Comb wax mediates the acquisition of nest-mate recognition cues in honey bees

(kin recognition/Apis mellifera/ontogeny/hydrocarbons/eusociality)

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ABSTRACT Honey bees, Apis mellifera, acquire nest-mate recognition cues from wax, the predominant material used in nest construction. Exposure of a newly emerged worker bee to wax-comb substrate significantly reduced the acceptability of that worker to sister bees. Cues acquired from the comb provided colony-specific information about the identity of worker bees; moreover, the effect of comb exposure has been previously shown to override individually produced cues. Food odors (anise oil), when dissolved in paraffin wax, affected worker-recognition characteristics but food odors did not affect these characteristics when fed to bees in sugar candy. Paraffin wax alone did not affect the recognition cues of bees, showing that the wax can be a neutral medium for the transmission of cues. The wax comb in the colony and the hydrocarbon outer layer of the bee cuticle may be a continuous medium for any hydrocarbon-soluble substances used by honey bees in nest-mate recognition; if so, a mechanism by which environmental cues are acquired by honey bees is provided.

Kin and social-group recognition have recently been perceived as factors of major importance in animal behavior and evolutionary biology. Recognition of kin allows animals to bias their behavior to relatives and consequently facilitates kin selection. Ants (1) and bees (2) may simultaneously use recognition cues that are produced by individual workers (self-produced cues) and cues that are acquired from the environment. Acquired cues are particularly interesting, as they give all individuals in the social group a common, unifying, recognition cue. The process of acquisition of environmental cues in social insect recognition systems is not yet well understood (3; for reviews of kin recognition, see ref. 4).

We tested mechanisms by which honey bees (Apis mellifera) might require cues. We demonstrate that beeswax, the primary nesting material of honey bees, mediates acquisition of acquired cues in honey bees. Paraffin wax, when substituted for naturally produced wax, also mediates cue acquisition. Thus, our results provide a key for understanding the complex recognition system of the honey bee.

Nest-mate recognition allows intruders to be discriminated and expelled from social-insect colonies. In this context recognition cues common to all colony members offer the most efficient discriminatory means. Such discrimination cues may be produced by the organisms or acquired from the environment. This form of kin recognition does not require cues to be correlated on the population level with genotype.

Kin recognition may also be important in intracolonial discrimination during feeding and grooming and in preferential rearing of larvae (5-8). In this case, self-produced cues that carry information about genealogical background are most efficient. Bees in social groups can discriminate kin from unrelated individuals (5) and super-sisters (mean r = 0.75, see ref. 18 for complete definition) from half-sisters (7).

In eusocial insects intracolony (subgroup) and nest-mate recognition (group member versus nonmember discrimination) systems may function independently and simultaneously. The former should rely on self-produced cues and the latter on acquired cues. Insight into how each system evolved and how they interact can be gained from studies of the mechanistic bases for each system.

Kalmus and Ribbands (9) showed that honey bees could discriminate nest mates at the colony entrance. Subsequent investigations showed that nest-mate discrimination cues develop when a bee is exposed to the colony environment (10, 11). The source, within the colony environment, of the acquired cues used in the nest-mate recognition system is not fully understood.

Our research purpose was to explore how environmental cues, such as food odors, can be acquired by individual worker honey bees and can influence their recognition characteristics. We tested three hypotheses related to the acceptance of an unfamiliar honey bee by other bees: (i) acceptance is influenced by exposure to comb substrates after adult emergence, (ii) environmental odor cues can be used as recognition cues when transmitted in a wax medium, and (iii) recognition cues derived from comb can override those produced by the bees themselves.

MATERIALS AND METHODS

The experimental design used in these experiments was similar to that used by us in previous work (5, 10, 12) and adapted by others (11). In this design, donor and recipient groups of newly emerged adult bees were established in the laboratory. The groups differed in relatedness (e.g., the bees in one group were either sisters or nonsisters of the other bee group) and/or in some treatment hypothesized to affect recognition cues. At a specified age (usually 5 days after adult emergence) (12), a bee was removed from a donor group and introduced into a recipient group. The behavioral interactions between the donor bee and recipient bees were then observed and scored for 5 min or until rejection, whichever came first. An interaction was scored as a rejection by the recipient bees when biting and stinging of the donor bee occurred; acceptance was the absence of biting and stinging during the 5-min observation period.

To test the effects of comb on recognition, virgin comb was obtained by placing new wax comb foundation in 15 colonies. The queens in these colonies were naturally mated, probably with a large number of males, and the assumed relatedness among colonies was 0.0. While the comb foundation was in the colony, additional comb was constructed and exposed to the colony environment but was not used for food storage or for brood rearing. In addition to the virgin comb, brood comb containing pupae was collected from each colony and main-
tained at 35°C. Bees were collected as they emerged in the laboratory, placed in containers, and given sugar candy (5% pollen) (wt/wt) and water ad lib. Groups of 140 sister bees were established from each colony in 1.0-liter plastic containers with an 8 \times 10-
\text{cm} piece of virgin comb either from their own or from an unrelated colony. Bees in containers with each comb type were marked with enamel paint for identification, a procedure previously shown to have no effect on recognition (12).

The colonies were randomly paired for experiments to assess the effects of comb on recognition characteristics of workers. Each pair of colonies was used to produce four types of recipient groups \((n = 10\) per group, see Results\). Recipient bees could be from the same or a different colony and maintained on comb from the same or different colony as donor bees. Responses by recipients to bees from the same versus another colony were used to test genetic effects (environmental odor effects were controlled by maintaining bees in identical laboratory setups). Responses to bees maintained on comb from the same or a different colony were used to test for colony–comb effects.

Recipient groups were established by splitting the larger groups of 5-day-old sister bees into groups of 10 bees 2–10 hr before testing. Recipient bees were randomly selected from the large groups on comb and placed into a 0.24-liter cardboard container. Donor bees were tested at 5 days of age and were maintained on comb from their natal colony until testing. Only one donor was added to each of the recipient groups, and each recipient group was used only once. Each replicate was observed for 5 min and scored for acceptance or rejection of the donor bee. Rejection was scored when the donor bee was bitten or stung by one of the recipient bees.

The data were recorded as number of acceptances out of 10 introductions. The observer was blind with respect to the identity of the recipient colony and type of treatment. In a separate experiment individual bees that had been exposed to comb for 5 days were placed with groups of 10 bees never exposed to comb; in the control for this experiment both the recipient and the donor groups were exposed to comb. Analysis of the main experiment was done using a two-way analysis of variance on transformed proportions to analyze genetic effects, colony–comb effects, and their interaction.

To test the effects of an artificial odor in wax medium, a mixture of 10 ml of anise oil in 180 g of paraffin wax was poured to cover the bottom of .35-liter cardboard containers. Paraffin wax was used because beeswax might contain odorants that would confound results. An equal number of control containers received the same amount of paraffin wax without anise oil. Four different colonies were used as sources, and an approximately equal number of tests was done from each colony through the experiment to control for colony effects. Groups of 10 bees were established in each container upon emergence.

Donors were maintained under the same conditions as recipients. However, all donors were marked with Testors enamel (Rockford, IL) for later identification. Half the donor containers received scented wax and the other half received unscented wax. Five-day-old donors were introduced to recipient groups for a 5-min period as described above. Recipient groups were from the same genetic source as donors throughout this experiment. Because the interaction between genetic effects and external odor effects was not tested in this experiment, \(x^2\) tests were used to test for differences between the experimental results and controls. Tests for the effects of paraffin wax on the acceptability of bees were performed following the same protocol.

Two experiments tested whether food odors not incorporated into wax may influence acceptance. (i) Anise oil was mixed in sugar candy (without pollen) in the same concentration as in the wax experiments and provided to groups of bees ad lib; controls received candy without anise. (ii) Honey candy or sugar candy was provided to groups. In this experiment both related and unrelated bees were tested. No wax was present in the rearings in either experiment, and testing and maintenance were the same as for the previous experiment.

## RESULTS

In an initial experiment, bees maintained on comb and introduced to sister bees without comb were usually rejected (mean acceptance = 1.3 of 10 donor bees by recipient groups; SE = 0.30, \(n = 6\) colonies), but when the recipient bees were reared on the same comb, the acceptance rate was high (mean acceptance = 7.3; SE = 0.21, \(n = 6\); Mann–Whitney test between bees with and without comb, \(P < 0.01\)).

To further test the effects of source of comb and relatedness on worker honey bee acceptance, these factors were tested in a 2 \(\times\) 2 design:

<table>
<thead>
<tr>
<th></th>
<th>Same</th>
<th>Different</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sister</td>
<td>6.8 ± 0.70</td>
<td>5.1 ± 0.80</td>
</tr>
<tr>
<td>Non-sister</td>
<td>6.2 ± 0.66</td>
<td>3.6 ± 0.62</td>
</tr>
</tbody>
</table>

Worker bees were maintained for 5 days on comb from their natal colony or another colony; after 5 days they were introduced to groups of 10 bees that had been exposed to the same or different comb from the same or different genetic source. Each set of 10 such introductions was treated as a replicate: the mean number (with SE) accepted out of 10 is shown above \((n = 11\) for each cell). We found a strong statistically significant effect of comb source on the acceptance of bees (Table 1). There was no overall effect of siblingship in this experiment, and the interaction term was not significant. The comb-mediated odors appear to override self-produced odors; non-sister donor bees maintained on the same comb as the recipient bees were accepted at nearly the same rate (no significant difference) as sisters. Thus, in the behavioral context of nest-mate recognition, cues derived from comb masked the effects of cues produced by the bees themselves.

Anise odors presented in a paraffin wax medium significantly altered recognition cues of related worker honey bees. In these experiments there was a lower acceptance rate for bees raised on different-odor wax than on same-odor wax (Fig. 1). Acceptance when both donor and recipient bees were reared on unscented paraffin wax alone was the same as previously published results (5) for related bees. Anise-scented sugar candy in the absence of wax had no effect on acceptance (Fig. 1). Honey presented in food (Table 2) also had no effect; in this experiment the effect of relatedness was significant.

Presence or absence of paraffin wax in containers had no effect on the acceptability of bees into groups with different treatment regimes. Sixty-four percent \((n = 50)\) of the donor bees were accepted when the treatments in the donor and recipient containers differed (donor = paraffin wax and recipient = no paraffin wax or vice versa); 77.5% \((n = 40)\) were accepted when the containers were the same. This difference is not significant \((\chi^2 = 1.93)\).

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>(F^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comb</td>
<td>1</td>
<td>50.20</td>
<td>9.53*</td>
</tr>
<tr>
<td>Genetic</td>
<td>1</td>
<td>11.43</td>
<td>2.17</td>
</tr>
<tr>
<td>Interaction</td>
<td>1</td>
<td>2.43</td>
<td>0.46</td>
</tr>
<tr>
<td>Within groups</td>
<td>40</td>
<td>5.27</td>
<td></td>
</tr>
</tbody>
</table>

\(df\), Degrees of freedom; MS, mean squares; \(F\), F-ratio. *\(P < 0.01\).
Odor in Recipient Group

<table>
<thead>
<tr>
<th>Odor in donor group</th>
<th>Wax only</th>
<th>Anise only</th>
<th>Sugar only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wax only</td>
<td>64.9 (57)</td>
<td>45.6 (57)</td>
<td></td>
</tr>
<tr>
<td>Anise and wax</td>
<td>52.1 (48)</td>
<td>89.4 (47)</td>
<td></td>
</tr>
<tr>
<td>Anise only</td>
<td></td>
<td></td>
<td>62.8 (43)</td>
</tr>
<tr>
<td>Sugar only</td>
<td></td>
<td>73.4 (41)</td>
<td>73.7 (38)</td>
</tr>
</tbody>
</table>

Fig. 1. Results are shown as percent acceptance; sample sizes are shown in parentheses. Comparison of odor effects in the presence of wax (same odor vs. different odor) was significant ($\chi^2 = 16.672$, $P < 0.001$). Comparison of odor effects without wax (same odor vs. different odor) was not significant ($\chi^2 = 0.02$).

DISCUSSION

We conclude that wax is a major source of recognition cues in the honey bee. Naturally produced comb modifies the recognition characteristics of individual workers. Artificial wax, impregnated with a scent, also has the capability of modifying worker recognition cues. These results for honey bees parallel those of Gamboa et al. (13), who found that paper wasps can both learn and acquire nest-mate recognition cues from the comb.

The comb effects in our experiments could be explained either by genetically correlated differences among wax combs produced by different colonies or by environmental odors adsorbed into the comb (14). Wax is present throughout the normal life cycle of honey bees and therefore is an efficient medium for transmittal of both types of odor cues.

Our experimental methods are somewhat artificial, involving young bees and laboratory assays of behavior. Breed et al. (10) have shown that the behavior of 5-day-old bees is similar to that of older bees and that the behavioral results obtained in the laboratory are similar to those obtained in parallel experiments at colony entrances. Laboratory experiments allow more precise control of individual variables than field experiments permit.

Wax-mediated cues may be partially derived from food odors. Kalmus and Ribbands (9) reported that food odors applied to entire colonies affected nest-mate recognition in the honey bee. Our experiments suggest that food odors are only a small component of recognition characteristics in the absence of wax, becoming important when incorporated into a wax medium. Food alone may not be an adequate cue for nest-mate discrimination. Individual foragers returning to a colony with a load of foreign (to the colony) food might acquire new cues from that food; thus bees with foreign food cues would run the risk of rejection.

Gamboa et al. (13) have proposed a “scented candle” model for nest-mate recognition. In their model, cuticular hydrocarbons provide a sink for environmentally or genetically derived cues. Like the wax in a perfumed candle, the cuticular hydrocarbons continuously release trapped substances, but the rate of release is slow enough to provide a constant recognition label. Our results show that honey bees probably have the functional basis to support such a model. The interaction between the cuticular hydrocarbons, environmental odors, and the wax comb provides a way of scenting the “candle.” The overriding effect of cues from wax is similar to the effect of acquired cues found in the wasp Polistes fuscatus (13).

Volatile from wax samples (detectable by GLC analysis) differ among colonies and among bee races (14). However, ultimately it will be of interest to determine the relative roles in the recognition system of substances from the wax glands, materials from the mandibular glands added to the wax as it is manipulated into comb, and odors adsorbed into the wax from the environment. In any case, wax-mediated cues probably provide a unifying recognition characteristic for the colony, which could be used in nest-mate discrimination at the colony level (15).

Wax-mediated cues from the environment, wax-mediated cues produced by bees in the colony, and self-produced recognition cues coexist in the honey bee. Breed et al. (5) found that bees in genetically mixed groups could discriminate genetic subgroups in the absence of wax. Several investigators (6–8) have demonstrated that such subgroup discrimination persists when wax is present in observation hives; this discrimination occurs in the contexts of feeding adults, grooming, and larval care. In the experiments reported here, the behavioral context (introduction of unfamiliar bees to established groups) stimulates responses similar to colony defense; persistent genetic discrimination based on self-produced cues might be demonstrated by observations in another behavioral context.

It is interesting to speculate on the evolutionary origins of nest-mate and kin recognition cues (13, 16). If, as seems likely, polyandry were derived subsequent to eusociality in the ancestry of the honey bees, then wax-mediated nest-mate recognition may be a secondary adaptation, in response to selection for a colony-level recognition system. When high genetic diversity due to polygyny or polyandry is present, genetic recognition systems fail, because in all the individual colony samples, recognition alleles will approach the population sample of alleles (15). In many polygynous species, for example, the nest-mate recognition system functions weakly or not at all (15). In the honey bee, which has a high degree of polyandry (17), the use of wax-mediated cues may compensate for genetic diversity found among the workers. Alternatively, nest recognition by solitary bees may have led to nest-mate recognition; not enough is known about nest recognition by solitary bees to evaluate this possibility. In either case, wax-mediated recognition cues that function in spite of lowered relatedness among the workers can operate at a colony level.

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