



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

Nadine C. Chapman*, James Makinson, Madeleine Beekman, Benjamin P. Oldroyd

Behaviour and Genetics of Social Insects Laboratory, School of Biological Sciences A12, University of Sydney

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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.

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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

* Correspondence: N. C. Chapman, Behaviour and Genetics of Social Insects Laboratory, School of Biological Sciences A12, University of Sydney, Sydney 2006, Australia.

E-mail address: nadine.chapman@bio.usyd.edu.au (N.C. Chapman).

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

we tested each colony with 10 non-nestmate test bees that came from queenless worker-laying colonies (queenless-non-nestmate) in addition to the 10 queenright-non-nestmate bees. This allowed us to determine whether non-nestmates with active ovaries (originating from laying-worker queenless colonies) were more likely to be rejected than non-nestmates without active ovaries (collected from queenright colonies). On day 52 ca. 100 workers were taken from each colony via aspiration and killed by freezing. Ovaries were dissected according to Oldroyd et al. (2001) and classified as being reproductively active if they contained eggs of any size, and nonreproductive if they did not have eggs. This enabled us to determine whether there was a difference in ovary activation rates in queenright and queenless colonies.

Colonies were provided with empty honey comb and extra space for brood or honey as required. Queenless colonies were provided with empty drone comb for the workers to lay in from week 4 onwards. Queenless colonies were inspected regularly, but not daily, for worker-laid eggs. Worker-laid eggs were found on inspection on day 30 in colony 5, day 35 in colony 1, and on day 38 in colonies 9 and 10. Queenright colonies were checked regularly for queen presence.

Data were recorded as the proportion of the 10 presented bees that were accepted by the test colony. We analysed the proportion of accepted workers using repeated measures ANOVAs with day as the within-subject variable, and test worker origin (nestmate or non-nestmate) and queenstate of test colony (queenright or queenless) as the main effects. Prior to analysis we transformed the data with an aresine-square-root transformation which improves the fit of proportional data to a normal distribution (Zar 1996). Mauchly's test was performed to confirm that the assumption of sphericity (equality of the variances of the differences between levels of the repeated measures factors) was not violated. As the assumption of sphericity was not violated ($P > 0.05$) results are reported with sphericity assumed. As the interaction between state and origin was always insignificant it was removed from the model.

We compared the transformed mean number of bees accepted per category using *t* tests of the least-square means and their associated SEs.

RESULTS

When all colonies were queenright (days 1–4), those that were assigned to become queenless and those that were to remain queenright behaved the same (Table 1); queenright-non-nestmate workers were rejected significantly more than nestmates and there was no effect of queenstate (queenstate here referring to queenstate assigned after day 4). Nestmate workers were accepted 94% of the time, and queenright-non-nestmates were accepted 84% of

Table 1
Repeated measures ANOVA of the aresine-square-root transformation of the proportion of nestmate and queenright-non-nestmate bees accepted for days 1–4 when all colonies were queenright

Source	df	Mean square	F	P	Power
Between-subjects effects					
State	1	180.58	0.92	0.353	0.15
Origin	1	1079.74	5.49	0.033	0.59
Error	15	196.85			
Within-subjects effects					
Day	2	77.60	0.33	0.721	0.09
Day×State	2	542.42	2.31	0.117	0.43
Day×Origin	2	139.69	0.60	0.558	0.14
Error (day)	30	234.91			

Origin (nestmate or queenright-non-nestmate) of offered workers and queenstate (state) that the colony was assigned after day 5 of the experiment are the main effects. Day is the within-subject variable.

Table 2

Repeated measures ANOVA of the aresine-square-root transformation of the proportion of nestmate and queenright-non-nestmate bees accepted for days 8–24 of the experiment, when no colonies had worker-laid eggs

Source	df	Mean square	F	P	Power
Between-subjects effects					
State	1	870.59	1.97	0.181	0.26
Origin	1	5300.91	11.97	0.004	0.90
Error	15	442.92			
Within-subjects effects					
Day	7	1721.74	6.59	<0.001	1.00
Day×State	7	3.77.19	1.44	0.196	0.59
Day×Origin	7	314.70	1.21	0.305	0.50
Error (day)	105	261.38			

Queenstate of the focal colony (queenright or queenless) and origin (nestmate or queenright-non-nestmate) are the main effects. Day is the within-subject variable.

the time. Thus our experimental colonies were homogeneous regarding guarding behaviour prior to manipulation.

On days 8–24 when four of the nine colonies were queenless but did not have worker-laid eggs, there was no effect of the queenstate of the focal colony on rates of test worker acceptance, but there was an effect of test worker origin (Table 2, Fig. 1). The power to detect a significant effect of queenstate was low (Table 2). None the less, queenless colonies accepted significantly fewer queenright-non-nestmates than nestmates ($t_6 = 2.92$, $P = 0.026$), while queenright colonies did not make this distinction significantly ($t_8 = 1.98$, $P = 0.083$; Fig. 2). Queenright and queenless colonies accepted equal proportions of nestmates ($t_7 = 0.37$, $P = 0.72$) and queenright-non-nestmates ($t_7 = 1.59$, $P = 0.155$; Fig. 2). There was a significant effect of day, but no significant interaction between day and queenstate or day and origin (Table 2).

For days 38–52, after the appearance of worker-laid eggs in all queenless colonies, there was a significant effect of queenstate and origin on the rates of acceptance (Table 3, Fig. 1). Statistically, queenless and queenright colonies accepted equal proportions of nestmates ($t_7 = 2.34$, $P = 0.052$) and queenright-non-nestmates ($t_7 = 1.60$, $P = 0.153$; Fig. 3). Queenless colonies accepted significantly fewer queenright-non-nestmates than nestmates ($t_6 = 2.73$, $P = 0.034$), while queenright colonies did not differentiate significantly between the two sources ($t_8 = 2.27$, $P = 0.053$; Fig. 3). There was an effect of day, but no significant interaction between day and origin or day and queenstate (Table 3, Fig. 1).

On days 43–52 when worker-laid eggs were present in all four queenless colonies there was a significant effect of state, origin and day, but no significant interaction between day and origin or day and queenstate (Table 4, Fig. 1). Queenright colonies accepted significantly more queenright-non-nestmates than queenless-non-nestmates during this period ($t_8 = 3.05$, $P = 0.016$; Fig. 4). Queenless colonies accepted equal proportions of queenright-non-nestmates and queenless-non-nestmates ($t_6 = 0.78$, $P = 0.465$; Fig. 4). Queenless colonies accepted significantly more queenless-non-nestmates than queenright colonies ($t_7 = 3.82$, $P = 0.007$); there was also a trend for queenless colonies to be more accepting of queenright-non-nestmates than queenright colonies, but there was no significant difference in acceptance rates ($t_7 = 1.77$, $P = 0.120$; Fig. 4).

No workers with active ovaries were found in the five colonies with a queen ($N = 492$) and 56% of workers in the four queenless colonies had active ovaries ($N = 401$).

DISCUSSION

We predicted that queenless colonies would be less accepting of non-nestmates than queenright colonies, as a defence against WRP, and that non-nestmate workers from queenless colonies would be

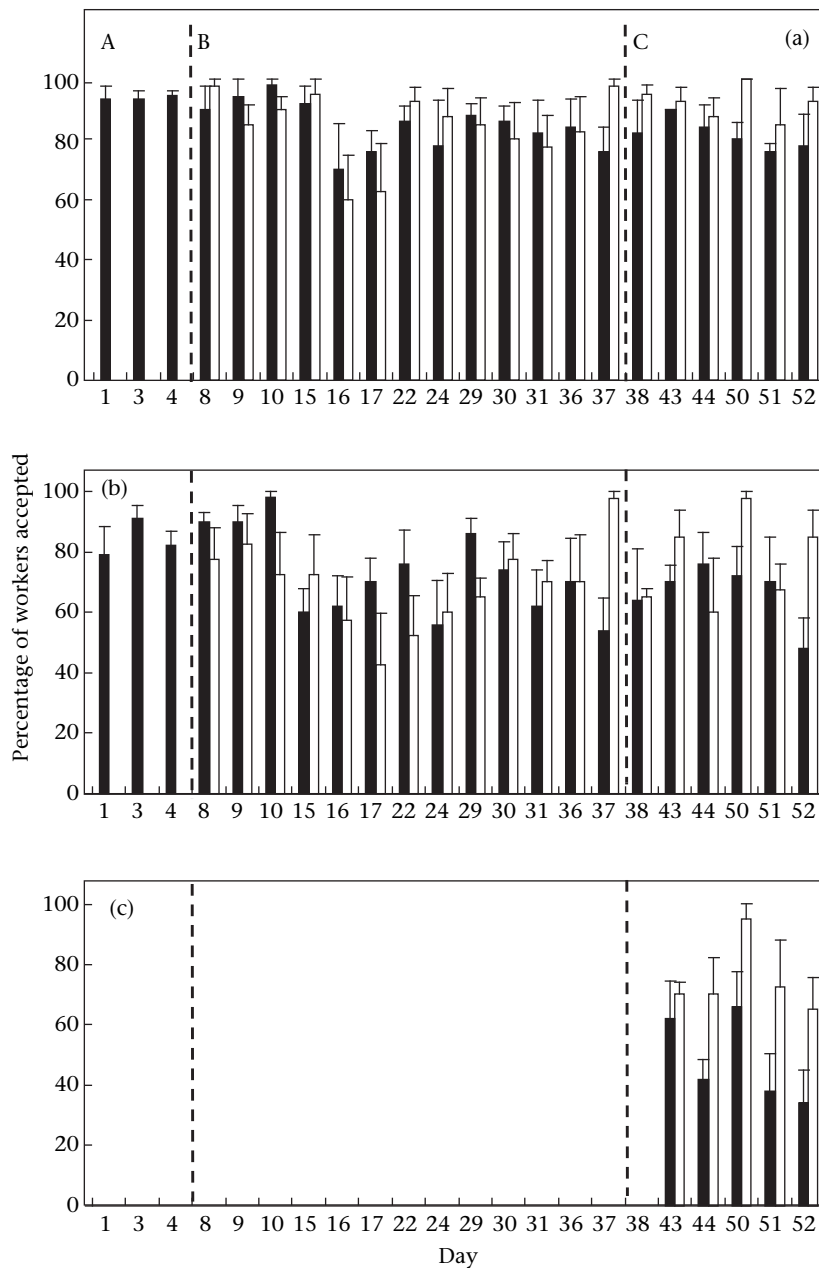


Figure 1. Mean \pm SE of the untransformed average percentage of (a) nestmate, (b) queenright-non-nestmate and (c) queenless-non-nestmate bees accepted into queenright (black) and queenless (white) colonies. In period A all nine test colonies were queenright. Four of the nine test colonies were queenless in periods B and C. Laying workers were present in all four queenless colonies in period C. Dashed lines indicate divisions between periods.

less likely to be accepted than non-nestmate workers from queenright colonies. These predictions were broadly upheld by our experiment. Queenless colonies rejected significantly more queenright-non-nestmates than nestmates, while queenright colonies did not differentiate significantly between these sources, presumably because field conditions were favourable. Queenright colonies rejected significantly more non-nestmate workers from queenless colonies, which are more likely to become reproductive parasites, than non-nestmate workers from queenright colonies.

Our study has shown that queenright honeybee colonies have the ability to distinguish between potential robbers (queenright-non-nestmates; inactive ovaries) and potential parasites (queenless-non-nestmates; active ovaries) and nestmates. This implies that guarding indeed has a dual purpose. Honeybee colonies respond to increased threat and likelihood of robbing by adjusting

their acceptance threshold so that fewer potential robbers (queenright-non-nestmate workers) are admitted. Similarly, guards are also less likely to admit potential parasites (queenless-non-nestmate workers), which are likely to have active ovaries. This suggests that a second mechanism, guarding, acts in concert with worker policing to reduce the incidence of worker reproduction in queenright honeybee colonies.

Queenless laying-worker colonies did not differentiate between non-nestmates from queenless or queenright colonies, while queenright colonies did. We note that it may be better for queenless colonies to turn away all non-nestmates regardless of their current reproductive potential. This is because all non-nestmates are potential parasites in a queenless colony. Queenright colonies may more readily discern queenless-non-nestmates than queenright-non-nestmates as being foreign. No workers are

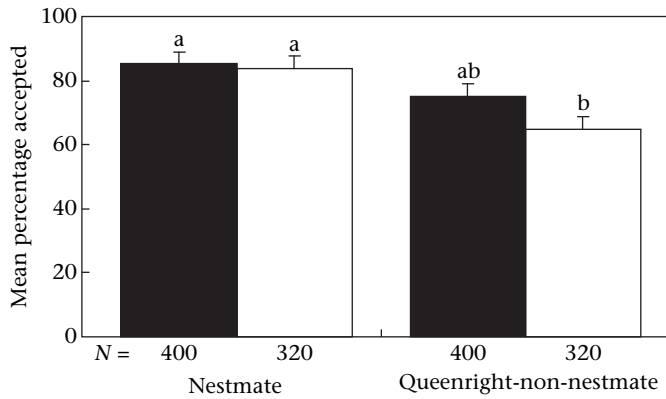


Figure 2. Mean + SE of the untransformed average percentage of nestmate and queenright-non-nestmate test bees accepted into queenright (black) and queenless (white) colonies on days 8–24 of the experiment when no worker-laid eggs were present in any of the queenless colonies. Bars with different letters above them are significantly different at the 5% level as determined with *t* tests.

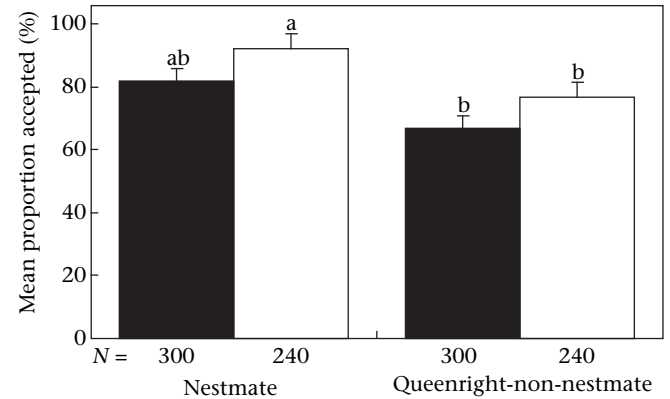


Figure 3. Mean + SE of the untransformed average percentage of nestmate and queenright-non-nestmate test bees accepted into queenright (black) and queenless (white) colonies on days 38–52 of the experiment when all queenless colonies had worker-laid eggs present. Bars with different letters above them are significantly different at the 5% level as determined with *t* tests.

expected to have active ovaries in queenright colonies, and so any worker with active ovaries is clearly a non-nestmate. In contrast, in queenless colonies not all workers activate their ovaries, and hence ovary activation does not provide a reliable cue for discerning nestmate from non-nestmate.

While every non-nestmate is a potential reproductive parasite in a queenless colony, the reproductive status of the non-nestmate is arguably more important in the period between a colony first becoming queenless and when the colony starts to produce worker-laid eggs. Workers with active ovaries are known to reduce the rate at which other workers activate their ovaries (Sakagami 1958; Velthuis et al. 1965; Velthuis 1970; Jay & Nelson 1973; Robinson et al. 1990). Most mature drone offspring arise from eggs laid during the first few days of worker oviposition (Page & Erickson 1988). A non-nestmate worker with active ovaries that joins a queenless colony is therefore poised to produce eggs quickly, while simultaneously reducing the opportunity for natal workers to produce eggs. Thus in newly queenless colonies guards may be particularly alert to the entry of non-nestmates with active ovaries. We did not investigate the reaction of queenless colonies to queenless-non-nestmates in the period before the queenless colonies produced eggs, but we speculate that queenless colonies would be more discerning of queenless-non-nestmates than queenright-non-nestmates during this time.

The guarding assay used here is artificial in that test bees were immobilized, and therefore did not behave as they would when

attempting to enter an unrelated colony in nature. None the less it is the behaviour of guards, and not that of the test worker, that is of interest, and the procedure provides an objective means of assessing the relative vigilance of guards under queenright and queenless conditions. Non-natal workers have been found in queenright colonies of all honeybee species that have been investigated thus far (e.g. Moritz et al. 1995; Pfeiffer & Crailsheim 1998; Neumann et al. 2000; Paar et al. 2002; Jensen et al. 2005; Nanork et al. 2005, 2007). Therefore, guards do encounter non-nestmates in nature.

A previous study (Chapman et al. 2008) concluded that guarding behaviour evolved solely as a defence against robbing and not as a defence against WRP. Our previous study, conducted on *A. cerana*, only considered the response of test colonies rather than both the queenstate of test bees and the queenstate of the focal colony. The change in the experimental design between the two studies is the most likely cause of the difference in outcomes. Thus the two studies are not indicative of a real biological difference between *A. cerana* and *A. mellifera*.

Our findings are in line with the predictions of the acceptance threshold model (Reeve 1989), which argues that as the costs and benefits of rejection change, so too does the response. There are several other notable examples of animals adaptively adjusting their acceptance thresholds in response to changing threat levels.

Table 3

Repeated measures ANOVA of the arcsine-square-root transformation of the proportion of nestmate and queenright-non-nestmate bees accepted for days 38–52 of the experiment, when all four of the queenless colonies had worker-laid eggs present

Source	df	Mean square	F	P	Power
Between-subjects effects					
State	1	2476.02	8.17	0.012	0.76
Origin	1	3924.68	12.94	0.003	0.92
Error	15	303.23			
Within-subjects effects					
Day	5	409.04	1.56	0.183	0.52
Day×State	5	582.41	2.22	0.061	0.69
Day×Origin	5	191.81	0.73	0.603	0.25
Error (day)	75	262.63			

Origin (nestmate or queenright-non-nestmate) and queenstate of the focal colony (queenless or queenright) are the main effects. Day is the within-subject variable.

Table 4

Repeated measures ANOVA of the arcsine-square-root transformation of the proportion of queenright-non-nestmate and queenless-non-nestmate bees accepted for days 43–52 of the experiment, when four of the nine colonies were queenless and had worker-laid eggs present

Source	df	Mean square	F	P	Power
Between-subjects effects					
State	1	4431.03	14.58	0.002	0.95
Origin	1	2205.05	7.26	0.017	0.71
Error	15	303.89			
Within-subjects effects					
Day	4	1270.00	4.03	0.006	0.89
Day×State	4	470.26	1.49	0.216	0.44
Day×Origin	4	73.86	0.23	0.92	0.10
Error (day)	60	315.51			

Origin (queenright-non-nestmate or queenless-non-nestmate) and queenstate of colony (queenless or queenright) are the main effects. Day is the within-subject variable.

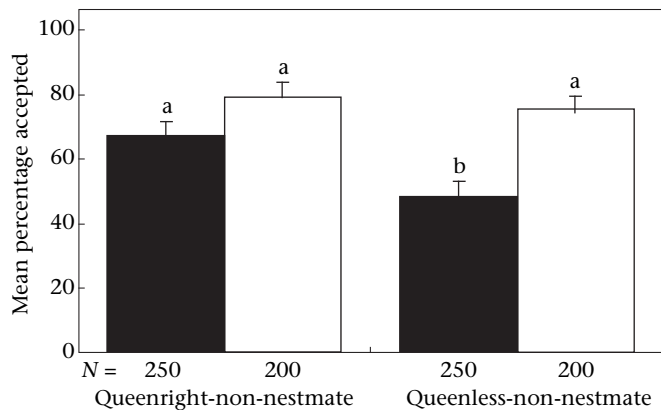


Figure 4. Mean + SE of the untransformed average percentage of queenright-non-nestmate and queenless-non-nestmate test bees accepted to queenright (black) and queenless (white) colonies on days 43–52 of the experiment when all queenless colonies had worker-laid eggs present. Bars with different letters above them are significantly different at the 5% level as determined with *t* tests.

When a worker of the slave-making ant *Polyergus rufescens* is placed in an arena with workers of its host, *Formica rufibarbis*, during times of host searching and raiding, *F. rufibarbis* workers taken from areas that are parasitized by *P. rufescens* are more aggressive towards *P. rufescens* workers than at other times (D'Etterre et al. 2004), suggesting that they adaptively adjust their acceptance thresholds depending on the level of threat. *Formica rufibarbis* workers taken from regions where parasitism does not occur do not respond in this way, suggesting that they do not adjust their acceptance thresholds seasonally, because there is no threat of parasitism (D'Etterre et al. 2004). In several bird species, as the risk of brood parasitism by cuckoos declines, their hosts become more permissive of nonmimetic eggs (e.g. Davies et al. 1996; Brooke et al. 1998; Lindholm & Thomas 2000; Cruz et al. 2008). Populations that are free of cuckoos show less rejection of model eggs than parasitized populations, and the response of hosts fluctuates with size of cuckoo population and time of season (e.g. Davies et al. 1996; Brooke et al. 1998; Lindholm & Thomas 2000; Cruz et al. 2008).

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