RAPID COMMUNICATION

Metabolic Test of Volatile Odor Labels as Kin Recognition Cues in Honey Bees

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ABSTRACT The ability of worker honey bees to discriminate between kin and non-kin was revealed using a metabolic bioassay. Groups of workers (test group) exposed to odors of unrelated bees (odor group) showed short-term increases in metabolic activity. The magnitude of the metabolic reaction was closely correlated with the genetic relationship between odor and test groups. Worker test groups reacted to both worker and drone odor groups whereas drone test groups did not respond in this bioassay. The results support the theory of inclusive fitness.

Nestmate and kin recognition in social Hymenoptera are central features in evolutionary sociobiology, especially when focusing on the theories of inclusive fitness as defined by Hamilton ('64a,b). In kin selection theory the evolution of sterile worker castes strongly depends on the ability of the workers to discriminate between related and unrelated individuals. Several behavioral studies in honey and sweat bees suggest that genetically determined odor labels are used as recognition cues by individual workers (Greenberg, '79; Breed, '83; Getz and Smith, '83; Page and Erickson, '84, '86; Getz et al., '86). Behavioral observations, however, are potentially susceptible to subjective indices and often difficult to reproduce. Until now there has been no experiment which rigorously excluded stimuli other than volatile odors as recognition cues. In addition, honeybees frequently do not operate as individuals but only within the context of a group. For example, the alarm reaction, which is important in preventing intrusion of non-nestmates into the colony, can only be elicited by pheromones when bees are in groups (Southwick and Moritz, '85). Reactions of groups of bees to alarm pheromones could be accurately quantified in a metabolic bioassay. Bees exposed to the pheromone show a typical increase in metabolic activity which is closely correlated to behavioral patterns of the alarm reaction (Moritz et al., '85). In this study we test, in a similar quantitative approach independent of potentially subjective behavioral indices, the ability of groups of workers to distinguish between drones and workers of varying genetic relationships. We also rigorously exclude stimuli other than volatile compounds as recognition cues in this metabolic bioassay.

MATERIALS AND METHODS

Three unrelated breeder colonies supplied drones and queens for 12 experimental colonies in an incomplete diallel with two replicates for each mating type as shown in Table 1. Queen excluders at the flight entrances prevented drifting of drones in all colonies. Semen of large numbers of drones (more than 100 per breeder colony) was pooled and 1:10 (semen:diluent) diluted in a 0.05 M Tris-HCl buffer (pH 8.7) containing 0.1% lysine, 0.1% arginine, 1.1% NaCl, 0.1% glucose (Moritz, '84). The semen was thoroughly mixed and reconcentrated by centrifugation (1,000 gav). This procedure ensured a homogenous mixed semen pool (D) of drones of each queen breeder colony (M) which could be used as genetically identical "males" for each mating type (Moritz, '83).

Virgin queens (Q), offspring of the corresponding breeder queen, M, were then instrumentally inseminated with this mixed

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TABLE 1. Three unrelated breeder colonies (M₁, M₂, M₃) supplied drones and queens for 12 experimental colonies

<table>
<thead>
<tr>
<th>Queen mother</th>
<th>M₁</th>
<th>M₂</th>
<th>M₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₁</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M₂</td>
<td>T₁₁a, T₁₁b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M₃</td>
<td>T₁₃a, T₁₃b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

An incomplete diallel mating scheme with one replicate per cell was performed in order to produce the experimental colonies Tᵢₘ, where i is the index for the queen mother and m is the index for the corresponding sire queen (mixed semen pool).

TABLE 2. Pedigree for experimental colony T₁₂

<table>
<thead>
<tr>
<th>Queen mother</th>
<th>Drone mother</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₁</td>
<td>100 Drones</td>
</tr>
<tr>
<td>Queen Q₁</td>
<td>Inseminated with semen pool D₂</td>
</tr>
<tr>
<td>Colony T₁₂</td>
<td>15 Test groups per colony</td>
</tr>
</tbody>
</table>

Fig. 1. Diagram of the experimental setup. Air is pumped through the test groups (TEST) and the oxygen concentration is measured after absorption of CO₂ (in 20% NaOH) and water vapor (in CaCl₂). The direction of the air flow is indicated by arrows. The combined oxygen consumption of test and control group (CONTROL) or test and odor group (ODOR) is monitored (channel 1). The oxygen concentrations in odor and control groups are monitored separately (channels 2 and 3) to determine the actual oxygen consumption of each test group.

These test groups were kept separately in the dark (30°C, 60% relative humidity, candy ad libitum) in ventilated air tight containers (200 ml) prior to the test. Fresh air was pumped through these containers for 6 h in order to dilute possible hive-specific compounds not produced by the bees. Kalmus and Ribbands (’52) showed that such environmental odors might override possible genetic effects.

In order to quantify the reaction of honey bees to odors we measured the rates of oxygen consumption of test groups exposed via an air flow-through system (Fig. 1) to the odor of other honey bee groups (odor groups). These odor groups were taken from colonies of various degrees of relationship. Each test group was initially exposed to a group of worker bees from its own colony (control group) until all three groups (i.e., test, odor, and control) attained resting metabolism. The test group was then exposed for 60 sec to air passing through the odor group. The difference between resting level and maximal metabolic activity (ΔO₂) was used to quantify the reaction of the test group. Care was taken that test, odor, and control bees were not exposed to each others’ odors before they were tested.

The asymmetrical pedigree coefficient of relationship, G (Pamilo and Crozier, ’82) had to be modified to quantify the relationship between groups. G*, the pedigree coefficient...
Table 3. Possible relationships ($G^*$) of a worker test group of colony $T_{12a}$ to worker and drone odor groups used in the experiment

<table>
<thead>
<tr>
<th>Odor group from colony no.</th>
<th>$G^*$ for Worker group</th>
<th>Drone group</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{12a}$</td>
<td>0.500</td>
<td>0.500</td>
</tr>
<tr>
<td>$T_{12b}$</td>
<td>0.750</td>
<td>0.125</td>
</tr>
<tr>
<td>$T_{13a,b}$</td>
<td>0.250</td>
<td>0.500</td>
</tr>
<tr>
<td>$T_{23a,b}$</td>
<td>0.750</td>
<td>0.250</td>
</tr>
<tr>
<td>$T_{33a,b}$</td>
<td>0.250</td>
<td>0.250</td>
</tr>
<tr>
<td>$T_{32a,b}$</td>
<td>0.500</td>
<td>0.125</td>
</tr>
</tbody>
</table>

Similar relationships can be computed for any other test group.

Results

Figure 2 shows a typical response of a test group to an unrelated odor group. Each of 15 test groups per colony was exposed to a set of 4 drone or 5 worker odor groups covering all degrees of relationship shown in Table 3. Between these exposures the test group was exposed for at least 10 min to the odor of the control group. The sequence of odor groups was randomized for each test series. The absolute metabolic reaction of test groups to odor groups was highly colony specific (ANOVA, $F = 22.0; p < 0.01$). Therefore, the average reaction to unrelated odor groups was set equal to 1 to compare the results from test groups of different colonies. The closer the relatedness of the tested worker bees to the worker odor group, the smaller is the reaction of the test group. Plotting relationship versus metabolic reaction reveals that 96% of the variance is explained by regression ($r^2 = 0.96$; Fig. 3). Worker bees also did respond to volatile compounds of drone odor groups. Also here more than 90% of the reaction is explained by regression of reaction on the relatedness ($r^2 = 0.98$). A crucial result is the difference in reactions shown by worker test groups to brother drones and sister workers of the same colony. The reaction to the drone odor ($22.01 \pm 1.95 \mu l O_2/bee/min$) is significantly higher than to the sister workers ($4.08 \pm 0.96 \mu l O_2/bee/min, t = 10.7, p < 0.01$) and in the same range as reactions to worker groups with $G^* \equiv 1$. The relationship of a worker group to a brother drone group is $G^* = \frac{1}{2}$ whereas the relationship of two sister worker groups of the same colony is close to $G^* = 1$. 

Fig. 3. Regression of group relationship on relative reaction of worker test groups ($O_{drone} = -0.85 \pm 0.06, p < 0.001$; $O_{worker} = -0.94 \pm 0.05, p < 0.001$). Drone odor groups, $\bigcirc$; worker odor groups, $\bullet$. Each point represents the mean of 12 test groups $\pm$ SE.
Drones did not respond to odors of workers or drones in this metabolic bioassay. In no case did drone test groups \((n = 12)\) show a change in metabolic activity when exposed to odor groups of any of the experimental colonies.

**DISCUSSION**

The metabolic reaction used in our bioassay is meaningful only in the light of previous behavioral studies which clearly revealed the potential of honey bee workers to discriminate between related and unrelated nestmates (Boch and Morse, '82; Breed, '81, 83; Breed et al., '85; Page and Erickson, '86; Noonan, '85). Getz et al. (86) was able to condition workers on the odors of super- and half-sisters. Our bioassay quantitatively supports the behavioral findings and clearly demonstrates that worker bees use highly volatile odors in discriminating related and unrelated drones and workers. Therefore odor cues seem to be crucial for the identification of kin in honey bees. Though environmental odors have been suggested as nestmate recognition cues (Kalmus and Ribbands, '52), they are apparently overridden by genetically determined volatile compounds in our bioassay. As all tested queens were introduced into nonrelated colonies chosen randomly out of a large population, environmental cues should result in random distributed reactions and a regression from reaction on relationship should not significantly differ from 0. Reasons for the low environmental effect in this study might be that all colonies were placed in the same apiary, foraging on similar nectar and pollen sources. Furthermore, the test groups were flushed with fresh air prior to the test for 6 h which might dilute possible environmentally determined hive odors.

The negative results of the drone test groups may be in line with field observations in which drones were less accurate in returning to their home colony and show a large tendency to drift between colonies (Rinderer et al., '85). In terms of inclusive fitness theory the selective advantage for drones recognizing kin should be small since they do not reduce their individual fitness in favor of related nestmates. However, we realize that the negative result in this particular bioassay for drones does not mean that in general they are unable to discriminate odors of different colonies or groups of workers. Under natural conditions they show a high capability of detecting low pheromone concentrations (Pain and Ruttner, '63; Renner and Vierling, '77). Shellman et al. (85) showed that males of *Polistes fuscatus*, a social wasp, do recognize their male nestmates.

Though honey bee workers have the potential for discriminating kin, this does not tell us the selective importance of this character under natural conditions. Breed et al. ('84), for example, could not find any evidence for queen larva discrimination in cross-fostering experiments. Moritz and Hillesheim ('85) suggested that natural selection in honey bees simultaneously operates individually on queens, drones, and workers as well as on the colony level. The role of kin selection for social evolution as a major selective force cannot be definitely determined with our or similar experiments. However, since only detailed behavioral observations or a highly sensitive experimental setup reveal properties of kin recognition, we think that kin selection in honey bees at the present state of evolution plays only a minor role. Nevertheless, kin recognition will have a selective advantage in socially structured populations as predicted by inclusive fitness theory. A central question, however, remains open: Did sociality evolve because of kin selection or have kin recognition mechanisms evolved as a result of sociality?

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


