Absence of nepotism in the harassment of duelling queens by honeybee workers

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Nepotism shapes interactions among the members of almost every animal society. However, clear evidence of nepotism within highly cooperative insect societies, such as ant, wasp and honeybee colonies, is rare. Recent empirical findings suggest that nepotism occurs within honeybee colonies where kin-selection theory most strongly predicts its existence: during the lethal queen–queen duels that determine which of several young queens will become the colony's next queen. In this study, I test whether worker bees act nepotistically by hindering duelling queens that are distantly related to themselves. I accomplished this by observing labelled workers harassing duelling queen bees in observation hives and subsequently by determining worker–queen relatedness using DNA microsatellites. I show that the workers that harassed duelling queens were neither more-closely nor more-distantly related to them than were workers selected randomly from the colony. Thus, workers did not behave nepotistically by hindering half-sister queens more than full-sister queens. These results demonstrate that under certain conditions, natural selection limits the evolution of nepotism within animal societies despite strong theoretical predictions for its existence.

Keywords: honeybee; intra-colony nepotism; queen duel; aggression; microsatellites

1. INTRODUCTION

Conventional formulations of kin-selection theory (Hamilton 1964; Maynard Smith 1964) predict that nepotism, the preferential treatment of more-closely related individuals over more-distantly related individuals, will be a common product of evolution. Indeed, nepotism has been found to shape interactions among the members of almost every animal society (Hepper 1991). However, clear evidence of nepotism within highly cooperative insect societies, such as ant, wasp and honeybee colonies, is rare (Queller *et al.* 1990; Breed *et al.* 1994; Bernasconi & Keller 1996; DeHeer & Ross 1997; Keller 1997; Strassman *et al.* 1997; Visscher 1998; Osborn & Oldroyd 1999; Hannonen & Sundström 2003). Recent work with honeybees (Tarpy & Fletcher 1998), suggests that nepotism occurs within honeybee colonies during events associated with queen replacement, where kin-selection theory most strongly predicts its existence.

The predictions of kin-selection theory for nepotism during honeybee queen replacement are strong. In the simplest form of nepotism, an actor, A, dispenses some benefit, b , to one of two potential recipients, C or D, who are related to A by r_{AC} and r_{AD} , respectively. Kin-selection theory predicts that nepotism will be favoured when b is large or when the difference between r_{AC} and r_{AD} is great. Both of these conditions are met during the annual replacement of a honeybee colony's queen. A honeybee worker could potentially influence the outcome of queen replacement in favour of a super-sister queen, which shares any given allele with a probability of 0.75 (rather than the 0.50 typical of mammalian siblings), over a half-sister queen, which shares any given allele with a prob-

ability of 0.25. Here, the difference in relatedness between the potential recipients, $r_{AC} - r_{AD} = 0.50$, is extraordinarily large (as large as the difference between a mammal's sibling and an unrelated individual). The potential benefit of nepotism, b , is also great; the queen that replaces the previous queen of the colony gains status as the sole reproductive of an established colony rather than death.

Strong empirical support for nepotism within honeybee colonies undergoing queen replacement has proved elusive. Evidence that workers preferentially rear super-sister queen larvae was reported by Page *et al.* (1989), but subsequent reanalysis (Oldroyd *et al.* 1990) revealed no statistical significance and general current opinion (Breed *et al.* 1994; Keller 1997; Visscher 1998) is that nepotism during queen rearing is either weak or absent (but see Osborn & Oldroyd 1999). However, this debate largely neglected the possibility of worker nepotism after queen emergence, during the series of lethal queen duels that determine which one of 15–25 adult queens will replace the previous queen of the colony. Worker participation in these duels has been well documented (e.g. Huber 1792; Weaver & Weaver 1980; Gilley 2001), but the adaptive significance of the workers' behaviour is unknown. A recent investigation by Tarpy & Fletcher (1998) shows that in duels among newly emerged queens, queens that are sisters of the colony's workers have an advantage over unrelated queens. Though the use of unrelated queens does not allow one to rule out as an explanation for these results the workers' use of genetic nestmate recognition cues, the results do suggest that worker nepotism might exist during the duels between queens that follow their emergence. Here, I test this possibility by observing labelled workers harassing queens in observation hives and subsequently by determining worker–queen relatedness using DNA microsatellites.

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Table 1. Distribution of patriline among the random sample of 50 bees from each colony.

colony	proportion of bees in random sample from patriline															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A	0.21	0.17	0.15	0.11	0.08	0.06	0.06	0.06	0.04	0.04	0.02	—	—	—	—	—
B	0.28	0.14	0.12	0.08	0.08	0.08	0.06	0.06	0.04	0.02	0.02	0.02	0.00	0.00	0.00	—
C	0.70	0.30	—	—	—	—	—	—	—	—	—	—	—	—	—	—
D	0.24	0.19	0.19	0.17	0.15	0.04	0.02	0.00	0.00	—	—	—	—	—	—	—
E	0.16	0.14	0.10	0.10	0.10	0.80	0.06	0.06	0.04	0.04	0.04	0.04	0.02	0.02	0.00	0.00

2. METHODS

(a) Behavioural observations

Behavioural observations were conducted in Ithaca, New York, during the summers of 1999 and 2000. Five honeybee colonies, each headed by a naturally mated queen, were each housed in a three-frame observation hive with removable glass sides. Each colony was allowed to begin preparations for swarming and the associated queen rearing which precedes queen duels. Approximately one month before the new queens began to emerge, 0-day-old worker bees were removed daily from each hive, labelled with a number tag and an abdominal paint mark for individual identification, and returned to their hives (total: $n = 14\,598$). As each young queen emerged, the identities of the labelled workers that harassed her were noted. Harassment consisted of ‘clamps’ and ‘grabs’ (Gilley 2001), two frequent, conspicuous and discrete worker–queen interactions. A ‘clamp’ was recorded when a worker closed her mandibles on a queen’s legs or wing bases, often getting dragged around the colony if the queen moved. Clamps were assumed to be costly to the queens because queens are sometimes stung by their opponents while immobilized by the clamping of many workers. A ‘grab’ was recorded when a worker excitedly touched a queen with her antennae and front legs, typically opening and closing her mandibles against the surface of the queen’s thorax and abdomen, and sometimes even briefly mounting the queen. Grabs were assumed to be costly to the queens because queens retreated rapidly from grabs and because grabs very often precede clamps. The identities of harassing workers were noted for harassment that followed a focal queen’s visit to a queen cell and for harassment throughout the hive. When approximately 30 labelled workers had been observed harassing a queen, she was removed and stored at $-20\text{ }^{\circ}\text{C}$. After completion of the queen replacement process in each colony (i.e. when only one queen remained alive in the colony), all the labelled workers (total: $n = 5439$) and the remaining queen were collected and stored at $-20\text{ }^{\circ}\text{C}$ for subsequent genetic analysis.

(b) Genetic analyses

DNA microsatellites were used to determine the paternity of the workers from three groups of bees: the harassed queens ($n = 27$), the recovered labelled harassing workers ($n = 555$) and a random sample of recovered labelled workers of the same age as the harassing workers ($n = 250$, i.e. 50 for each colony). Workers of the same age as the harassing workers were used in the random sample because if harassing is an age-related behaviour, then random fluctuations in colony demographics could result in false positive or false negative errors. The identities of the workers in the random sample were determined by constructing an age distribution of the harassing workers from each colony, then randomly selecting a group of 50 workers from that colony with an identical age distribution. To extract the DNA,

one-half of each bee’s thorax was ground, the cells lysed and the DNA purified using a Qiagen DNeasy Tissue Kit. Six microsatellite loci were used to determine paternity: Ap36, Ap43 (Garnery *et al.* 1998); CD (Rowe *et al.* 1997); and A29, A76, B124 (Estoup *et al.* 1994). The forward primer for each locus was fluorescently labelled on the 5’ end. Polymerase chain reactions (PCR) were carried out in 10 μl volumes (7.22 μl of H_2O , 1.00 μl of PCR buffer, 0.30 μl of MgCl_2 , 0.08 μl of dNTP, 0.15 μl of forward primer, 0.15 μl of reverse primer, 0.10 μl of Taq polymerase, and 1.00 μl of DNA template) using a 50 s denaturing step ($95\text{ }^{\circ}\text{C}$), a 60 s annealing step ($44\text{--}54\text{ }^{\circ}\text{C}$) and 60 s extension step ($72\text{ }^{\circ}\text{C}$). PCR products were visualized and recorded using an ABI Prism 377 DNA Sequencer with the ABI Prism 377-96 2.5 Data Collection Package and Virtual Filter Set D. At each locus, the paternal allele for each bee was determined by elimination of the known maternal allele. The patriline of each bee was then determined by using each bee’s composite genotype from all six loci.

3. RESULTS

I observed in five colonies of honeybees the interactions of the workers with newly emerged duelling queens. As previously observed (Gilley 2001), beneficent interactions (e.g. trophallaxis and grooming) were uncommon during duels, whereas harassment of the queens ($n = 27$) by the workers was a common event which occurred after a focal queen’s visit to a queen cell and also throughout the hive. Repeated harassment of queens by particular workers was infrequent; we observed most workers harassing a single queen a single time. The distribution of patrilines within the random sample of 50 workers from each colony is shown in table 1. Figure 1 shows the relationships between each queen and the workers that harassed her, and the relationships between each queen and the randomly selected workers. If workers were behaving nepotistically by harassment of a queen, then the proportion of half-sisters among the harassing workers should have been larger than the proportion of half-sisters among the group of randomly selected workers. This was never the case. With two exceptions (queens C2 and D2), the relationship between the queen and the workers was independent of whether or not the workers harassed the queen (G -tests of independence using Yates’ correction for continuity). These two cases do not fulfil the predictions of nepotism because workers that harassed the queen were, on average, more-closely related to the queens than were the randomly selected workers.

To check more rigorously for possible nepotism, I calculated the degree of nepotism shown towards each queen by subtracting the proportion of half-sisters in the group

Figure 1. A comparison of worker–queen relatedness between harassing workers and randomly selected workers. The first circle for each queen represents the proportion of harassing workers that were her half-sisters (unshaded region) versus super sisters (shaded region). The second circle for each queen represents the proportion of randomly selected workers that were her half-sisters (unshaded region) versus super sisters (shaded region). *n* refers to the number of aggressive workers sampled. The number of workers in the random sample was always 50. The last column provides the *p*-value for a two-tailed *G*-test of independence using Yates’ correction for continuity. Three queens (queens B1, B3 and B4) could not be compared because no full sisters were found among the randomly selected workers. No comparisons yielded *p*-values below 0.0019, required for significance with a Bonferroni correction for an experiment-wise type I error rate of 0.05.

of randomly selected workers from the proportion of half-sisters in the group of workers that harassed the queen. This resulted in a normally distributed (experiment-wise) variable with possible values between –1.00 and 1.00, where 1.00 represents the strongest preference to harass half-sister queens and –1.00 represents the strongest pref-

erence to harass full-sister queens. Figure 2 shows the mean value of this variable for each colony. The degree of nepotism did not differ significantly from 0 for any of the five colonies in this study (two-tailed *Z*-tests: *n* = 8, 5, 3, 5, 6). The overall degree of nepotism observed in this study (the grand mean for all five colonies) was –0.0082,

Figure 2. The degree of nepotism observed in five honeybee colonies. Each bar shows the mean degree of nepotism in one colony, measured for each queen as the difference between the proportion of half-sisters in the harassing workers and the proportion of half-sisters in the randomly selected workers. The mean is based on eight, five, three, five and six queens in colonies A, B, C, D and E, respectively. Error bars indicate ± 1 s.e.m. The p -values above each bar indicate the significance of a two-tailed Z -test. No colony's mean differs significantly from 0.

also not significantly different from 0 (two-tailed Z -test: $n = 5$, $p = 0.8443$). When the same analysis was done by using only workers that grabbed queens (i.e. when workers that clamped queens were removed from the analysis), the results were similar: the grand mean was -0.0052 .

To determine the probability of a type II error in this study, I performed a power analysis on the data presented in figure 2. The chance of a type II error becomes significant at the $\alpha = 0.05$ level only if the true mean of the degree of nepotism is between -0.080 and 0.080 (mean under $H_0 = 0.0$; measured mean = -0.0164 , $n = 27$). To investigate the chance of a false negative more rigorously, I performed a Monte Carlo simulation, again using the data in figure 2, iterated 1000 times. In this simulation, the chance of a type II error became significant at the $\alpha = 0.05$ level only when the true strength of nepotism, measured as the reduction in probability that super-sister versus full-sister workers harass a queen, fell below 0.16. Thus, this experiment had a greater than 95% chance of detecting all but the weakest nepotism, which if it did exist, would be of questionable biological significance.

4. DISCUSSION

Despite the potential benefits of nepotism, workers in this study did not assist full-sister queens by preferentially harassing half-sister queens during queen duels. Power

analyses show that this result is unlikely to be a false negative resulting from the experiment's inability to detect moderate levels of nepotism. The lack of nepotism in worker harassment suggests that nepotism is unlikely to exist during queen duels and that strong nepotism may be absent from the queen replacement process in honeybee societies. Worker nepotism might exist during queen duels in the form of worker-queen interactions other than harassment, such as trophallaxis or queen immobilization. However, both of these interactions are infrequent, brief and are even absent during some duels; harassment of queens by the workers is the only consistent, persistent worker-queen interaction during queen duels (Gilley 2001). If harassment of queens effectively protects queen cells from destruction by emerged queens (as originally suggested by Huber (1792)), then workers might act nepotistically during queen duels by preferentially guarding the cells of full-sister queens. This hypothesis is not supported by the pattern of queen-cell visitation by workers (brief visits to many cells; D. C. Gilley, unpublished data), but merits further investigation.

The lack of nepotism demonstrated by the worker honeybees in this study provides insights into the conditions under which natural selection may limit the evolution of nepotism. Natural selection among groups of organisms may favour groups that lack nepotism when nepotism results in costly conflicts within the society (Keller 1997; Keller & Reeve 1999), e.g. if all workers attacked half-sister queens then no queens would survive. Natural selection among individuals within groups may favour individuals that obscure the kin recognition cues necessary for nepotism when the costs of advertising kinship are high (Keller 1997), e.g. if a honeybee queen advertises her kinship then she may be attacked by all half-sister workers, most of the colony. Finally, natural selection among genes within a genome may favour genes that suppress genes for nepotism when nepotism benefits only a portion of the genome, e.g. maternally inherited honeybee genes might suppress the nepotism favoured by paternally inherited genes. To understand fully the conditions under which natural selection favours the evolution of nepotism within a society, we need to untangle the selective forces operating at each of these levels.

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