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Honeybee, *Apis mellifera*, guards use adaptive acceptance thresholds to limit worker reproductive parasitism

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To protect their colonies from robbing by conspecifics, honeybees have evolved nest-guarding behaviour. Guards adjust their acceptance threshold so that, as the likelihood of robbing increases, fewer non-nestmates are admitted. In addition to the possibility of robbing, queenless colonies may be infiltrated by reproductively parasitic non-nestmates. We tested the hypothesis that queenless colonies would be more discriminatory of non-nestmates than queenright colonies. As predicted, queenless colonies accepted significantly fewer non-nestmates (from queenright colonies) than they did nestmates, whereas queenright colonies did not differentiate significantly between the two sources. This trend continued once laying workers became active in queenless colonies. Thus there is evidence that queenless colonies are more discerning against potential reproductive parasites than queenright colonies. We also tested the hypothesis that as the likelihood of an intruder being a reproductive parasite increased, guards would become less permissive of allowing it entrance to the colony. Queenright colonies accepted significantly more non-nestmates from queenright colonies (no active ovaries) than they did non-nestmates from queenless colonies (many with active ovaries). However, queenless colonies did not make this distinction. We suggest that to queenless colonies all non-nestmates are potential parasites.

Animals require the ability to distinguish competitors, predators, prey, potential mates and their kin. The cues that animals use to distinguish individuals from these different groups may overlap, leading to the potential for costly recognition errors. Conspecific acceptance threshold theory assumes that an animal evaluates the number of 'markers' that it shares with a second individual, and adjusts its behaviour towards that individual based on the degree of similarity and the context (Getz 1981; Waldman 1987; Reeve 1989). For example, in the cockroach *Blattella germanica*, siblings are preferred as social partners, but nonsiblings are preferred as mating partners (Lihoreau & Rivault 2009). Thus the amount of variation deemed acceptable, or the acceptance threshold, is context specific (Getz 1981; Waldman 1987; Reeve 1989) and animals adjust their acceptance threshold accordingly.

The contents of a honeybee (*Apis* spp.) colony are nutritionally valuable, and attacks against a colony to gain these resources may result in its death (Winston 1987; N.C. Chapman, personal observations). To defend their colony from robbing by conspecifics, cavity-nesting species deploy guard workers at the entrance to their nest. These middle-aged bees inspect a proportion of the workers entering the colony and often reject workers from other colonies that attempt to enter (Butler & Free 1952; Herman & Blum 1981; Moore et al. 1987). To do this, guard workers must be able to differentiate between nestmates and non-nestmates. It is thought that odours from the comb and pheromones from the queen are integrated into the cuticular hydrocarbon profile of workers, and that subtle differences between the average profiles of workers from different colonies enable nestmate recognition (e.g. Breed et al. 1988, 1992, 1998). Workers attempting to enter a colony that differ from a colony's recognition signature above the acceptance threshold (Getz 1981; Waldman 1987; Reeve 1989) are usually ejected from the colony entrance (Seeley 1985; Winston 1987).

There are two possible errors associated with guarding: a guard may mistakenly reject a nestmate or mistakenly accept a non-nestmate. These two errors, and thus a guard's acceptance threshold, must be balanced based on the likelihood of a non-nestmate attempting to enter a foreign colony, and the costs of allowing such entry, which vary with changing conditions (Reeve 1989; Downs & Ratnieks 2000; Couvillon et al. 2008). When foraging conditions are good, because of high availability of nectar and pollen in the field, few bees attempt to rob conspecific colonies (Downs & Ratnieks 2000). This is because there is likely to be less

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risk associated with collecting food from flowers than with attempting to gain entry to a foreign colony containing an unknown quantity of resources (Downs & Ratnieks 2000). Thus the likelihood of guards encountering non-nestmates under these conditions is low, the risk of robbing is low and the cost of allowing some non-nestmates to join the colony is also low. For these reasons the maximum dissimilarity from self that is acceptable should increase in times of floral abundance and may even result in 100% of non-nestmate workers being allowed to enter a colony in an experimental setting (Downs & Ratnieks 2000).

When robbing becomes profitable, because of a dearth of forage, the number of non-nestmates attempting to gain entry into colonies increases (Downs & Ratnieks 2000). The acceptance of a large number of robbers comes at significant cost to a colony, which can rapidly lose stored food. Extensive robbing may result in the colony's death by starvation and population depletion arising from worker–worker combats (Winston 1987; N.C. Chapman personal observations). Thus, when guards encounter a large number of non-nestmates attempting entry, they adjust their acceptance threshold and reject more non-nestmates. In one study, 25% of non-nestmates were accepted when conditions were poor, compared to 100% when conditions were good (Downs & Ratnieks 2000). Under poor foraging conditions it may be better to reject all non-nestmates and incur the smaller cost of mistakenly rejecting a few nestmates because of the decreased acceptance threshold.

Inclusive fitness theory (Hamilton 1964a, b) explains why, in honeybee colonies that have a queen, workers do not normally lay eggs themselves but raise the queen's offspring. In temperate strains of *A. mellifera* fewer than 1 in 10 000 workers have fully formed eggs in their ovaries if a queen is present (Ratnieks 1993). Worker reproduction is tightly regulated because the few eggs that are laid by workers are recognized as such and are policed (eaten; Ratnieks & Visscher 1989; Ratnieks 1993, 1995; Visscher 1996; Wenseleers et al. 2004a, b; Beekman & Oldroyd 2005). Thus, in temperate *A. mellifera* colonies with a queen, and the three other *Apis* species investigated thus far, workers do not contribute significantly to the production of males (Visscher 1989; Halling et al. 2001; Oldroyd et al. 2001; Wattanachaiyingcharoen et al. 2002), which arise from unfertilized eggs.

In contrast to the situation when colonies contain a queen, hopelessly queenless colonies (a colony that has no queen and no chance of raising a new one) are vulnerable to worker reproductive parasitism (WRP) by workers from other nests. Workers in a queenless colony activate their ovaries, produce eggs and, critically, must decrease the rate at which they police these eggs (Miller & Ratnieks 2001; Nanork et al. 2005, 2007) to raise reproductive drones before the colony perishes. If workers removed all worker-laid eggs, then the colony would produce no offspring; if they removed none then the colony may be heavily parasitized by unrelated workers if these workers gained entry to the colony. Non-nestmate workers have been found to have higher reproductive success than nestmates in queenless colonies of three honeybee species (Nanork et al. 2005, 2007; Chapman et al. in press). Although some eggs produced by non-nestmates may be removed, the higher reproductive success of non-nestmates compared to nestmates (Nanork et al. 2005, 2007; Chapman et al. in press) proves this mechanism, if it exists, to be inefficient.

A possible mechanism by which queenless colonies could defend themselves against WRP is to co-opt the pre-existing mechanism, guarding, to reduce the rate at which unrelated workers join a colony (Chapman et al. 2008). Just as guards adjust their acceptance threshold depending on the likelihood and cost of robbing, so too they could become less accepting of non-nestmate workers when the threat of WRP increases. The proportion of non-nestmate workers present in an *A. mellifera* colony declines significantly once it is made queenless (Chapman et al. in press). This suggests a shift in acceptance thresholds with increasing risk of WRP and the expectation that queenless colonies will reject significantly more non-nestmates than queenright colonies. If guard bees can recognize whether an intruder has active ovaries, this provides a mechanism by which guards could repel workers that have a greater likelihood of becoming parasites. Increased aggression towards workers with active ovaries has been reported (Sakagami 1954; van der Blom 1991; Visscher & Dukas 1995; Dampney et al. 2002), but has not been shown in guards (Beekman et al. 2002).

We examined guarding vigilance in queenright and queenless *A. mellifera* colonies, and the acceptance rates of non-nestmates taken from queenless colonies that had actively laying workers, to test the hypotheses that (1) queenless colonies, which are vulnerable to the threat of WRP because of the absence of policing, reject significantly more non-nestmates than queenright colonies, and (2) non-nestmate workers from laying-worker queenless colonies, which are more likely to have active ovaries and to become reproductive parasites, are rejected significantly more than non-nestmate workers from queenright colonies.

METHODS

We used 10 colonies (1–10), each consisting of four combs (one foundation comb and three brood/honey combs) and headed by a naturally mated queen of Italian lineage. The experiment was conducted at the University of Western Sydney Apiary, Richmond, NSW, Australia in spring/summer 2008. An extended entrance board was placed at the entrance to each colony to facilitate observations of the interactions of guard bees with test individuals.

Blind tests of acceptance thresholds began on 28 October 2008 using the procedure of Downs & Ratnieks (1999). We aspirated approximately 20 returning foragers from the entrance of a focal colony using a pooter. We then transferred the workers to a sealable plastic bag along with a small folded piece of paper that identified the origin of the sample. We also collected workers from an unrelated (non-nestmate) colony in an identical plastic bag using the same method. We cooled the bees in an ice box so that they could walk but not fly. We then tested the acceptance of the nestmate and non-nestmate workers by the focal colony. Using forceps we placed a worker on the entrance board of the colony and observed the reaction of the guards. If the test worker was bitten, had its legs, antennae or wings pulled, or was removed from the colony, then this was recorded as 'rejection'. Otherwise the bee was recorded as 'accepted'. All test bees were approached by guards. We observed each test worker for the duration of any interaction until it was either rejected or the guard(s) stopped inspecting it (rarely more than 5 min). We then removed the test bee from the entrance board. We then offered the colony a worker from the other bag, continuing to alternate between bags until a total of 10 workers from each bag (nestmate or non-nestmate) had been tested. This was repeated in each of the other colonies. No test bee was used more than once and the observer was unaware of the contents of each bag (nestmate or non-nestmate) until all observations were completed. After observations on day 4 we removed the queen from half of the colonies (1, 5, 8, 9 and 10) selected at random, and confirmed the presence of the queen in the remaining colonies. We removed queen cells from the queenless colonies on days 8, 10 and 17. Queenless colony 8 was found to have a queen of unknown origin on day 30; this colony was therefore excluded from the experiment.

We tested the colonies 2–3 days a week for 8 weeks, during which period non-nestmate test bees always came from queenright colonies (queenright-non-nestmate). Additionally, on days 43–52