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En garde: rapid shifts in honeybee, *Apis mellifera*, guarding behaviour are triggered by onslaught of conspecific intruders

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In a recognition context, discriminating agents decide whether to accept or to reject. In the honeybee, entrance guards distinguish between nestmates and intruders. Those below a threshold of dissimilarity are accepted. However, the threshold is dependent on ecological conditions and may shift to become either restrictive or permissive, depending on the frequency of intrusion and cost of admitting an intruder. Previous research on the honeybee has shown that both the number of guards and their acceptance threshold to conspecific non-nestmates can change dramatically over weeks owing to changing nectar availability and robbing intensity. This project investigated whether these changes could also occur rapidly, over minutes, in response to sudden increases in conspecific intruders (robber bees). We induced high levels of intrusion at nest entrances and determined changes in the number of guards, the number of fights per guard, and the acceptance thresholds of guards. Our results show a rapid response within 15 min. At the level of individual guards, acceptance declined from 83 to 55% for nestmates and 67 to 43% for conspecific non-nestmates. Also, per individual guard, mean fights increased from 0.005 to 0.06 fights/guard. At the colony level, the mean number of guards at the entrance rose from 1.9 to 2.3, and overall acceptance in a 3-min trial declined from 74 to 52% for nestmates and 59 to 30% for conspecific non-nestmates. These results show that honeybees can make rapid behavioural shifts at both the colony and the individual levels.

Keywords: adaptive response; *Apis mellifera*; nestmate recognition

Animals may increase their fitness by making appropriate recognition decisions. For example, recognizing kin may provide both direct and indirect positive benefits (for reviews, see Hamilton 1964; Fletcher & Michener 1987; Waldman 1988; Hepper 1991; Holmes 2004; for some examples, see Cheney & Seyfarth 1982, vervet monkeys; Mateo 2003, rodents; Mehdiabadi et al. 2006, social microbe, *Dictyostelium purpureum*; and Schausberger 2007, predatory mites, *Phytoseiulus persimilis*, in which kin are helped more than nonkin). Alternatively, it is equally important to recognize potential threats and to act aggressively (Sherman et al. 1997), which is especially relevant for vulnerable locations like a nest entrance. This is evident within the social insects, as they normally possess entrance guards to recognize and exclude intruders while allowing nestmates to enter (termites, Wilson 1971; ants, Hölldobler & Wilson 1990; wasps, Gamboa et al. 1996; and honeybees, Butler & Free 1952; Moore et al. 1987; Breed et al. 1990). Honeybees are a good model system in which to study recognition because colonies and individual bees show adaptive shifts in their recognition behaviour in response to changing threats.

Honeybees from different colonies will rob from one another's honey supplies (Free 1977), which could lead to

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the death of the victim colony. This makes the guard and her recognition abilities critical for colony survival. The threat of robbing from conspecific non-nestmates is greater when nectar is in short supply (Seeley 1985). During these times, victim colonies respond adaptively, and at the colony level, more guards are recruited to the nest entrance (Downs & Ratnieks 2000). However, each individual guard will also respond. Guards base their acceptance or rejection on a comparison between the odour of each incomer with a colony odour 'template' (Getz 1982; Lacy & Sherman 1983; Couvillon et al. 2007). They accept individuals that are below a threshold of dissimilarity (Reeve 1989; Crozier & Pamilo 1996). When nectar is in short supply and robbing is a threat, guards adopt a non-permissive acceptance threshold and reject many incomers, which include almost all conspecific non-nestmates and even some nestmates (Downs & Ratnieks 2000).

In a field study in southern Florida, U.S.A., these changes in guard number and in individual guard acceptance thresholds occurred over several weeks during the gradual shift from nectar dearth to nectar abundance. As citrus trees came into bloom, robbing by conspecifics diminished from a high frequency to zero (Downs & Ratnieks 2000). These results represented the first field data to support the predictions of the acceptance threshold model, which describes a context-dependent response to recognition that is based on the frequency of intruder contact and cost of errors (Reeve 1989). However, it was not previously known if honeybee guards are also capable of responding rapidly, in minutes, to increased threats of robbing.

Here we induced sudden increases in the number of conspecific robbers at hive entrances during a period of nectar dearth. Within minutes, there was an increase in the number of guards and in the number of fights per guard and the acceptance threshold of each individual guard quickly became less permissive. The honeybee colonies showed a rapid, adaptive response to increased numbers of robbers, and this response occurred at both the individual and the colony levels.

METHODS

Study Details

We conducted this study in an apiary in Sheffield, U.K., using a behavioural assay of discrimination by natural entrance guards (Downs & Ratnieks 2000; M. Couvillon, G. G. F. Roy, & F. L. W. Ratnieks, unpublished data). We captured returning foragers without pollen in individual vials and chilled them until they could move but not fly. We then allowed them about 1 min to warm to ambient temperature and placed them, one at a time using forceps, on the entrance platform (approximately 20×10 cm) of a discriminator colony. We then observed the reaction of the guards for 3 min. The entrance observer, blind to the source of the introduced bee (Gamboa et al. 1991), scored the introduction as a rejection if the guards stung, grappled, pulled, or bit the introduced bee. We scored the introduction as acceptance if the introduced insect was left alone or allowed to enter the hive after being inspected by one or more guards. We classed any bee that was not inspected during the 3 min on the platform or that entered the colony without inspection as an acceptance. In most cases (>95%), introduced bees were immediately contacted by guards and either accepted or rejected. We used seven hives in total, five of which acted as discriminator hives and received nestmate and non-nestmates introductions. The remaining two hives served as either the robber hive (hive X) or a source of control non-nestmates worker bees (hive Y).

Data Collection

Quantifying guard numbers and intruder intensity

We collected data from 2 to 24 October 2006, from 1030 to 1600 hours, on days when the temperature was at least 13 °C and foragers were active. This time of year was ideal for the experiment. The autumn weather was warm enough for the bees to fly, but there was little nectar available. The lack of nectar meant that it was easy to stimulate robbing (see below) and that guards were unlikely to accept all intruders (Downs & Ratnieks 2000). The prerobbing acceptance levels were similar to those previously reported for this time of year (Downs et al. 2000; Couvillon et al. 2007, unpublished data).

To investigate changes in guarding intensity and natural intrusions, it was necessary to quantify the numbers of guards and natural fights (as a proxy for intrusion) between guards and intruders observed on the entrance platforms at the start of each series, both before and during the robbing. We identified guards by their characteristic posture of standing with raised forelegs and forward-facing antennae (Butler & Free 1952) and other behavioural idiosyncrasies, specifically not flying away and behaving aggressively towards bees attempting to enter the hive. We counted fights for two reasons. First, an increase in fights on the platform was a measure of increased robbing. Second, the total number of fights and the number of fights per guard were measures of the colony and individual level responses. In particular, it was important to determine whether the increase in counted fights was due simply to more guards (colony response) or to each individual guard rejecting/fighting more (individual response).

Quantifying acceptance threshold of guards

We determined acceptance of both nestmate and non-nestmates workers both before and after the induction of frequent robbing by hive X. In one series of introductions, each of the five discriminator hives (A–E) received in random order one nestmate, one non-nestmate from the robbing hive (X), and one non-nestmate from the control hive (Y), making a total of 15 introductions. We used non-nestmates from two hives, one of which was induced to rob, to determine if guards learn to recognize and selectively reject workers from the robber hive.

We performed four trials, each taking 2 consecutive days. The first day established baseline, prerobbing, acceptance levels through six series of introductions. On

the second day, we completed two additional series in the morning, before high robbing was induced, to confirm that the guards were accepting at levels similar to the previous day. We then immediately stimulated robbing by placing a shallow dish with 100 ml of 2 M sucrose solution on the entrance platform of the robber hive (hive X). The platform was covered with a piece of glass to prevent foragers from other hives from finding the syrup. Foragers from hive X, upon discovering this rich and nearby food source, would re-enter their hive and perform recruitment dances (von Frisch 1967), recruiting many foragers to leave the hive and to examine the nearby area, which included the five discriminator hives located 1–4 m away. Data collection resumed 15 min later. We performed eight postrobbing series of introductions. We refilled the 2 M sucrose solution as needed throughout the afternoon to maintain high robbing levels. At the end of the day, we removed the sucrose solution.

We then left the hives undisturbed for at least 1 week before performing the next trial, to allow guarding behaviour to return to prerobbing levels. Based on the life cycle of honeybees (Seeley 1985) and evidence that guard turnover happens frequently, with each guard working on average for a day (Breed et al. 1992), the guards present in each trial should be different so that the data from different trials are independent. The use of five discriminator hives also ensured adequate replication. A total of 960 bees were introduced throughout the entire experiment.

Comparing acceptance of 'first contact' guards with overall acceptance

For over half of the trials, in addition to the overall 'verdict' (accept/reject) in the 3 min following the introduction of a nestmate or non-nestmate, we also separately recorded the reaction of the first guard that contacted the introduced bee. This was done, as well as scoring the increase in fights per guards, to investigate differences in individual- and colony-level responses. We wished to determine whether the cumulative decrease in the proportion of nestmates and conspecific non-nestmates accepted was due to changes in the number of guards (i.e. more guards make it harder for conspecifics to be accepted) or shifts in the threshold of individual guards towards being less permissive.

Statistics

We used a generalized linear mixed model (GLMM) with binomial error structure and the free software environment R for statistical computing and analysis. This allows for both fixed and random factors to be investigated and is well suited to nonparametric data. To test for the effect of frequent robbing on acceptance, we compared the acceptance of introduced nestmates and non-nestmates before and after robbing was induced. The dependent variable was whether or not an introduced bee was accepted. The model is presented in more detail in the supplementary online information (Table S1).

RESULTS

Guards became significantly less accepting after robbing by hive X was induced (GLMM: $t_{946} = -5.44$, $P < 0.0001$, Fig. 1, Supplementary Table S1). This decrease in overall acceptance was seen for both nestmate (from 74 to 52%) and conspecific non-nestmate (from 59 to 30%) bees (Fig. 1). This effect was verified in the model by the nonsignificant interaction between nestmate status and robbing (GLMM: $t_{945} = -0.78$, $P = 0.44$, Supplementary Table S1), meaning that robbing caused a decrease in acceptance of both nestmates and non-nestmates. Additionally, acceptance or rejection of the introduced bees by the first guard to contact it did not significantly differ from the overall acceptance or rejection of the introduced bee (nestmates: $\chi^2_1 = 0.004$, $P = 0.95$; non-nestmates: $\chi^2_1 = 0.306$, $P = 0.58$), showing that the acceptance threshold of individual guards became less permissive when robbing increased. In particular, the increased rejection of introduced bees was not simply because there were more guards and a greater chance of being rejected. We were able to pool the data across trials in this analysis because the GLMM showed that there was no effect of trial or type of non-nestmate (see below).

As shown above, guards showed a highly significant ability to discriminate nestmates from non-nestmates (GLMM: $t_{946} = -6.07$, $P < 0.0001$, Fig. 1). However, there was no significant difference in the acceptance of non-nestmate worker bees from hive X, the robbing hive, versus hive Y, the nonrobbing hive (GLMM: $t_{629} = -0.71$, $P = 0.48$, Supplementary Table S1). There was also no effect of trial (GLMM: $t_{469} = -0.90$, $P = 0.37$) or day (GLMM: $t_{469} = 0.57$, $P = 0.57$), which allowed us to remove these

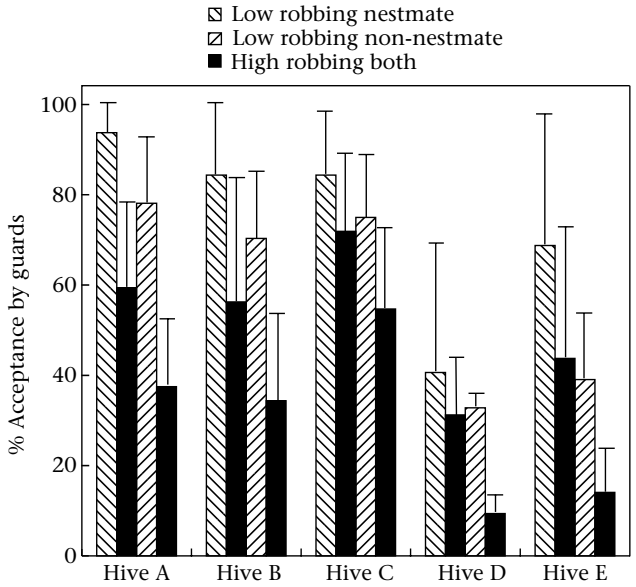

Figure 1. Overall guard acceptance per hive over 3 min significantly declines after robbing from a nearby hive is stimulated. Data are pooled across trials. For each hive, the first two bars are for nestmates and the last two bars are for non-nestmates. Error bars indicate the standard deviation between trials of the means calculated from the pooled data.

factors from the model. The nonsignificance of day indicates that the two prerobbing series performed on the second day of each trial were not significantly different from the six prerobbing series of the first day of the trial. There was an effect of hive, with two hives being significantly less accepting, so hive was retained as a factor in the model. However, even though two hives were more discriminating, all five discriminator hives displayed the significant trend of decreasing acceptance following the increase in robbing, showing that we observed a general result (Fig. 1).

The number of guards at a hive entrance increased significantly, but only by approximately 20%, from an average of 1.9 before robbing was induced to 2.3 after ($t_{159} = 2.89$, $P = 0.004$, Fig. 2). The number of simultaneous fights on the entrance platform increased approximately 1000%, from an average of 0.005 to 0.06 fights per guard at any one time (Wilcoxon signed rank: $T = 99.0$, $N = 14$, $P = 0.004$, Fig. 3). Therefore, both guard number and fights per guard increased with robbing.

The effect of robbing was seen immediately. Average overall acceptance decreased from 80 to 58% for nestmates and 61 to 34% for non-nestmates in series 7 and 8, the last prerobbing series performed on the second day, versus series 9 and 10, the first performed postrobbing series (Wilcoxon signed rank: $T = 53.0$, $N = 10$, $P = 0.01$).

DISCUSSION

The results show clearly that honeybee colonies and individual bees can make rapid adaptive shifts in guarding behaviour in response to sudden increases in robbing intensity. Frequent robbing caused guard numbers to increase significantly, although only by 20%. However, we saw a significant, 10-fold increase in the number of fights per guard, indicating that the increase in fights was not just from more guards being present on the platform. Additionally, our technique of inducing robbing provides a novel method of changing the frequency of interactions with undesirable recipients, which is predicted to be a catalyst for shifting acceptance thresholds (Reeve 1989; Downs & Ratnieks 2000). Context-dependent shifts in acceptance have been previously shown (Starks et al. 1998; Holen & Johnstone 2004; Liebert & Starks 2004). However, our study shows that the changes might occur in a matter of minutes for both the individual guard and the colony as a whole.

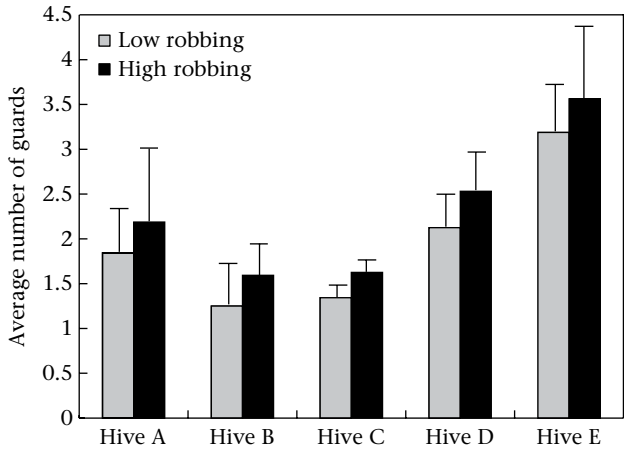

Figure 2. The average number of guards significantly increased in all five hives after robbing. Data were averaged across all four trials. Error bars indicate the standard deviation between trials.

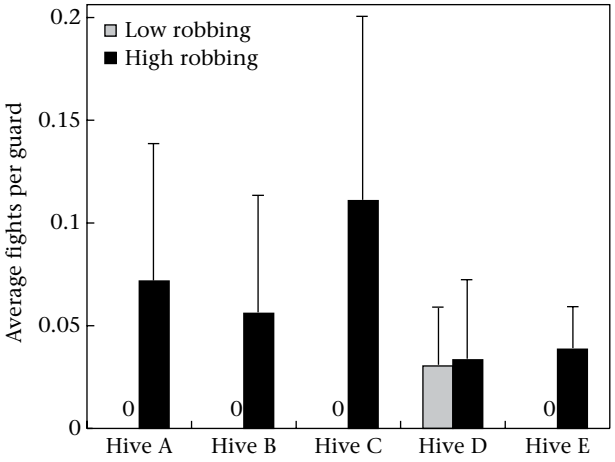

Figure 3. The average number of fights per guard increased in all five hives. '0' indicates where there were no fights. For the purposes of this graph, data were pooled for trials, and error bars indicate the standard deviation between trials.

The likelihood of acceptance of introduced bees by the guards became significantly less, resulting in 22% fewer nestmates and 29% fewer conspecific non-nestmates being accepted. This decrease in overall acceptance (i.e. whether an incoming bee was accepted over 3 min by all guards) was also reflected in the behaviour of the first guard to contact the incomer. Acceptance decreased by 20 and 16%, for nestmates and conspecific non-nestmates, respectively. These proportions were not significantly different from the overall decrease, showing that the overall decreased acceptance was not just due to more guards being around potentially to reject. Given that there was only a 20% increase in the number of guards, the main component of the increased overall rejection of incomers was the less permissive acceptance threshold of individual guards.

As expected, the less permissive acceptance threshold following the increase in robbing resulted in the rejection of more nestmates as well as non-nestmates. This is expected because nestmates and conspecific non-nestmates should have overlapping cue distributions (Lacy & Sherman 1983; Reeve 1989; Couvillon et al., unpublished data). A restrictive acceptance threshold is predicted to affect both nestmates and non-nestmates, resulting in an increase in rejection errors of nestmates and a decrease in acceptance errors of non-nestmates (Couvillon & Ratnieks 2008).

Our results showed no difference in the treatment of non-nestmates from the robbing hive (X) versus those from the nonrobbing hive (Y). This may be because guards were unable to distinguish these two categories of workers. However, even if they could be distinguished, a more adaptive response may simply be to become more globally rejecting, as the presence of a robber is usually indicative of a time period when robbing is likely, which is predicted

to cause the acceptance threshold to shift to a less permissive position (Reeve 1989; Sherman et al. 1997). The latter seems more likely, especially as it was noted how nestmate acceptance also decreased.

Having a response at both the level of the individual and the colony level is a property frequently described in social insects (Robinson 1992; Gordon 1996; Bonabeau et al. 1997; Jones et al. 2004). Each individual insect responds to her local cues; however, there emerges at the colony level a response that is not the simple summation of the interactions (Camazine et al. 2001). The upregulation in defensive behaviour described here is a good example of this property.

Once an intruder is detected, does an individual guard change her own behaviour, or is a signal given to the colony that increased security is needed? That we observed an individual and a colony level response suggests both might occur. A guard might herself monitor the frequency of intrusions and change her behaviour, depending on experience. Additionally, however, the increase in guard number makes it likely that signalling is occurring, probably via alarm pheromones. When a guard bee is disturbed, she often will respond by raising her abdomen and extruding her stinger, a behaviour known to release alarm pheromone (Free 1987). One response from the release of alarm pheromone is the increase of bees at the entrance (Maschwitz 1964). A recruited guard, upon detecting the pheromone, might immediately adopt a nonpermissive acceptance threshold. One way to test this hypothesis is to divide guards into one group that will be contacted by several intruders and another group that will not be contacted. The latter group could be isolated while the first group experiences intruder introductions. If the noncontact guards then display an a priori nonpermissive acceptance threshold, this would suggest that intruder monitoring is not essential and, instead, that the need for increased security is communicated in other ways.

Honeybees live in fluctuating environments, especially regarding nectar availability, which translates into different levels of threats to security. These circumstances select for workers that are able to respond rapidly on multiple levels to changes in the defensive environment. Nestmate recognition is a good model for studying multilevel organization in honeybees because responses can be quantified. Further research is needed to understand the mechanisms involved in this complex response.

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.anbehav.2008.08.002.

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