

Honeybee guards do not use food-derived odors to recognize non-nest mates: a test of the Odor Convergence hypothesis

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Honeybee (*Apis mellifera*) colonies rob honey from each other during periods of nectar shortage. Persistent robbing can kill weak colonies. Primarily responsible for preventing robbing are guard bees. Previous research has shown that the probability of both nest mate and non-nest mate workers being accepted by guards at the nest entrance increases as nectar availability increases. The mechanism responsible for this change in guard acceptance can be explained by two competing hypotheses: Odor Convergence and Adaptive Threshold Shift. In this study we tested the Odor Convergence hypothesis. The acceptance by guards at the nest entrance of workers transferred between four colonies that had been fed either odorless sucrose syrup (two colonies) or diluted heather honey (*Calluna vulgaris*) (two colonies) was measured for 3 days before feeding and during 2 weeks of feeding. Despite the large sample sizes, the probability of guards accepting non-nest mates was not affected by the similarities or dissimilarities in food odor between guards' and non-nest mates' colonies. This finding contrasts with the accepted wisdom that food odors are important in nest mate recognition in honeybees and the data, therefore, strongly reject the Odor Convergence hypothesis. *Key words*: Conspecific discrimination, environmentally-acquired odors, adaptive threshold shifts. [*Behav Ecol* 12:47–50 (2001)]

Recognition is fundamental in maintaining the integrity of most social groups. Nest mate recognition is particularly important in social insects because neighboring colonies often harm each other. Harm occurs in various ways such as territorial competition (African weaver ant *Oecophylla longinoda*; Hölldobler and Wilson, 1978), cannibalism (*Formica polyctena*; Driessen et al., 1984; *Polistes chinensis antennalis*; Kasuya et al., 1980) and slave capture (*Myrmecocystus mimicus*; Hölldobler, 1981). Honeybee (*Apis mellifera*) colonies rob honey from each other during periods of nectar dearth (Winston, 1987). Robbing can be fatal because colonies with inadequate honey stores starve during periods of nectar shortage (Winston, 1987). Primarily responsible for preventing robbing are guards (Butler and Free, 1952). This temporal caste of workers (Winston, 1987) patrol the nest entrance, inspect entering bees, and exclude non-nest mates while allowing nest mates to enter the nest (Butler and Free, 1952). The ability of guards to discriminate between nest mates and non-nest mates has long been recognized (e.g., Edwards, 1908) and much studied (review, Breed, 1998) making the honeybee a key model system for studying nest mate recognition.

To discriminate between nest mates and non-nest mates, guard honeybees antennate and lick workers encountered at the nest entrance (Butler and Free, 1952), thereby assessing the entering bee's odor recognition cues. The cues used to discriminate between nest mates and non-nest mates are suggested to originate from various sources (queen: Moritz and Crewe, 1988; Saleh-Mghir and Darchen, 1990; Breed et al., 1992; stored food: Ribbands et al., 1952; Ribbands et al., 1952 cited in Wilson, 1971; comb wax: Breed et al., 1988, 1995) but are not self-produced (Downs and Ratnieks, 1999). Two early

studies have repeatedly been cited as evidence to support the importance of food derived cues (Ribbonands et al., 1952; Ribbands et al., 1952 cited in Wilson, 1971). Ribbands et al. (1952) de-queened a colony and divided it into three groups, two of which were not fed and one fed with honey and treacle. Foragers from each hive were then trained to a syrup feeder and marked with different color paints (*Zwei-Völker-Versuche* technique; von Frisch and Rösch, 1926). The three hives were opened between nine and 17 days later and the number of marked bees counted. Marked bees were classified as "correctly" marked (i.e., nest mates) if they had a paint spot appropriate for that hive, or "incorrectly" marked (i.e., non-nest mates) if they had a paint spot of another hive. There was no significant difference in the number of correctly and incorrectly marked workers found in the two unfed groups. There were, however, significant differences between the fed and unfed groups, although the differences between the fed and unfed groups were not consistent. In the first comparison, the unfed group had proportionally more incorrectly marked bees than the fed group, where in the second comparison, the unfed group had proportionally fewer incorrectly marked bees than the fed group. The differences between fed and unfed groups were attributed to different colony odors arising from the different food odors. The importance of food-derived odors was further supported by a study which showed that non-nest mate acceptance increased when hives were located in an area where a single plant, heather (*Calluna vulgaris*), was the major forage source (Ribbonands et al., 1952 cited in Wilson, 1971).

Similarity in colony odors caused by foraging on the same plant species is referred to as Odor Convergence (OC) (Downs and Ratnieks, 1999). The conclusions of Ribbands et al. (1952) that nest mate recognition is greatly affected by food odors are widely known, largely because they are summarized in Wilson (1971). Wilson gives the impression that the data strongly support the importance of food-derived odors in nest mate recognition. However, the data in the original paper are less clear. For example, it is unclear whether

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Received 8 November 1999; revised 30 April 2000; accepted 26 May 2000.

both the feeding and hive relocation studies (Ribbands et al., 1952; Wilson, 1971) investigated non-nest mate acceptance in the context of robbing or drifting of bees between nearby hives. Furthermore, the data set was small and uneven samples were compared (e.g., 43 and 14 were compared to 11 and six). Thus the conclusions drawn from the statistical analysis must be considered with some hesitation. These studies, in particular the feeding study, should therefore be viewed critically; they suggest that the role of food-derived odors in nest mate recognition is in need of further study. Furthermore, the data collected from colonies in an area experiencing a heather nectar flow are also consistent with a second hypothesis, Adaptive Threshold Shift (ATS) (Downs and Ratnieks, 2000; Reeve, 1989). The ATS hypothesis predicts that as the cost and frequency of robbing declines, the acceptance threshold of guard bees becomes more permissive (Reeve, 1989). This shift is adaptive because the cost of rejecting nest mates, such as time wasted in entering the hive, is reduced at a time when the cost of accepting non-nest mates is low (because robbing does not occur when nectar is abundant). Downs and Ratnieks (2000) provide data strongly supporting the ATS hypothesis; over several weeks guards became more accepting of both nest mates and non nest mates as foraging conditions changed from nectar dearth to abundance. Eventually, all bees, both nest mates and non-nest mates, were accepted. However, because the nectar came from blooming citrus it was not possible to categorically reject the OC hypothesis. Because bees from different hives were all foraging mainly on citrus, they would presumably all acquire citrus odor (Downs and Ratnieks, 2000).

Perhaps as a result of the acceptance of these early studies (Ribbands et al., 1952; Wilson, 1971) as proof that food odors are used in honeybee nest mate recognition, further experiments to investigate the importance of food-acquired odors have been scant. One set of studies has shown that treating individual nest mates or non-nest mates with floral oils does not affect their probability of being accepted in comparison to untreated workers (Bowden et al., 1998; Downs et al., 2000), although the time taken for a treated non-nest mate to be rejected is increased (Downs et al., 2000). In contrast, Saleh-Mghir (1992) showed that sister groups of bees held in laboratory cages freely accepted workers transferred between groups fed the same-flavored honey, but rejected sisters from groups fed different-flavored honey (Saleh-Mghir, 1992). The apparent discrepancies between the results of these experiments (Bowden et al., 1998; Downs et al., 2000, versus Saleh-Mghir, 1992) may either be because floral odors are only used in recognition when entire colonies or groups, rather than just individual workers, have been exposed to them, or because food-acquired cues are only used for recognition when bees are kept under laboratory conditions that limit acquisition of odors cues.

This study was designed to test the Odor Convergence hypothesis. To do this we fed free flying honeybee colonies syrup, with or without strong natural floral odors, and measured the acceptance of non-nest mate workers transferred between colonies exposed to either the same or different food odors. If food odors are incorporated into colony odors and used for nest mate recognition, the Odor Convergence hypothesis predicts that workers transferred between colonies fed odored syrup would have a higher probability of being accepted than workers transferred between colonies fed odored and odorless syrups. Our results show that food odors do not affect the acceptance probability of non-nest mates. Food odors, therefore, do not appear to be used to assess worker affiliation and the Odor Convergence hypothesis is rejected.

MATERIALS AND METHODS

Study organism

The study was conducted in an apiary in Sheffield, UK, during July 1999. The four study hives were arranged in a row with approximately 2 m between neighboring hives to minimize drifting of foragers between colonies. The colonies were of mixed European race (*Apis mellifera*) and were queenright throughout the study. The colonies were of approximately equal population (20,000–30,000 bees) and were housed in standard Langstroth equipment (2 “deep” boxes of c. 45l each). Each hive had a standard “long” bottom board that extended 5 cm beyond the hive entrance. This facilitated the introduction of bees and observation of guarding.

Experimental design

We were specifically interested in the behavior of guard bees toward non-nest mate workers from colonies exposed to either a natural strong food odor or to odorless sucrose syrup. To create this odor dichotomy, we fed two experimental colonies with diluted heather honey and two with sucrose syrup. We then transferred workers between the heather-fed colonies (H to H), between sucrose-fed colonies (S to S), from heather-fed colonies to sucrose-fed colonies (H to S), and from sucrose-fed colonies to heather-fed colonies (S to H), and observed the behavior of guards that contacted the introduced bee. The OC hypothesis predicts that the acceptance of H to H would be greater than S to S, and that both H to S and S to H acceptance would be lower than H to H and S to S. Prior to feeding the colonies we measured the acceptance of non-nest mates for each colony for 3 days (days one, four, five; rain prevented data collection on days two and three) allowing us to monitor changes in acceptance caused by feeding. Feeding began at the end of day five and acceptance data were collected for the next 13 days. The 2 week duration of feeding was comparable to the duration of the nectar flow caused by a single plant species (Seeley, 1995) and was also similar to the duration over which previous work (Downs and Ratnieks, 2000) had shown substantial changes in acceptance thresholds.

Feeding the colonies

A standard 5 l syrup feeder (a plastic bucket with a 5 cm diameter hole covered with fine mesh in the center of the lid) was used for each hive. The bucket was filled with the appropriate syrup and inverted over the inner cover hole of the hive. The inner cover is a wooden board that sits on top of the combs underneath the outer lid. Bees could then suck out the syrup through the holes in the wire mesh of the feeder. We fed two colonies with sucrose syrup (60% sugar, measured with a refractometer) and the other two colonies with diluted heather (*Calluna vulgaris*) honey (56% sugar). The honey used had been made the previous year as comb honey and was removed from the combs immediately prior to dilution. We used heather honey because it is well known for its strong and distinct odor and because previous work (Ribbands et al., 1952, cited in Wilson, 1971) measured guard acceptance during a heather nectar flow. Sucrose syrup is odorless and the hives fed sucrose acted as both a control to test for the effects of syrup feeding itself and to compare S to S, H to S and S to H against H to H. The study was conducted before the local heather began to bloom so that no natural heather nectar was available for the colonies to collect. The hives were fed ad libitum throughout the study. Each colony drank approximately 15 l of syrup over 2 weeks, which simulates a substantial nectar flow (Seeley, 1995).

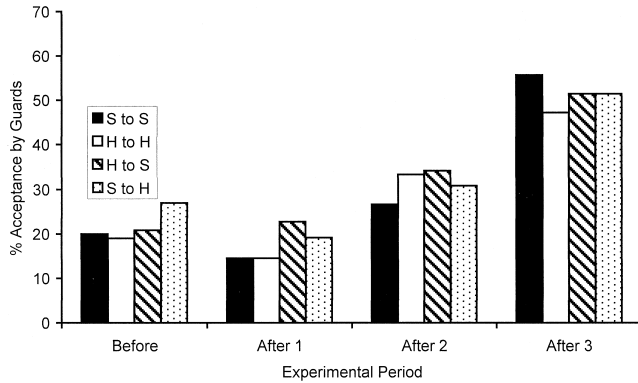


Figure 1

The acceptance by guards of non-nest mates transferred between colonies which were fed either the same or different odored syrups (S = sucrose syrup; H = diluted heather honey). The data are presented in four time periods, one before (days one to five) and three after feeding (days six to 18), and each period gives results from 3 days of data collection. The total number of introduced bees in each experimental period are: Before, $n = 312$; After 1, $n = 312$; After 2, $n = 360$; After 3, $n = 216$. There was no significant difference in guard acceptance within any of the experimental periods ($\chi^2: p \gg .05$).

Measuring guard acceptance

Guard acceptance was measured using a standard bioassay in which previously chilled foragers are introduced to a hive entrance (Downs and Ratnieks, 1999, 2000; Downs et al., 2000). The behavior of guards towards the introduced bee is then observed. When the introduced bee is bitten or stung this is classed as rejection. When the introduced bee is inspected (licked and antennated by guards) and allowed to enter the nest, or remained on the entrance for 5 min without being rejected, this is classed as acceptance.

Each colony usually received 10 workers from the other three colonies, resulting in a total of 120 introductions per day (30 per colony). However, on days five and six lack of personnel meant that each colony received only six workers from the other three colonies, resulting in a total of 72 introductions per day (18 per colony). At the beginning of the study (days one to 11) introductions were made day, weather permitting (rain on days two and three prevented study). At the end of the study (days 14, 16, and 18) introductions were made every 2 days.

The experiment was conducted blind as recommended for recognition studies (Gamboa et al., 1991). Assistants introducing the bees were given unmarked plastic vials each containing a bee and were unaware of the colony origin of that bee. In addition, they did not know which colonies had been fed honey and which sucrose.

RESULTS

All four colonies accepted non-nest mate workers from the three other colonies equally (data for all days: Hive A, $\chi^2 = 0.68$; $p = .71$; Hive B, $\chi^2 = 0.44$; $p = .80$; Hive C, $\chi^2 = 3.25$; $p = .2$; Hive D, $\chi^2 = 0.70$; $p = .70$). Since no colony had an acceptance bias towards bees from any other colony either before the feeding or during any part of the study, we pooled the data across colonies when comparing acceptance in the four combinations (H to H, S to S, H to S, and S to H) to test for an effect of food odors on acceptance.

When analyzing the data for an effect of food odors, we divided the data into four 3 day periods (Before Feeding, After Feeding 1, After Feeding 2, and After Feeding 3). There

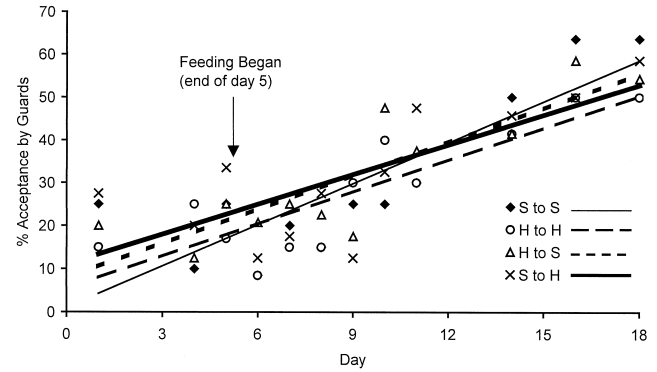


Figure 2

The change in guard acceptance of non-nest mates transferred between colonies during the study. Feeding of sucrose syrup and dilute heather honey began after data collection on day five. Guard acceptance of non-nest mates from all colonies increased significantly following feeding ($p < .002$). The change in guard acceptance was uniform between the colonies (ANCOVA: $p = .67$).

was no significant difference in the acceptance probabilities in any of the four transfer combinations (H to H, S to S, H to S, and S to H), during any period (Figure 1; Before, $\chi^2 = 2.69$; $df = 3$; $p = .44$; After 1, $\chi^2 = 2.00$; $df = 3$; $p = .57$; After 2, $\chi^2 = 1.23$; $df = 3$; $p = .73$; After 3, $\chi^2 = 4.26$; $df = 3$; $p = .24$).

Although syrup odor did not affect the probability of acceptance, regression analyses showed that there was a significant and gradual increase in acceptance following feeding (H→H: $r = .981$; $df = 1,7$; $p < .001$; S→S: $r = .923$; $df = 1,7$; $p < .001$; H→S: $r = .869$; $df = 1,7$; $p = .002$; S→H: $r = .908$; $df = 1,7$; $p = .001$; Figure 2). There was, however, no significant difference in the change in acceptance between the different transfer combinations (H to H, S to S, H to S, and S to H) over time (ANCOVA: $F = 0.51$; $df = 1,43$; $p = .67$).

DISCUSSION

All four colonies showed a gradual increase in non-nest mate acceptance following feeding (Figures 1 and 2). The increase in acceptance was universal and not directed toward non-nest mates from colonies fed particular syrup. For example, colonies fed diluted heather honey accepted workers from honey-fed and sucrose-fed colonies equally (H to H = S to H). Similarly, colonies fed sucrose solution accepted workers from sucrose-fed colonies and honey-fed colonies equally (S to S = H to S). The absence of an effect of feeding strongly odored heather honey, combined with the large data set, strongly suggest that food odors played no role in honeybee nest mate recognition and specifically rejects predictions of the OC hypothesis. The pooling fallacy does not apply to our study (i.e., data are pooled to create significant results) because there were no significant differences between the individual colonies. Furthermore, there was no significant treatment effect despite the large sample size, which is the opposite of the situation created by the pooling fallacy. Because feeding increased acceptance and, in particular, because acceptance was not affected by the similarity or difference in food odors between the colony of guard or intruder, our data support the ATS hypothesis (Downs and Ratnieks, 2000; Reeve, 1989). In light of the results of this study, we suggest that data previously considered as evidence to support the use of food-acquired odors in nest mate recognition are actually due to adaptive shifts of the acceptance thresholds of guards (Ribbands et al., 1952, cited in Wilson, 1971). This is because these studies

confounded the odor effects with feeding effects. For example, feeding and natural increases in nectar availability may reduce robbing, thereby causing a more permissive guard acceptance threshold.

Although this study shows that food odors acquired from nectar (syrup) do not affect acceptance, it does not exclude the possibility that floral odors present in pollen or on the plant tissue that a bee contacts when foraging may contribute to colony odor. Experiments to test the latter possibility would need to treat foragers or hives with flower odors while keeping nectar (syrup/honey) odors constant.

Significant effects of food-acquired odors on intracolony recognition have also been used as evidence for the importance of food odors in honeybee nest mate recognition. As previously mentioned, sister groups kept isolated in the laboratory freely accept each other when they are fed the same-flavored honey but reject sisters who have been fed different-flavored honey (Saleh-Mghir, 1992). Although these data show that food-odors can affect recognition, the experimental conditions reduce the availability of odor cue sources and prevent normal guard behavior. The data do demonstrate, however, that under specific conditions food-derived odors can be used for recognition by honeybees. But under the natural conditions of our study, honeybees presumably obtained sufficient recognition cues from other sources, such as comb wax (Breed et al., 1988, 1995), so that guards did not use food odors during discrimination. Although guards were able to detect and respond to floral oils without this affecting their acceptance threshold, as shown by the increase in rejection time (Downs et al., 2000) but not rejection probability of introduced workers treated with floral oils (Bowden et al., 1998; Downs et al., 2000). This situation is analogous to studies that have demonstrated the use of self-produced heritable cues in the laboratory (Breed, 1983; Getz and Smith, 1983), yet under natural conditions self-produced cues are overridden by environmentally-acquired cues (Downs and Ratnieks, 1999). It seems that self-produced cues are only used when acquisition of a normal complement of environmental odors is prevented. As with a previous study (Downs and Ratnieks, 1999), this study highlights the caution that must be taken when interpreting laboratory recognition studies and the need for these to be complemented by studies of recognition in natural contexts.

This study was funded by a BBSRC studentship (97/A1/S/03803) (SGD). The authors thank Dan Parkinson and Andy Martin (funded by a Nuffield Summer Research Bursary) for their help with introducing the bees. We also thank the referees for their useful suggestions and comments. This study is dedicated to the late Roger A. Morse of Cornell University.

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