Comb wax effects on the ontogeny of honey bee nestmate recognition

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Abstract. We addressed the general question of how kin recognition cues develop by investigating cue differentiation between colonies of the honey bee, Apis mellifera. In honey bee colonies, exposure to the wax comb is a critical component of the development of kin recognition cues. In this study, we determined how the cues develop under natural conditions (in swarms), whether the genetic source and age of the wax affect cue ontogeny, and whether exposure to wax, as in normal development, affects preferential feeding among bees within social groups. Cue development in swarms coincided with wax production, rather than with the presence of brood or the emergence of new workers; this finding supported previous observations concerning the importance of wax in cue ontogeny. Effective cue development required a match between the genetic source of the workers attempting to enter the hive, the wax to which they were exposed, and the guards at the hive entrance. The wax must also have been exposed to the hive environment for some time. Cues gained from wax did not mask or override cues used in preferential feeding interactions; this finding supports the contention that two recognition systems, one for nestmate recognition and the other for intra-colonial recognition, are present. The results fit a general model for cue development in nestmate recognition that relies on the use of nesting materials as an intermediary; the evolutionary significance of this model is discussed.

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Social recognition, including discrimination of kin from non-kin, has considerable significance in animal behaviour (Hepper 1991). In many species recognition plays a critical role in early development and the shaping of parent–offspring, sibling–sibling or other group interactions. Young animals often present recognition cues that are learned by parents or siblings. These cues may allow parents to discriminate their offspring from other juveniles (Beecher 1991) or may facilitate grouping of siblings (Blaustein & Waldman 1992). Some social insects may, however, be unique in modifying the recognition cue phenotype ontogenetically in order to facilitate communication.

Why modify cue phenotypes ontogenetically? In social insects, workers need to be admitted to the colony after foraging. Due to the large numbers of colony members and turnover of defenders, colony defence and controlled admission to the colony is simplest if all colony members carry the same recognition cues, like a uniform identity badge. Developmental modification of recognition cues by exposure to a cue source common to all colony members, such as nesting material, is a parsimonious mechanism for giving all colony members similar recognition cues (Gamboa et al. 1986a). After reproduction by swarming, however, a new twist is added to the problem; entrance guards at the old and new colonies may need to discriminate between genetically similar individuals that had originally been exposed to the same nest environment.

Thus, while cue modification is most commonly modelled as a process of individual ontogeny after an adult worker emerges in a colony, a parallel process may occur in the cue differentiation between mature workers after swarming. To date, little is known about the divergence of cues between parent and offspring colonies in
swarm-founding species of social insects; the question of how nestmate recognition develops in social units that result from the fission of a single colony is intriguing. We explored how this cue development in individuals culminates in nestmate recognition cues that diverge between colonies of the honey bee, *Apis mellifera*.

Our approach to this question is grounded in one of the two well-documented examples of cue phenotype modification by nesting materials. In honey bees, *A. mellifera*, recognition cues are acquired from comb wax (Breed et al. 1988, 1995a). In this species, adults undergo a period of cue acquisition after emergence; contact with the nesting material is a necessary component of acquiring the colony’s cue identity. The studies leading to the discovery of the critical role of comb wax cues in honey bee nestmate recognition have been reviewed by Getz (1991) and Smith & Breed (1995). Initially, Breed (1983) showed that newly-emerged adult bees are more likely to be accepted by guards if they are related to those guards. Guards reject all older bees unless they are first exposed to that colony’s nest (Breed et al. 1988); this finding suggested a search for recognition cue sources within bee colonies. Exposure to comb wax is a major source of recognition cues (Breed et al. 1995a); exposure masks other cues that might be used by guards (e.g. cuticular hydrocarbons: Breed 1983; Page et al. 1991) and alters the surface chemical profile of bees. The other well-documented example of cue modification during development is in paper wasps, *Polistes* sp., in which cues come from the paper nest (Gamboa et al. 1986a, b).

We examined nestmate recognition between natal nests of honey bees and the swarm colonies that they produce. Observations on development of recognition differences between swarms and their natal nest led us to determine whether exposure to comb from a colony during development is sufficient to gain entrance to the colony, or whether other factors, such as genetic relationship to the guards or exposure to odours from the environment is also critical. Finally, having found a key role for comb in divergence of cues of the swarm, we explored how cues acquired from comb during development may affect social interactions within colonies. These experiments give new insight into nestmate recognition in swarm founding eusocial insects and into how acquired cues are used in nestmate recognition.

**METHODS**

Bees used in these experiments came from the University of Colorado apiaries, Boulder, Colorado. We collected combs containing pupae from colonies and maintained them at 32°C in the laboratory until adult emergence. We placed the emerging adults into 0.47-litre cardboard cups and maintained them with sugar candy and water ad libitum. Comb construction in colonies was stimulated by removing frames of comb and replacing them with empty frames without foundation. We collected newly-constructed comb daily by cutting pieces under construction from the added frames. We cut old comb, constructed in the colonies from which it was obtained, by slicing pieces of darkened comb from frames removed from the brood area of the colony. Darkening of comb indicates use for food storage and brood rearing. In all experiments, except those involving different colour phenotypes, the observer was blind with respect to either the hypothesis being tested or the source of bees and the treatment.

**Experiment 1: Divergence of Recognition Cues Between Parental and Daughter Colonies**

For collection of natural swarms, we allowed apiary colonies to engage in pre-swarm behaviour. We observed the departure of two natural swarms from their source colonies; the swarms were captured at the apiaries and established in hives with unused frames and a supply of sugar water (1:1, v:v).

To test our assumption that bees from the swarm would be compatible with guards from the parental colony but not with other colonies, we presented swarm bees to guard bees from each colony in the apiary. The procedure for guard-bee testing followed that of Breed et al. (1992). Guards were identified by their behaviour at the nest entrance (Moore et al. 1987). We removed five guards with forceps and placed them in a cardboard cup with a clear plastic lid. One of the worker bees was then introduced into the cup and the reaction of the guards was recorded. Biting or stinging was scored as a rejection. If no biting or stinging took place, the replicate was scored as an acceptance. We terminated observations after 5 min. No introduced bee or group of guards was used more than once. We carried out these tests...
for each colony present, three per colony. Once
the compatibility of the parental colony with the
swarm was verified, reciprocal transfers of forag-
ers into containers of guards were performed over
3–4 weeks to assess acceptance levels of the
workers by guards from the other colony.

We created two artificial swarms by enclosing
the queen from a colony in a cage and suspend-
ing her in an unused hive body (following the
set-up used for natural swarms). Approximately
half of the workers were shaken into the new nest
and left overnight to establish themselves. We
released the queen from the cage 24 h after
separation from the parental colony. The original
colony was left with eggs to enable the process
of rearing emergency queens. Testing then pro-
ceeded as with natural swarms, spanning 3–4
weeks after separation. Controls, consisting
of donor bees introduced to guards from their
natal colony, were also run during the testing
period.

Experiment 2: Effects of Comb on Acceptability
of Daughter Colony Bees

We used three of the four swarms from the
above experiment to examine the effects of expo-
sure to comb on acceptability of foragers to
non-nestmate guards from the parent colony.
Newly constructed comb from the daughter col-
yony was collected using the methods described
above. We placed 10 foragers from the parental
colony in a cardboard cup with a 2.5 × 2.5-cm
piece of daughter comb, with food and water
added ad libitum. The bees were then transported
to the laboratory and kept for 24–72 h in a heated
room. (Length of exposure primarily depended
upon weather conditions and subsequent testing
conditions.) We then removed the foragers from
the laboratory and returned them to the apiary.
Testing followed the procedure outlined above,
with foragers introduced into cups containing
guard bees. Recipient cups held guards from
either the daughter or the parental colony. Con-
trols consisted of two separate tests. We first
introduced foragers from the parental colony with
no exposure to the daughter comb to guards from
the same colony. The second control test involved
parental colony foragers, also not exposed to
comb, placed in cups with guards from the
daughter colony.

Experiment 3: Effects of Comb and Familial
Source on Acceptability of Foragers

For each of five colonies selected for use in this
experiment, new comb was collected as described
above. We also collected old comb, that is comb
that had been used for brood rearing and food
storage, from each colony and cut it into 2.5-cm²
pieces for use. Pupae from each colony were
allowed to eclose in the laboratory (Breed 1983);
upon adult emergence, we placed groups of 10
bees in cardboard cups in the laboratory. Each
cup contained 10 bees from the same source col-
yony and treatment: no comb, old comb from the
same hive as the bees, new comb from the same
hive, old comb from a different hive (not the hive
of the guard or the forager), or new comb from a
different hive. After 5 days of exposure to the
comb in the laboratory, we transported the bees
to the apiary and tested for acceptability to guard
bees in either their natal colony or another colony.
Introduction of laboratory bees to entrance
guards followed the method described above. No
introduced bee or group of guards was used more
than once. For each colony, we tested guards
with each of the possible combinations of source
bees (same colony, different colony) and comb
(new, old, or none). In control trials, we collected
foragers from the same colony as the guards.

Experiment 4: Feeding Preferences in Laboratory
Groups of Bees

We conducted tests of the effect of comb wax on
intra-group feeding preferences using two proto-
cols following the techniques of Breed et al.
(1994). Both protocols are based on observations
of groups of 10 bees, half of which belong to one
phenotypic group and half to another. Control
bees were not exposed to comb, and treatment
bees were continuously exposed to old comb;
feeding rates were recorded within and between
phenotypic groups. To test the hypothesis that
comb exposure affects feeding preferences we
compared the control (no comb present) and
treatment (comb present) feeding rates.

In the first protocol, we placed five bees from
one colony and five from another in a cardboard
cup on the day of their emergence, as described
above. In controls, only food and water were
provided. The treatments had in addition a
2.5-cm² piece of old comb. Each bee was
numbered with a ‘bee tag’ for individual identification. Five days after placement in the cup, we observed the bees for 30 min and the following behavioural acts were recorded: soliciting food, giving food, taking food and rejecting a solicitation. These observations were blind with respect to the source colony of the bees. For the second protocol, we chose a single colony that produced workers of highly variable colour phenotype. Bees of extreme black or extreme yellow phenotypes were selected after adult emergence (Breed et al. 1994). We placed five bees of each type into cups and the method for the first protocol was followed. In this case, however, blind observations were not possible.

**RESULTS**

**Experiment 1: Divergence of Recognition Cues Between Parental and Daughter Colonies**

Incompatibility between the parental and daughter colonies developed soon after division of the colonies (Fig. 1). By 3–7 days after swarming, the rejection rate for swarm bees was significantly higher than it had been on the day of swarming (day 0, $\chi^2 = 8.92, P = 0.002$). By 8–13 days after swarming, rejection was significantly higher than it had been at 3–7 days ($\chi^2 = 11.03, P = 0.0009$); by 8–13 days following division, 60% of foragers that transferred between the colonies had been rejected. This rate did not significantly change over the following 30 days (the 20+ column in Fig. 1). Comb construction in the daughter colonies began soon after division, but egg-laying was delayed by 10–14 days, and brood emergence occurred well after maximum incompatibility was reached. There was no significant heterogeneity between the four pairs of colonies in this developmental pattern (Kolmogorov–Smirnov test, $P > 0.05$). The rapid development of nestmate discrimination between a daughter colony and its parental colony coincided with the construction of a new comb in the daughter nest (Fig. 1), implicating the presence of this comb wax as the source of differentiated recognition cues.

**Experiment 2: Effects of Comb on Acceptability of Daughter Colony Bees**

All control foragers introduced into groups of guard bees from the same colony were accepted by the guards (Table I). After at least 24 h of exposure to comb collected from the other nest, the acceptance rate was significantly lower (77.5%, $\chi^2 = 10.8, P = 0.001$). Foragers removed from the parental colony and tested with daughter guards were accepted only 32% of the time. Treatment with comb from the daughter nest significantly increased the level of acceptance by the guards from that nest to 49.5% ($\chi^2 = 5.7, P = 0.0166$). These results show that exposure to the comb is an important factor in the cue divergence of parental and daughter colonies.

**Experiment 3: Effects of Comb and Familial Source on Acceptability of Foragers**

Control bees had no exposure to comb; in the controls, only 4.8% of the introduced bees were accepted by guards, although they were sisters to
the guards. The combination of being from the same colony and being exposed to old comb from that colony was the only effective treatment in making bees acceptable to guards, raising the acceptance rate to 24% (Table II). This treatment was significantly different from the control ($\chi^2 = 23.1, P < 0.0001$). No other treatment was significantly different from the control (chi-square test). The same colony/old comb treatment was also significantly different from all other treatments (e.g. compared with same colony/new comb, $\chi^2 = 28.4, P < 0.0001$). Although the acceptance rate of sister bees exposed to old comb is not high, these results indicate the importance of a combination of three factors in recognition: genetic cues of workers, acquisition of cues from comb and environmental effects on that comb.

**Table I.** Effect of comb on the acceptability of foragers from the parental colony into the parental or the swarm colony

<table>
<thead>
<tr>
<th>Type of recipient bees</th>
<th>Treatment of foragers</th>
<th>Per cent accepted</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guards from same colony (control)</td>
<td>No comb</td>
<td>100.0</td>
<td>40</td>
</tr>
<tr>
<td>Parental guards</td>
<td>Comb from swarm</td>
<td>77.5</td>
<td>120</td>
</tr>
<tr>
<td>Swarm guards</td>
<td>Comb from swarm</td>
<td>49.5</td>
<td>48</td>
</tr>
<tr>
<td>Swarm guards</td>
<td>No comb</td>
<td>32.0</td>
<td>90</td>
</tr>
</tbody>
</table>

**Table II.** The effect of source colony and comb exposure on the acceptability of worker honey bees to entrance guards

<table>
<thead>
<tr>
<th>Control (same colony, no comb)</th>
<th>Same colony, old comb</th>
<th>Different colony, old comb</th>
<th>Same colony, new comb</th>
<th>Different colony, new comb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rejected</td>
<td>158</td>
<td>95</td>
<td>111</td>
<td>145</td>
</tr>
<tr>
<td>Accepted (%)</td>
<td>8 (4.8)</td>
<td>30 (24.0)</td>
<td>6 (5.1)</td>
<td>4 (2.7)</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Comb wax is important in the ontogeny of nestmate recognition cues in the honey bee (Breed et al. 1992, 1995a). Our current major findings are as follows. (1) Exposure to comb wax is correlated with the appearance of differing recognition cues between a parental and daughter colony. (2) Bees from the parental colony can be manipulated by exposure to comb from the daughter so that they are more acceptable to the daughter and less acceptable to their own colony; this establishes a causal link between comb and recognition. (3) Both genetic similarity and exposure to old comb from a colony are required for a worker to be acceptable to that colony’s guards. (4) Exposure to comb wax does not extinguish phenotypically-based feeding preferences in small groups of bees.

**Experiment 4: Feeding Preferences in Laboratory Groups of Bees**

In this experiment, trophallaxis preferences were assessed among bees that comprised either of the two colour phenotypes from the same colony or of bees from two different colonies. The use of the black and yellow phenotypes from within a colony allows tests for phenotype preferences among sister bees derived from an open-mated queen (Breed et al. 1994). Bees preferentially engaged in trophallaxis with bees of the same phenotype (colour or colony) in both controls and treatments (Table III). Exposure to comb had no significant effect on any of the behavioural acts.
membership in the daughter and parental colonies had not been explored until the present study.

Upon the first construction of comb wax in the daughter nest (experiment 1), acceptance levels began to change, eventually reaching a plateau near the end of the testing period. The final level of incompatibility was not as complete as that found between two unrelated colonies (Breed et al. 1992), perhaps reflecting the genetic similarity of the workers and the components of variation in the wax produced by them (Breed et al. 1995b). The comb in the old colony was influenced by the cumulative effects of a number of worker generations and perhaps by shifting genetic effects if queens swarmed or were superseded. We showed in experiment 3 an important effect of age of the comb: cues found in the new wax of the daughter colony differed from those in the older comb wax located in the original nest. This difference alone may be enough to drive the differentiation between the two groups of bees.

Because swarms may nest near their source colony and may compete with the parental colony for resources, we predicted prior to experiment 3 that colony differentiation would eventually develop. Based on our experience with comb wax, we predicted that comb wax would be critical in discriminating between the two colonies. We were surprised, however, by the rapidity with which the intolerance developed. Robinson & Dyer (1993) found that at least some of the foragers that reoriented to the swarm’s nesting location retained a memory of the location of the natal nest, but there was no evidence for interchange of materials, such as food or bees between natal and daughter colonies.

In an evolutionary context, this result is easy to understand. As stated above, swarms may settle near the parental colony. Our result is consistent with the hypothesis that there is strong selective pressure for rapid incompatibility between colonies, even if they are closely related. Inter-colonial compatibility persists for long periods after fission in stingless bees (e.g. Trigona cupira; Wille & Orozco 1975) and is associated with a continuing flow of bees and materials between the two colonies, indicating that different patterns of colony fission may yield differences in recognitive function.

A parallel exists between parental colony–daughter colony relations in honey bees and the genetic effects on recognition found among related non-nestmates in Polistes wasps (Bura & Gamboa 1994). Related (nieces and first cousin) but non-nestmate wasps were tolerated in roughly the same frequency as our worker honey bees from related colonies. This result suggests the same sort of interaction between cues produced by individuals and cues from nesting materials.

### Table III. Preferential feeding within phenotypes in laboratory groups of honey bees

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Different colonies</th>
<th>Colour phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No comb present</td>
<td>Comb present</td>
</tr>
<tr>
<td>Feeding between phenotypes</td>
<td>12.3 ±1.05</td>
<td>13.5 ±1.58</td>
</tr>
<tr>
<td>Paired t, P</td>
<td>2.93, 0.0062</td>
<td>2.10, 0.0468</td>
</tr>
<tr>
<td>Feeding within phenotype</td>
<td>16.4 ±1.40</td>
<td>16.25 ±1.94</td>
</tr>
<tr>
<td>N</td>
<td>33</td>
<td>24</td>
</tr>
</tbody>
</table>

Animal Behaviour, 55, 1

- Comb Exposure and Cue Divergence in Swarm Bees

The study of compatibility between parental and daughter colonies (experiment 1) was, necessarily, correlative. Experiment 2 was important in determining whether a causal relationship existed between comb wax, which was implicated by the time sequence (Fig. 1) and by cue divergence. By manipulating comb exposure, the cue divergence was partially reversed; nestmates from the parental colony were rejected by their own guards when exposed to comb wax from the daughter’s nest. Similarly, exposure of parental worker bees to daughter comb increased the acceptability of those workers to daughter guards. The bidirectionality of this effect builds a convincing case for the importance of comb exposure in driving the rapid development of parental colony/daughter colony differences.
Breed et al. (1988, 1995a) argued that cues found in the comb wax partially override the cuticular hydrocarbon signatures used in discrimination in a laboratory setting (Breed et al. 1994). The daughter experiments here support this conclusion, showing an interaction between genetic relationship and comb-derived cues.

Wax Exposure and Acceptability of Foragers to Guards

Our experiment with comb and workers from mature, unrelated colonies (experiment 3) underscores the importance of the interaction between genetic background and comb exposure. This laboratory treatment successfully mimicked, even though partially, the effects of exposure to the nest in gaining acceptability. The critical components of the mimicry were a genetic match with the guards and exposure to old comb from the same colony. Even with these factors, the rate of acceptance was much lower than the normal field condition, in which guards accept all nestmates. Presumably a developmental environment in which workers are subjected to more jostling with other workers, and in which workers move around on larger areas of comb, would result in a higher rate of acceptance. The small areas of comb used in this experiment may not be adequate to represent the entire suite of comb-derived cues in a large nest.

Feeding Preferences Within Colonies

The experiment on feeding preferences (experiment 4) gives further insight into the relationship between individual cuticular hydrocarbon signatures and the comb-derived signatures. It is not our purpose here to address the controversy concerning nepotism in honey bee colonies (Smith & Breed 1995); we used feeding preferences between phenotypic groups (not patrilines) as a tool for testing hypotheses concerning the relationships between individual signatures and colony-level signatures. Feeding preferences among phenotypic groups within colonies persisted in the presence of comb. Like the other experiments reported in this paper, this result shows that the cuticular hydrocarbon cues persist as an independent source of information in the presence of cues obtained from the comb.

General Discussion

How do cues derived from comb integrate with other cues? Recognition cues in honey bees can be genetically determined individual cuticular hydrocarbon signatures (Page et al. 1991; Breed et al. 1992), hydrocarbons or oxygenated compounds from the comb wax (Breed et al. 1992), or a variety of compounds from floral sources (R. Bowden & M. D. B., unpublished data). No single factor (genetically determined cuticular signatures, exposure to comb wax, or exposure to floral cues) is responsible for nestmate recognition; rather, all three factors seem to work together.

Our results indicate that both cues from the comb and the cues that are produced by each individual are important. Genetic matching is unimportant to acceptance if there is a long period of exposure to the nest (Breed et al. 1988). Further understanding comes from the experience of beekeepers, who have found that unrelated bees may be placed successfully into colonies as pupae or young workers. If a bee’s entire adult experience is in a colony, then it becomes acceptable to that colony whether it is related or not. If development takes place in a colony then the colony is able to accommodate considerable genetic diversity. This could be because guards learn the range of metabolically derived cues in the colony, including those of the introduced bees, or because the comb cues are more important.

The difference between new and old comb is important, because it points to other odour sources, such as floral scents, brood pheromones or cumulative effects of generations of workers, in addition to comb constituents, as being the critical cues. This finding complicates the conclusions from earlier experiments (Breed & Stiller 1992; Breed et al. 1995a) that showed that various wax compounds are effective as nestmate recognition cues in laboratory bioassays, and that exposure to new comb in the laboratory has a strong effect on recognition.

In conclusion, we have shown that comb wax has a central place in the development of honey bee nestmate recognition cues. This conclusion is consistent with those of Breed et al. (1988, 1995a). We have also demonstrated an important function for individual cuticular signatures. Comb wax provides a unifying, colony-level, set of cues that guards can use in combination with individual signatures to identify nestmates accurately. Comb
wax does not, however, obscure cues that are useful in intra-colonial discriminations (Arnold et al. 1996). The next step is to determine which compounds in wax actually serve as recognition cues.

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