individual bumblebees discriminate between concentrations of food mixtures. Based on these findings, we suggest that *B. terrestris* can taste concentration differences of nutrients (most likely water-soluble amino acids) in pollen and use this information to assess the protein content of different plant species and select pollen of higher protein content. However, because the PER paradigm represents a comparatively simple learning task, it is not clear whether foragers use information on nutrient concentration differences to choose between pollen sources while foraging or whether such cues may (also or instead) be used by nurse bees for making choices about the kind of pollen to feed to the brood.

Interestingly, the preference for protein-rich pollen shown by bumblebees contrasts with a study on honeybees that found no behavioral preference for food sources rich in protein (Pernal and Currie, 2002). As honeybees have hitherto not been conditioned on chemotactile cues relating to food concentrations, we cannot rule out that they are principally able to perceive the difference and just do not translate it into foraging decisions. However, given the possibility for more direct feedback from larvae via nurses to foragers in honeybee colonies (Crailsheim, 1998), individual honeybee foragers may, in contrast to bumblebees, rely more on colony feedback than individual quality assessment when collecting resources from flowers (Camazine, 1993; Camazine et al., 1998; Pernal and Currie, 2001). In fact, Pernal and Currie (2002) found that honeybee foragers did not evaluate pollen quality (i.e. protein content), but instead relied on pollen odor when making foraging decisions, a cue that they relate more to foraging and recruitment efficiency than to food quality per se. The authors further suggested that honeybees may use odor intensity to assess the amount of pollen present in a flower (Pernal and Currie, 2002). However, this assumption is contradicted by Wright et al. (2005) and our findings, which show that bees were unable to differentiate between pollen odors and pollen:cellulose mixtures of different concentrations using only olfactory cues. We therefore suggest that (nutrient) concentration differences cannot be detected by smell alone, preventing bees from assessing the amount of pollen present in flowers before landing, but that they need direct contact, i.e. chemotactile cues, in order to do so.

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To conclude, individual bumblebee foragers are able to discriminate different concentrations of food mixtures using their sense of taste, while they may use olfactory cues to differentiate between pollen of different plant species (Dobson and Bergstrom, 2000). Combined with potential feedback mechanisms at the colony level (i.e. feedback from the larvae), bumblebees should therefore be able to carry out sophisticated regulation of the intake of nutrients into the colony once they find floral resources of appropriate quality. Which compounds are used for the assessment remains as yet unknown. To examine the role of amino acids as potential nutrient cues, we plan to test whether bumblebees are also able to discriminate between food of identical protein content but different amino acid composition and between single amino acids.

MATERIALS AND METHODS

Study animals and substances used

Four bumblebee colonies were obtained from Koppert B. V. (Berkel en Rodenrijs, The Netherlands) and housed in the original boxes (27×24×20 cm) provided by the deliverer. The boxes were kept in the laboratory under natural daylight conditions filtered through window glass and fed *ad libitum* with Apiinvert (i.e. a sugar solution containing sucrose, fructose and glucose; Südzucker, Mannheim, Germany) and honeybee-collected pollen (obtained from Naturwaren Niederrhein GmBH, Goch-Asperden, Germany).

The substances used for the PER conditioning were 1-nonanol (as a control for PER conditioning in general), the milk protein casein (both Sigma-Aldrich, Munich, Germany), unwashed and washed bee-collected pollen as well as hand-collected apple (*Malus domestica*, Rosaceae) and almond (*Prunus dulcis*, Rosaceae) pollen (obtained from Firman Pollen, Yakima, WA, USA). To remove sugar residues from pollen, which could elicit an immediate PER, pollen was washed prior to the experiments. It was ground and 200 g was dissolved in 600 ml of de-ionized water for 1 h. Following filtration, the insoluble residue was washed in 99% ethanol (Hartenstein, Würzburg, Germany) overnight, filtered again, dried in a climate chamber for 48 h at 30°C and finally ground again. The protein content of bee- and hand-collected pollen as well as casein was determined by ion exchange chromatography (IEC) and was about 10% for bee-collected pollen, 12% for hand-collected pollen and 54% for casein (see supplementary material Table S1).

Mixtures of different food concentrations (used for differential conditioning) were produced by mixing casein or pollen with cellulose (Macherey-Nagel, Düren, Germany). We used cellulose because it is odorless for bumblebees (Mapalad et al., 2008; Konzmann and Lunau, 2014). The following mixtures of pollen:cellulose and casein:cellulose were prepared by volume: 1:2, 2:1, 1:5, 5:1, 10:1 and 1:10. A 50 g sample of each mixture was mixed with 20 ml water to get a homogeneous mass, dried for 48 h at 30°C and finally ground.

1-Nonanol was only used for olfactory absolute conditioning. This substance has been successfully applied in previous PER conditioning with bumblebees (Sommerlandt et al., 2014) and therefore allowed for a comparison of our findings with previous results on learning performance in bumblebees.

Experimental setup

The experimental setup was adapted from Sommerlandt et al. (2014) and slightly modified. Each day, 30 workers of unknown age and different sizes were randomly taken from one of the four colonies and chilled on ice for 45 min. They were afterwards harnessed in tubes of 7 mm diameter and 35 mm length with a 'yoke' (made from a paperclip). The bee's head and front legs were loosened in the harness to enable a PER. Such fixed animals were allowed to drink *ad libitum* from a 0.5 mol l^{-1} sucrose solution (Sigma-Aldrich) before they were kept in darkness in a climate chamber for 25 h at 20°C and 70% relative humidity.

To avoid irritation of animals by any smell emanating from human hands, all experimenters wore latex gloves. Before each experiment, all individuals were tested for a general PER by touching their antennae with a toothpick covered with a 0.5 mol l^{-1} sucrose solution. Only those animals that showed a PER (approximately 40–50% per day, but with large variations between days) were used for the subsequent conditioning experiments. The conditioning experiments were performed using a standard protocol established for bees (e.g. Bitterman et al., 1983; Laloi et al., 1999). All yoked animals were placed under a lab fume hood to continuously remove odors. The test individual was placed separately and was allowed to rest for 15 s before the CS was applied for 6 s. After 3 s, an antenna was touched with the toothpick covered in sucrose solution for 3 s and the animal was allowed to lick. It could then rest for another 15 s before it was replaced by the next individual. The interval between two trials with the same individual (inter-trial interval, ITI) was 8 min (Bitterman et al., 1983; Sommerlandt et al., 2014). Each individual was used for one experiment only.

For testing olfactory learning, we used a 20 ml syringe equipped with a 2×20 mm filter paper. Nonanol was diluted 1:100 in paraffin oil (Wright et al., 2009) and 5 µl was applied to the filter paper. For the solid substances (pollen and casein), 0.3 g were placed on a previously humidified filter paper and a pin was mounted at the 4 ml mark on the syringe to allow the syringe to be pressed without touching the substances with the plunger and therefore pushing them out of the syringe. During the 6 s of conditioning, a stream of air was produced by pressing the syringe volume onto the bee's antenna.

For the chemotactile experiments, we used a stick made of copper holding a small copper plate [3×4 mm, adapted from Scheiner et al. (1999)] (see supplementary material Fig. S1). A 1 g sample of each pollen/casein:cellulose mixture was mixed with 2 ml of water, producing a sticky paste. About 50 mg of the test substance was then applied to the plate and moved towards the antennae at an angle of approximately 45 deg by means of a micromanipulator whenever the stimulus needed to be presented. This allowed the bee to directly contact the applied substance with the tip of its antenna (de Brito Sanchez, 2011; de Brito Sanchez et al., 2014). The sugar water reward was presented to one antenna 3 s after the antenna had touched the substance and only if it was still in contact with the substance, as the CS and US have to be presented simultaneously. Recording of the 6 s of stimuli presentation started as soon as the bee touched the substance with its antenna. Plates were cleaned in 99% ethanol after each trial.

Chemotactile experiments were only performed using differential conditioning and only with those substances that the animals were unable to differentiate by smell (as olfactory cues cannot be excluded when the bees are allowed to touch the stimuli). Thus, casein versus pollen and apple versus almond pollen were only tested in olfactory differential conditioning, as the bees already learned the difference using olfaction.

Absolute conditioning

To test whether our test substances can be perceived and learned, the bumblebees were trained with absolute conditioning. Each day, we randomly chose between 48 and 63 individuals and allocated them to three treatment groups, one test group ('paired') and two control groups ('control' and 'unpaired'). The test group was conditioned following the protocol described above. Bees in the control group received only airflow from an empty syringe as the CS, to test for any effect of the application procedure on learning. Individuals of each of these two groups encountered 10 trials. The unpaired group received either the CS or the US stimulus in a separate trial in a random order for 20 trials (10 CS and 10 US trials; Sommerlandt et al., 2014). Therefore, the number of stimuli presentations was similar across all three groups.

Differential conditioning

We used differential conditioning to test whether bumblebees were able to differentiate between different protein sources (i.e. pollen and casein) as well as different concentrations of pollen/casein:cellulose mixtures. Following the protocol described above, each individual was conditioned for 10 trials presenting a rewarded stimulus (CS+) and for another 10 trials presenting an unrewarded stimulus (CS−) in a pseudo-randomized order (CS+, CS−, CS+, CS+, CS−, CS+, CS−, CS−, CS+, CS−, CS+, CS+, CS−, CS−, CS+, CS−, CS−, CS+).

For each pairing, a second group of individuals was tested with the meaning of the stimuli (rewarded and unrewarded) being reversed.

Because water evaporated from the pollen/casein:cellulose paste, the food surface texture may actually have changed over the course of the experiment and might have been used as a discrimination cue by the bees. Water evaporation could also have altered the concentration of water-soluble amino acids, providing an alternative (or additional) discrimination cue not directly related to nutrient content. To rule out the possibility that the bees used tactile (i.e. mechanical) cues or changes in the concentration of water-soluble amino acids caused by water evaporation, we performed an additional control experiment to assess whether bumblebees are able to perceive differences in moisture content. To produce the stimuli, we prepared a paste of 10:1 pollen: cellulose mixture and tested fresh and dried paste (ca. 2 h at room temperature) in a differential conditioning experiment. Water loss was quantified by weighing ca. 50 mg of fresh and 2 h-dried food mixture. To further assess potential changes of surface texture of the stimuli caused by evaporation, we took photographs of fresh and dried 10:1 pollen:cellulose mixture under a stereo microscope (Leica M80, Wetzlar, Germany) equipped with a camera (Leica IC80HD) at highest magnification for visual inspection. The 10:1 versus 1:10 casein:cellulose mixture was additionally tested in darkness under red light (>640 nm, as bees cannot see red; Chittka and Waser, 1997) to verify that the bees did not use visual cues for discriminating different concentrations. Between 55 and 60 individuals were tested per group.

Data analysis

All statistical tests were performed using GraphPad Prism version 6.04 for Windows (GraphPad Software, La Jolla, CA, USA). For absolute as well as for differential conditioning, the number of positive responses per individual (i.e. the number of times an individual showed a PER) following a CS was used as a response variable, ranging between 0 and 10 for each bee.

For the absolute conditioning, a non-parametric Kruskal–Wallis ANOVA followed by a Dunn's multiple comparisons test was performed to compare the different groups (paired, control, unpaired), except for 1-nonanol, which comprised only two groups (paired and control) and was therefore analyzed with a Mann–Whitney *U*-test. An additional Kruskal–Wallis ANOVA followed by a Dunn's multiple comparisons test was used to compare the paired groups between different substances (1-nonanol, casein, washed pollen and unwashed pollen).

For the differential conditioning, a Mann–Whitney *U*-test was performed for each comparison of CS+ and CS− as well as their reversed meanings. As the type of CS+ and CS− did not affect the bees' learning performance (see supplementary material Tables S2, S3) the two groups tested on the same food pairing were combined.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

The study was conceived by S.D.L. and J.S. and performed by F.A.R. F.A.R., S.D.L. and J.S. wrote the manuscript.

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Supplementary material

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