

The importance of experience in the interpretation of conspecific chemical signals

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Abstract Foraging bumblebees scent mark flowers with hydrocarbon secretions. Several studies have found these scent marks act as a repellent to bee foragers. This was thought to minimize the risk of visiting recently depleted flowers. Some studies, however, have found a reverse, attractive effect of scent marks left on flowers. Do bees mark flowers with different scents, or could the same scent be interpreted differently depending on the bees' previous experience with reward levels in flowers? We use a simple experimental design to investigate if the scent marks can become attractive when bees forage on artificial flowers that remain rewarding upon the bees' return after having depleted them. We contrast this with bees trained in the more natural scenario where revisits to recently emptied flowers are unrewarding. The bees' association between scent mark and reward value was tested with flowers scented from the same source. We find that the bees' experience with the level of reward determines how the scent mark is interpreted: the same scent can act as both an attractant and a repellent. How experience and learning influence the interpretation of the meaning of chemical signals deposited by animals for communication has rarely been investigated.

Keywords Context learning · Interference · Cognition · Pheromone · Communication · Repellent · Attractant

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Introduction

Chemical signals used in insect communication were once thought to trigger a mainly hard-wired, genetically predetermined response (Karlson and Luscher 1959). We now know that the same chemical signals can elicit different behaviours in different contexts (Hölldobler and Wilson 1990; Keeling et al. 2004). However, very few studies have differentiated between substances which have fixed meanings (e.g. through genetically hard-wired responses) and those that can develop or change meanings through learning and experience. This distinction seems crucial if we are to understand what role chemical signals play in communication systems and how they evolved.

One behavioural context where the response to an insect chemical cue has been considered to be hard-wired is the scent marking of flowers by pollinating insects (Giurfa and Núñez 1992; Stout et al. 1998; but see Stout and Goulson 2002). Using scent marks to indicate a renewable resource's reward status is expected to reduce time and energy spent locating the food source as well as investigating emptied food sites (Giurfa and Núñez 1992). Although this topic has mainly been explored in insects, such as bees and ants, it is probably widespread throughout the animal kingdom (Harrington 1981; Henry 1977). Flowers generally offer small rewards, and bees need to forage on hundreds of flowers per foraging bout to fill up their honey crop (Ribbands 1949). Because nectar is a slowly replenishing resource, an obvious problem bees encounter is how to avoid revisiting flowers they already visited. Using spatial memory alone may not be sufficient given the enormous number of flowers they would need to remember, so instead bees use scent marks left by themselves and other visiting foragers to avoid visiting recently emptied flowers (Gawleta et al. 2005; Reader et al. 2005; Stout and Goulson 2001;

Williams 1998). Such scent cues are transient marks composed of volatile compounds possibly secreted from the tarsal glands (Schmitt et al. 1991), and relying on such marks has been shown to reduce time spent probing unprofitable artificial flowers (Giurfa and Núñez 1992). Bees can adjust their reliance on scent marks depending on the flower handling time (Saleh et al. 2006) and nectar secretion rates of the flower species, suggesting that the meaning of the scent marks can be learned (Stout and Goulson 2002).

In contrast to the repellent effect found in several publications, some studies have found that the scent marks serve to attract bumblebees to the flowers (Cameron 1981; Schmitt and Bertsch 1990). Several hypotheses have been suggested to explain these divergent observations. For example, Stout et al. (1998) postulate that two types of marks with different chemistry may exist, which may come from different glands where at least one is actively secreted and controlled. This is highly unlikely to be the only possible explanation because studies on bumblebees have shown that chemicals similar to tarsal gland secretions elicit both attractive and repellent properties (Goulson et al. 2000; Schmitt et al. 1991). Another conjecture made by both Stout et al. (1998) and Giurfa and Núñez (1992) is that for bumblebees and honeybees, fresh scent marks may be repellent, but once the volatile compounds evaporate, the remaining non-volatile components become attractive. In bumblebees, this is unlikely to be applicable because Stout and Goulson (2002) found that the repellent effects can last up to 24 h. We also find that the bees rely differently on the scent marks depending on the handling time of the flower, indicating that marks carrying similar amounts of scent can elicit different degrees of repellent responses in bees (Saleh et al. 2006). A third explanation is that bees are simply interpreting the scent as attractive or repellent depending on the reward levels of the food source.

Studies that have found the scent marks to be attractive in bumblebees have been conducted in the laboratory where the reward levels were 1 μ l of 50% sucrose per visit (Cameron 1981; Schmitt and Bertsch 1990). The bees received a reward each time they visited a scent-marked flower. Studies that have found the scent marks to be repellent have been conducted in both the laboratory (Saleh et al. 2006) and the field (Stout and Goulson 2001). The flowers in these studies were not rewarding upon immediate revisitation. This correlation is also found in honeybees. Studies that have found an attractive effect were carried out in the laboratory with either 50-ml feeders (Free and Williams 1983) or 200- μ l feeders (Williams and Poppy 1997). The repellent effect was found when bees foraged on natural flowers (Reader et al. 2005; Stout and Goulson 2001; Williams 1998) or on artificial flowers where immediate revisits were not rewarding (Giurfa and Núñez 1992). Therefore, bees may interpret the scent marks as attractive

when revisits yield rewards and as repellent when revisits do not yield rewards. We use bumblebees to investigate how the meaning of scent marks left on food sources can change depending on the reward levels at the food source. We show that the same chemical mark can have opposite meanings and that this is directly attributed to learning.

Methods

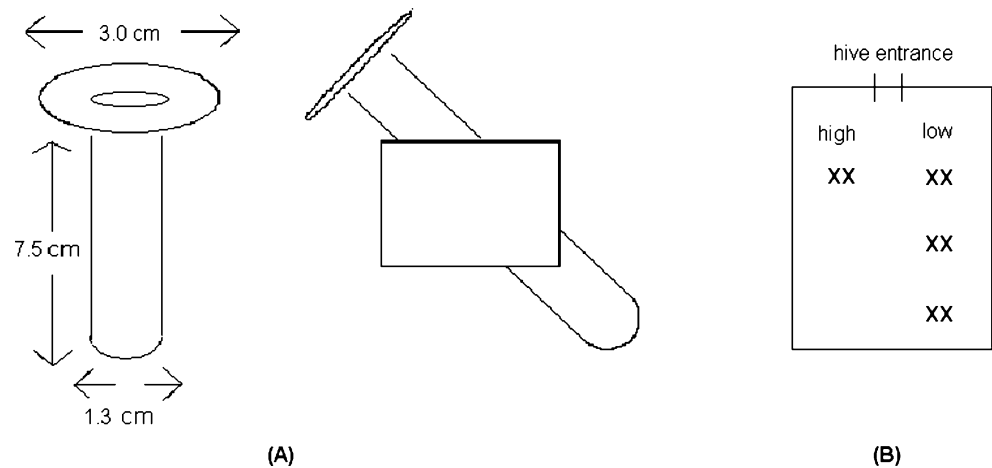
Colonies of *Bombus terrestris* were obtained from Koppert Ltd. (the Netherlands). Each colony was connected to a flight arena [100 (L) \times 40 (S) \times 70 (H) cm] by a transparent Plexiglas tube. Moveable cardboard flaps within the tube allowed only selected individual foragers into the flight arena. Approximately 8 g of pollen was fed directly into the nest every other day. Green cardboard was taped onto the arena floor to mimic the green foliage background found in most natural situations of bees foraging from flowers.

Flowers were designed from 5-ml polypropylene round-bottom tubes (13 \times 75-mm style; Plastiques Gosselin, Hazebrouck Cedex, France), which were 7.5 cm in length and 1.3 cm in diameter. Each flower was inserted into a brown wooden block (2.8 \times 2.7 \times 6 cm) at a 45° angle to facilitate access. A white filter paper collar of 3.0 cm diameter (3MM Qualitative, Whatman, W & R Balston Ltd., England) was placed around the top (see Fig. 1a).

For the high-reward treatment, the training phase consisted of bees being allowed to forage on two flowers for 15 bouts. One of these flowers contained 1 ml of 50% (v/v) (1.46 M) unscented sucrose solution and the other 1 ml of water. These flowers were placed side-by-side with wooden blocks touching, and their position was alternated between bouts. Their distance away from the hive entrance was also randomly changed between bouts (see Fig. 1b). Thus bees could not use spatial memory between bouts to locate the rewarding flower. Upon exiting the hive, the bees encountered two identical flowers, one of which was rewarding upon revisits. We expect any scent marks to indicate the position of the rewarding flower. These flowers were not changed between bouts to allow accumulation of any scent marks and to give the bees the opportunity to make the positive association between reward level and scent mark. Two flowers were used in this treatment because the bees generally filled up their crop from the first rewarding flower they encountered. This also ensured that the rewarding flower contained sufficient amounts of scent.

For the low-reward treatment, the bees were allowed to forage on six flowers with 30 μ l of 50% (v/v) unscented sucrose solution, where revisits did not yield rewards, for 15 training bouts. The flowers were changed after each bout, so only unmarked flowers were available for each new foraging bout. In this situation, we expect any scent

Fig. 1 **a** Test flowers used in the study. The tube is inserted into a wooden block making it stand at a 45° angle to facilitate access for the bees. **b** Arrangement of flowers during training phase. In the high-reward treatment, only two flowers were used; these were alternated in position relative to each other and distance from the hive entrance at each bout. For the low-reward treatment, six flowers were used, with their distribution as shown



marks to indicate that the flower had already been visited and was, therefore, empty. The flowers were placed side by side as in the high-reward treatment, forming three rows of two flowers each (see Fig. 1b).

The bees in the two treatments were subsequently tested in an identical situation where scent marks were collected from the same source. In test trials, the test bees encountered two identical flowers, where one was scent marked and the other was unmarked. To mark the test flowers, a clean, previously unused flower with 30 μ l of 50% unscented sucrose solution was placed inside a separate marking arena where non-test foragers were foraging. These foragers were allowed to feed on the flower, thereby scent marking it. The number of foragers marking the flower was recorded, and when all bees had left the flower, it was quickly removed and 30 μ l of water were placed at the bottom. This flower was presented to the test bee along with an identical unmarked flower also filled with 30 μ l of water. We conducted three test bouts for each bee, where each test bout was followed by two non-test bouts similar to the bee's training bouts. We have previously shown that bees will deposit and detect scent marks left by other foragers in this setup (Saleh et al. 2006). All flowers were handled with non-powdered gloves and there were 12 bees in each treatment. Only bees with inter-bout times of less than 5 min were used to ensure motivation to forage (very few bees failed this criterion), and each bee was only used once. Flowers were only refilled between bouts. A flower was discarded if the dispenser touched the inner walls of the tube when injecting the sucrose reward at the base of the flower; this was to ensure that bees were not detecting the presence or absence of sucrose through residue on the inner walls.

Data Analysis

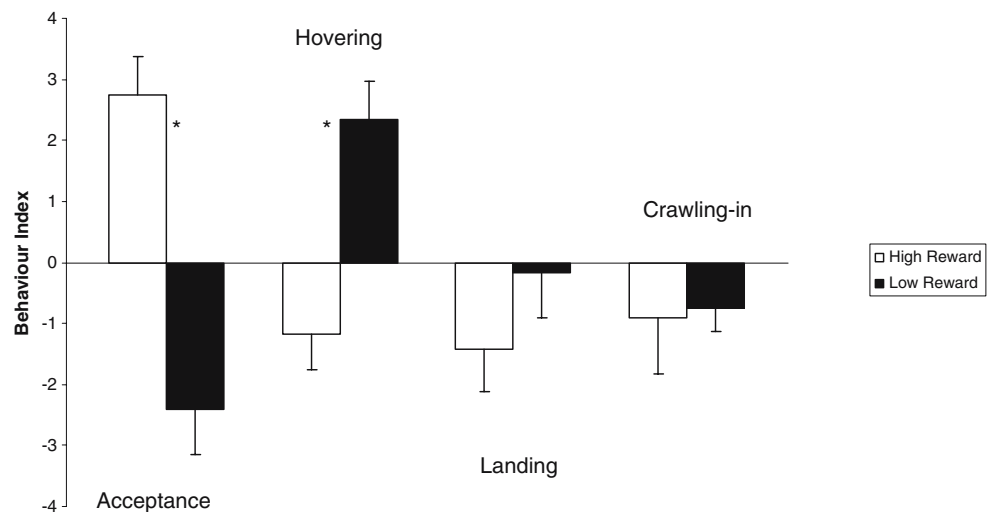
We investigated four different behaviours: (a) acceptance: the bee entered to the bottom of the flower; (b) crawling-in:

the bee entered halfway into the flower tube, crawled back out and flew off the flower; (c) landing: the bee landed on the filter paper of the flower with all six legs then flew off the flower and (d) hovering/rejection: bee hovered within 1 cm of the filter paper for more than 1 s then flew away from the flower. We added up the number of times each of these behaviours were used by each bee for the three test trials and used this sum for statistical analysis, with bee as the unit of replication. We conducted a two-sample *t* test with context (high-/low-reward level) as the factor and the behaviour index of each bee as the response. The behaviour index was calculated by subtracting the number of behaviours performed towards the unmarked flowers from those performed towards the marked flowers. Negative values indicate a preference for unmarked flowers, positive values indicate a preference for marked flowers and a value of zero indicates no preference. Bees in the high-reward treatment averaged 7.91 ± 1.13 (SE) visits to unmarked flowers and 7.16 ± 1.02 visits to marked flowers for the three test bouts. Those in the low-reward treatment averaged 10.23 ± 1.12 and 9.38 ± 0.94 visits to unmarked and marked flowers, respectively. There was no significant difference in the number of bees marking the test flowers between the two treatments (two-sample *t* test: $df=14$; $t=-0.67$, $p=0.51$). Therefore, any observed differences between the two bee treatments should not be due to differences in amount of scent mark deposited. We report averages of the behavioural indices as means \pm SE.

Results

Bees in the high-reward treatment accepted more marked flowers than those in the low-reward treatment, in which bees accepted more unmarked flowers (two-sample *t* test: $df=22$; $t=5.39$, $p<0.001$; see Fig. 2) (high-reward treatment = 2.75 ± 0.62 , low-reward treatment = -2.42 ± 0.73). Bees in the high-reward treatment were more likely to

Fig. 2 Bees showed a difference in acceptance and hovering rates towards marked and unmarked flowers depending on whether flowers they experienced prior to testing had high or low reward. There was no significant effect on crawling-in and landing behaviours. Mean behavioural indices are shown with standard error on mean. *Negative values* indicate a preference for unmarked flowers and *positive values* indicate a preference for marked flowers. *Asterisk* indicates significant difference ($p < 0.05$) between the two bee treatments for that behaviour



hover over, and subsequently reject, unmarked flowers than those in the low-reward treatment, which were more likely to hover over the marked flowers (two-sample t test: $df = 22$; $t = -4.02$, $p < 0.001$; see Fig. 2) (high-reward treatment = -1.17 ± 0.60 , low-reward treatment = 2.33 ± 0.63). There was no significant difference in the number of crawling-ins and landings performed by bees in the two treatments (two-sample t test: $df = 22$; crawling-in: $t = -0.17$, $p < 0.87$; landing: $t = -1.23$, $p < 0.23$; see Fig. 2) (crawling-in: -0.92 ± 0.90 and -0.75 ± 0.39 ; landing: -1.42 ± 0.70 and -0.17 ± 0.74 for high- and low-reward treatments, respectively).

Discussion

The bees in our study were tested in the same manner and with the same source of scent marks, where the only difference between the two groups was in their experience with high- or low-rewarding flowers. Therefore, any differences in behaviour are due to their previous experience with the flowers.

Bees in the high-reward treatment, where revisits were rewarding, accepted more scent-marked flowers compared with the unmarked flowers than those in the low-reward treatment. Thus scent marks, in this case, were perceived as an attractant. The opposite is true for bees trained in the low-reward treatment; these bees were more likely to accept unmarked flowers, suggesting that the scent mark in this context served as a repellent. Indeed, analysis of hovering/rejection behaviours indicates that bees trained in the low-reward treatment were more likely to hover over marked flowers. The opposite is true for those trained in the high-reward treatment; these bees were more likely to hover, or reject, unmarked flowers. Bees in both treatments usually hovered over flowers that did not offer reward. This may be an attempt to ensure that they have correctly detected the

presence or absence of scent mark in order to minimize erroneously entering into emptied flowers.

Although the attractant effect of foraging scent marks in bumblebees, honeybees and stingless bees has only been found on artificial feeders where revisits are rewarding, we do expect bees to have a use for an attractive scent in nature. For example, bees may want to scent mark bonanza food sites such as flowering trees (Seeley 1995), other bees' nest that are sometimes raided (Sakagami et al. 1993) or rotting fruit (used as food sources in foraging honeybees and bumblebees; Chittka, personal observations) in order to return to them later. To understand the true function of any of these chemical cues, we need to test them in experimental setups that mimic natural foraging conditions. This will allow us to identify exactly what role they play to foraging bees and what their evolutionary significance may be. We have some clues on some of the meanings the foraging scent mark can have. In addition to attractant and repellent effects, bees will rely on the scent marks to different degrees depending on the handling time of the flowers (Saleh et al. 2006). They also seem to adjust their reliance on scent marks depending on the nectar secretion rate of the flower species (Stout and Goulson 2002).

As bumblebees do not recruit to food sites, and forage in areas where they mix at random with non-colony members (Chapman et al. 2003; Thomson and Chittka 2001), it would seem that the attractant effect of the foraging scent mark could be used by a bee to signal a rewarding food site to itself. This can be advantageous because, although detectable to other bees and insects (Gawleta et al. 2005; Reader et al. 2005; Stout and Goulson 2001; Williams 1998), they would not be able to interpret its meaning unless they were also aware of the value of the food site. This is especially true if their interpretation of the scent is as a repellent due to the fact that pollinating insects mainly forage on low-rewarding flowers that replenish too slowly

for immediate revisits to be rewarding (Seeley 1995). This may act to reduce competition for the food source, allowing the bee to exploit it more thoroughly.

We do not know if the foraging scent marks have an innate meaning that can change with experience or if they acquire their meaning independently. Distinguishing between these two modes of learning is important. Nonetheless, both scenarios may involve simple associative learning. Yet simple associative learning does not exclude the ability of the association to influence biologically important behaviour. The foraging scent marks can increase the bees' foraging efficiency (Giurfa and Núñez 1992); therefore, they are able to spend more time investigating rewarding flowers. More food can directly impact the reproductive output of the colony (Pelletier and McNeil 2003; Schmid-Hempel and Schmid-Hempel 1998). Thus it is important to identify the versatile and learned cues used in communication.

Bees are very versatile learners. They can associate an odour with reward after only one rewarded experience (Menzel 1985), and they are capable of contextual learning (Chittka 1998; Chittka and Thomson 1997; Chittka et al. 1995; Collett and Kelber 1988; Lotto and Chittka 2005). Although we know that the same pheromonal cue can elicit different behaviours in a variety of different contexts (Keeling et al. 2004), we know very little about the role learning plays in the interpretation of marks left behind by the bee itself. Several studies using proboscis extension response (PER), where honeybees and wasps were harnessed and tested for their response to a conditioned stimulus, have shown that these insects can learn to associate alarm (Sandoz et al. 2001) and sex pheromone chemicals (Hartlieb et al. 1999) with food. This indicates that even substances that are innate or have a tendency to produce very specific behaviours can be associated with a different meaning.

This study has highlighted the importance of learning and experience in determining the meaning of the scent mark left on food sources. It has shown that bees will interpret foraging scent marks differently depending on the reward status of the food source where revisits do or do not yield reward. The evidence so far suggests that the use of foraging scent marks in bumblebees is very flexible, ultimately depending on the bee's personal experience with the mark. Flexible cues can play important roles in influencing an animal's behaviour and ultimately its fitness. Distinguishing between scents whose effects are highly flexible and those that are less flexible will give us a better understanding of the role chemical signals play in communication. We expect that learning and experience actually have a large role to play in the interpretation of chemical signals and may explain conflicting reports in the literature.

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