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Worker policing and worker reproduction in *Apis cerana*

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Abstract Workers of the Asian hive bee, *Apis cerana*, are shown to have relatively high rates of worker ovary activation. In colonies with an active queen and brood nest, 1–5% of workers have eggs in their ovarioles. When *A. cerana* colonies are dequeened, workers rapidly activate their ovaries. After 4 days 15% have activated ovaries and after 6 days, 40%. *A. cerana* police worker-laid eggs in the same way that *A. florea* and *A. mellifera* do, but are perhaps slightly more tolerant of worker-laid eggs than the other species. Nevertheless, no worker's sons were detected in a sample of 652 pupal males sampled from 4 queenright colonies. *A. cerana* continue to police worker-laid eggs, even after worker oviposition has commenced in a queenless colony.

Keywords *Apis cerana* · Worker policing · Oophagy · Laying workers · Worker sterility

Introduction

Worker policing describes any behaviour of social insect workers that lowers direct reproduction by other workers. Worker policing of male production by unmated hymenopteran workers is predicted to evolve due to the relatedness asymmetries that arise from polyandry and polygyny (Ratnieks 1988; Visscher 1996, 1998). In poly-

androus species such as honey-bees and wasps, these asymmetries result in workers being more related to the sons of the queen ($r=0.25$) than to the sons of other workers ($r<0.25$), most of which are half-sisters.

In the western honey-bee, *Apis mellifera* (Ratnieks and Visscher 1989), the dwarf honey-bee, *A. florea* (Halling et al. 2001), and the wasp *Vespula vulgaris* (Foster and Ratnieks 2001a, 2001b), an important component of worker policing behavior is oophagy of worker-laid eggs. Queens of these species are thought to mark their eggs with a pheromone. Workers lack this pheromone and other workers therefore eat their eggs (Ratnieks 1993, 1995).

Once an efficient mechanism of worker policing has evolved, the benefits to an individual of attempted personal reproduction are severely curtailed. Under these circumstances, workers are expected to evolve functional sterility in the presence of a queen or brood (Seeley 1985; Keller and Nonacs 1993). As predicted, workers of *A. florea* and *A. mellifera* show virtually no evidence of ovary activation in queenright colonies (Ratnieks 1993; Halling et al. 2001). Curiously, however, workers of the eastern hive bee *A. cerana*, have been reported to have high rates of ovary activation. Sakagami and Akahira (1958) reported that 10–20% of *A. cerana* workers in queenright colonies contained mature eggs in their ovaries. Bai and Reddy (1975) found little ovary development in workers from queenright colonies containing brood. They did, however, report that in queenless, broodless colonies, 72% of workers had activated ovaries. *A. cerana* workers begin laying 2–3 days after loss of a queen (Blanford 1923), much more quickly than workers of *A. mellifera* (6–30 days: Page and Erickson 1988). Furthermore, unlike *A. mellifera*, *A. cerana* workers may continue to lay after the introduction of a new queen (Sakagami 1958).

In a species with high rates of ovary activation in queenright workers, one might predict that policing behaviour would be less well expressed than in species like *A. florea* and *A. mellifera* where ovary activation is rare. The selective forces that promote worker policing run in

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parallel with forces that promote individual reproduction (Montague and Oldroyd 1998; Oldroyd et al. 1999; Barron et al. 2001). The outcome of these conflicting selective forces will depend on a number of factors, including the colony-level costs of widespread reproduction by workers, and the physiological ability of police workers to evolve strategies for policing new worker genotypes that can reproduce in queenright colonies. Even in species like *A. mellifera* in which selection appears to favour functional sterility, occasional colonies show high rates of worker reproduction (Oldroyd et al. 1994; Montague and Oldroyd 1998).

To investigate further the evolution of policing in *Apis*, we investigated rates of ovary activation in and the reproductive success of *A. cerana* workers.

Methods

Worker-policing assays

Colonies of *A. cerana* were collected from a research apiary maintained by Chulalongkorn University. These colonies had been wild-caught from Samut Songkhram Province of Thailand and were housed in single-story box hives. We investigated the ability of workers to detect and remove worker-laid eggs in both queenright and queenless colonies.

To obtain worker-laid eggs, several colonies were dequeened, and any queen cells that subsequently developed were removed to prevent any colony requeening itself. Worker oviposition commenced within a few days of dequeening. Queen-laid eggs were obtained from drone combs placed in queenright colonies.

To conduct a worker-policing assay, we used techniques described in Oldroyd and Ratnieks (2000). Eggs were harvested from drone combs containing eggs of the appropriate material. To do this, queen-laid (QL) eggs from the drone comb of queenright source colonies and worker-laid (WL) eggs from queenless source colonies were collected separately and placed vertically on microscope slides with the aid of modified forceps (Taber 1961; Ratnieks and Visscher 1989). Loaded slides were kept in a container under moist paper towelling to prevent the eggs drying out.

Several rows of cells within the test drone combs were marked with coloured drafting pins. In each row, 20 eggs from a single source colony were inserted into adjacent cells. (On three occasions during the queenright assays, only 18 or 19 eggs were available). For each trial, the test comb received both WL and QL eggs, each egg type being obtained from one to three colonies. Care was taken to place eggs upright in the base of the cell, similarly to those positioned by a queen. A marked row of 20 cells was left empty to test for any egg laying in the drone comb during the course of the experiment. Row allocation was arbitrary with respect to the type of egg (QL or WL). Before a test comb was reused, any eggs remaining from the previous trial were removed, and the comb was washed out with water to remove excess nectar and returned to its colony for 1–2 h.

Test combs with repositioned eggs were then placed into queenright and queenless discriminator colonies. In the queenright colonies, the number of eggs remaining was determined after 0.5, 1, 2, 3, 4 and approximately 20 h. In queenless colonies, the 0.5- and 3-h inspections were omitted because the colonies became agitated by the frequent disturbance.

Data were recorded as the proportion of the 20 eggs that were present at each inspection. They were analysed using repeated-measures ANOVAs after transforming raw data with an arcsine \sqrt{x} transformation which helps normalize proportional data such as these. However, survival data are inherently non-normal, and we recommend cautious interpretation of ANOVA results combined with careful reference to the figures.

During observations of transferred eggs, eggs were occasionally observed in rows of previously empty cells and new eggs sometimes appeared in cells that had held transferred eggs (i.e. an egg was seen in a cell that had previously been recorded as empty). This indicated that queens or workers were laying in the drone combs during the experiment. However, as the position of these eggs was random with respect to the type of eggs placed in a row, this could not have biased our results. No colonies were sufficiently populous to allow us to exclude the queen from the area where the bioassayed eggs were placed.

Policing in queenright colonies

Experiments in queenright discriminator colonies were conducted in March 1999 at the main campus of Chulalongkorn University, Bangkok, Thailand. Environmental conditions were excellent for bees in that good supplies of nectar and pollen were available. Eggs came from four queenless and six queenright colonies. All six queenright source colonies were also used as discriminator colonies, but no colony was used as both a source colony and a discriminator colony on the same day. (This scheme was made necessary by the availability of eggs.) Colonies were actively rearing drones, but no adult drones were present.

Policing in queenless colonies

Experiments using queenless discriminator colonies were conducted at the Tropical Fruits Research Station in Chanthaburi during April 2000. Environmental conditions for bees were moderate during this experiment, with somewhat limited floral resources. However, most colonies had significant amounts of drone brood. Two colonies were used as sources of WL eggs and two others as sources of QL eggs. Different source colonies were used on different days according to the availability of eggs. Three different colonies were used as discriminators. Assays were conducted over a period of 9 days. This allowed us to see if there was any change in policing behaviour as time progressed.

Ovary activation

To determine levels of worker ovary activation in queenright *A. cerana* colonies, random worker bees were collected from four colonies used in the queenright assays and stored in ethanol at -20°C . Queens were positively identified as being present when these samples were taken and the colonies had normal brood nests with all stages of brood present. Ovaries were dissected according to Dade (1977) and ovary development classified as ovarioles not distinguishable (inactive), ovarioles visible (inactive), small eggs present (<50% of full size, activated) and eggs >50% full size (activated).

We also collected workers from the three queenless discriminator colonies used in the second experiment in order to monitor the rate at which queenless *A. cerana* workers activate their ovaries. These workers were dissected immediately after killing by freezing. To do this, the thorax was pinned into a wax plate and the abdomen immersed in water. The worker was then grasped by the most posterior segment. A second pair of forceps was then placed between the third and fourth tergites. The last segment was then gently pulled so that the third and fourth segments became separated to reveal the ovaries. These ovaries were scored as inactive (no visible ova) or activated (clearly defined ova present in the ovarioles).

Maternity of drones

Drone pupae (total=652) and workers were collected from five queenright colonies. These bees were analysed using the microsatellite primers A28 (annealing temperature 55°C), B124 (57°C) (Estoup et al. 1994) and Ap43 (60°C) (Garner et al. 1998). DNA was extracted using a 5% Chelex solution (Walsh et al. 1991) and

diluted 1:2 vol in water before use as a PCR template. PCRs were conducted in 10- μ l volumes using 1.5 mM MgCl₂. PCR products were electrophoresed on an automated DNA analyser (Corbett Research Sydney) and the allele size scored in base pairs.

The genotype of the queen heading each colony was inferred from the worker genotypes (28 workers per colony), and the possible genotypes of her sons deduced. The actual genotype of the drones was then evaluated to determine if any were not sons of the queen (Oldroyd et al. 1994; Montague and Oldroyd 1998; Halling et al. 2001)

Results

Ovary activation

Of 800 workers examined from queenright colonies, 2 (0.25%) had full-size eggs, and 5.25% had activated ovaries (Table 1). The 2 bees with full-sized eggs were from the same colony. However, bees containing smaller eggs in their ovarioles occurred in all colonies at similar frequencies (Table 1). Most workers showed at least partial ovariole development.

In the queenless discriminator colonies, 1.35% of bees had activated ovaries on the day the experiment commenced (Fig. 1). This proportion climbed rapidly until after 5 days, 40–50% of workers had eggs present in their ovarioles (Fig. 1).

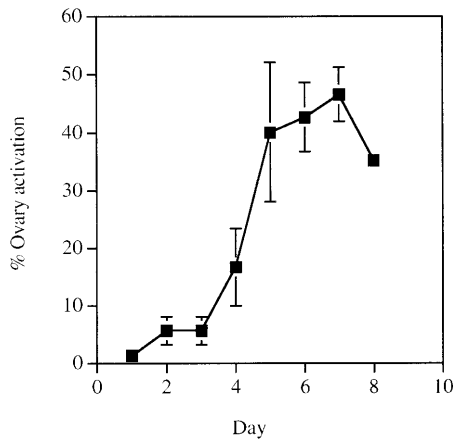


Fig. 1 Ovary activation in *Apis cerana* against the number of days since dequeening. Days 1–3 are based on 100 bees from each of three colonies. Days 4–10 are based on 50 bees from each of three colonies. Ovaries were designated as active if distinct ova could be seen in the ovarioles. Bars indicate standard errors of the means

Table 1 Rates of ovary activation in *Apis cerana* workers queenright colonies

Colony	Ovary activation			
	Ovarioles could not be discerned	Ovarioles visible	Eggs <50% full-sized (activated)	Eggs >50% full sized (activated)
1	32	156	12	0
2	49	136	15	0
3	53	141	6	0
4	60	131	7	2
Total	194	564	40	2
	24.25%	71.50%	5.00%	0.25%

Worker policing in queenright colonies

QL eggs survived much longer than WL eggs (Fig. 2). A repeated-measures ANOVA (Table 2) showed that, overall, WL eggs were removed significantly more quickly than QL eggs ($P < 0.001$; Table 2). Discriminator colonies differed significantly in their treatment of eggs ($P = 0.02$), but all discriminators removed worker-laid eggs more

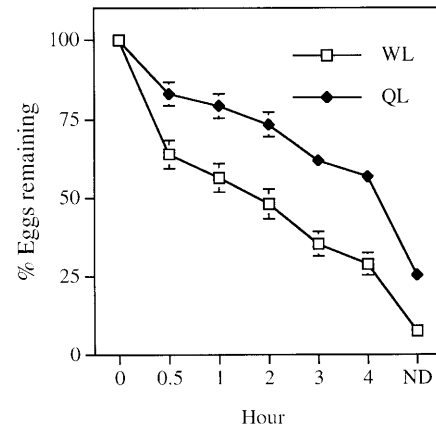


Fig. 2 Survival of worker-laid (WL) and queen-laid (QL) eggs in queenright discriminator colonies. Bars indicate SEs of least-square means. Data are pooled across discriminator colonies and days (ND next day, approximately 20 h)

Table 2 Repeated-measures ANOVA of the effect of egg maternity (worker or queen-laid), discriminator colony and time in hours from commencement on the proportion of eggs surviving in queenright discriminator colonies (experiment 1). All data have been transformed with an arcsine \sqrt{x} transformation

Source	df	MS	F	P
Between subjects				
Egg maternity (M)	1	13,259.30	31.55	<0.001
Discriminator (D)	5	1,346.13	3.20	0.02
M×D	5	996.99	2.37	0.06
Error	32	420.29		
Within subjects				
Time (T)	5	7,288.03	124.30	<0.001
T×M	5	53.75	0.92	0.47
T×D	25	141.14	2.41	<0.001
T×M×D	25	133.87	2.28	0.001
Error	160	58.63		

Table 3 Repeated-measures ANOVAs of the effect of egg maternity (worker or queen-laid), time in hours from commencement, discriminator colony and days since dequeening on the proportion of eggs surviving in queenless discriminator colonies (experiment 2). All data have been transformed with an arcsine \sqrt{x} transformation

Source	<i>df</i>	MS	<i>F</i>	<i>P</i>
Data pooled across the three discriminator colonies				
Between subjects				
Egg maternity (M)	1	17,886.39	38.64	<0.001
Day	5	9,609.90	20.75	<0.001
M×day	2	267.28	0.56	0.72
Error	28	462.86		
Within subjects				
Time (T)	4	5,648.92	99.62	<0.001
T×M	4	186.57	2.88	0.03
T×day	8	62.65	1.05	0.41
T×M×day	8	82.80	1.38	0.21
Error	112	59.90		
Data pooled across the 9 days (six measurements) of the experiment				
Between subjects				
Egg maternity (M)	1	19,400.98	9.80	<0.004
Discriminator colony (D)	5	1,199.49	0.61	0.55
M×D	2	853.75	0.43	0.65
Error	28	1980.26		
Within subjects				
Time (T)	4	5,967.56	99.62	<0.001
T×M	4	172.44	2.88	0.03
T×D	8	62.65	1.05	0.41
T×M×D	8	82.80	1.38	0.21
Error	112	59.90		

quickly than queen-laid eggs. The interaction between maternity and discriminator colony was not significant ($P=0.06$; Table 2), indicating there were no strong differences between discriminators in their preferential removal of WL eggs.

Worker policing in queenless colonies

We were unable to include both day and discriminator colony in the same analysis of variance. We therefore conducted two separate repeated-measures ANOVAs of transformed data (Table 3). The first (Table 3; pooled across discriminator colonies) showed that the maternity of transferred eggs had a significant effect on egg survival ($P<0.001$). Overall, QL eggs survived longer than WL eggs. Averaging across all experimental days and the three discriminator colonies, 23.7% of QL eggs remained in the discriminator colonies overnight, whereas only 9.9% of WL eggs did so. This effect varied by day ($P<0.001$). However, the greater survival of QL versus WL eggs was constant across all days of the experiment (Fig. 2) and there was no significant interaction between day and egg maternity ($P=0.72$; Table 3).

The second analysis (Table 3) pooled across the 9 days (six measurements) of the experiment. Again, the maternity of eggs had a strong effect on egg survival ($P=0.004$; Table 3 B). There was no significant difference between the three discriminator colonies ($P=0.5$), nor was there a significant interaction between discriminator and egg maternity ($P=0.65$; Table 3).

The survival of both QL and WL eggs declined over the course of the experiment, relative to the first 2 days (Fig. 3). This decline, first manifest on day 3, coincided with the onset of ovary activation in workers (Fig. 1) and the appearance of worker-laid eggs in the discriminator colonies.

Maternal origin of drones

Queen genotype was inferred (Oldroyd et al. 1996) for each colony from which drones were obtained (Table 4). A total of 652 male pupae from four colonies were analysed (Table 4). No drone genotype was observed that was not consistent with inferred queen maternity (Table 4). For each colony, all drone genotypes were found in the ratios expected under the hypothesis that the queen was the sole mother of all the males ($P\chi^2>0.51$ in all cases; Table 4).

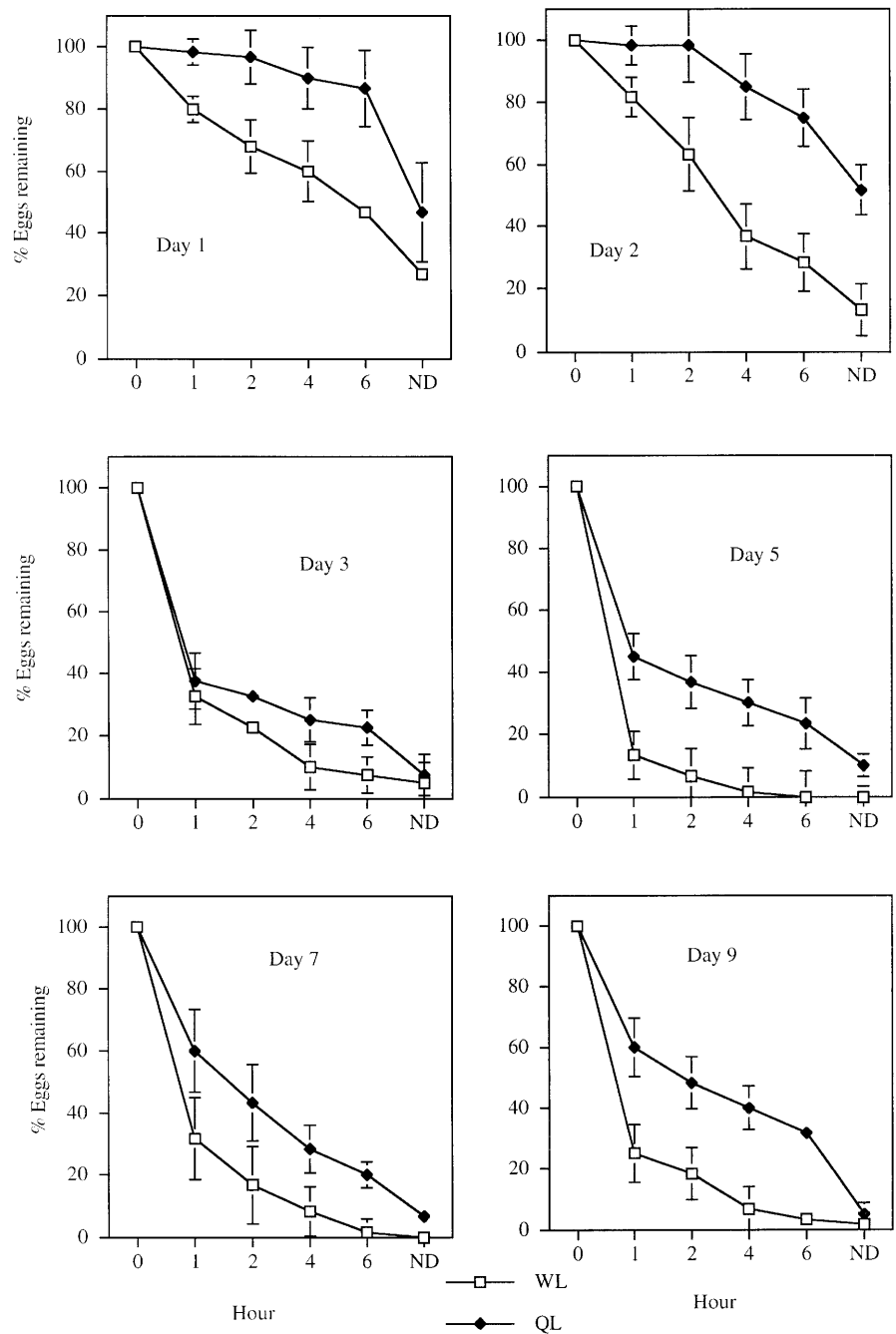
Drones arising from worker-laid eggs can have genotypes consistent with being queen-laid. This is because a worker's son has a 50% probability of carrying the copy transmitted to his mother from the queen. Furthermore, queens may mate with a proportion p_k drones carrying alleles identical to one of her own at any given locus (k). This leads to a non-detection error rate, P_{ND} , across the n loci studied that is estimated as:

$$P_{ND} = \prod_{k=1}^{11} (0.5 + 0.5p_k)$$

Thus the probability of detection is:

$$P_D = 1 - P_{ND} \text{ (Halling et al. 2001)}$$

Fig. 3 Survival of worker-laid (WL) and queen-laid (QL) eggs in three queenless discriminator colonies. Data are the means of the three colonies. *Bars* indicate the SEs of the least-square means. *Day* indicates the number of days since dequeening on day 0



We estimated p_k for each colony and locus using data from the 140 workers used to infer queen genotype, and then P_D for each colony. The average probability of detecting a son of a worker over all four colonies was 0.56 (i.e. there is a 44% chance that any one son of a worker would not be detected). Therefore in the sample size of 652 males, we would expect to see at least one worker-derived individual if the actual frequency was as low as 1 in 365 drones. If the actual frequency of worker-laid males was 5%, then the expected number of detected worker-laid males in a sample of this size is 18.2.

Discussion

Extreme polyandry appears to be a universal feature of the genus *Apis* (Palmer and Oldroyd 2000), suggesting that polyandry arose before the divergence of the extant species. Given that worker policing is also documented in *A. mellifera* (Ratnieks and Visscher 1989) and *A. florea* (Halling et al. 2001), the presence of well-developed worker policing based on oophagy in *A. cerana* provides further support for the hypothesis that policing behaviour is plesiomorphic in the genus (Halling et al. 2001). This concurs with theoretical predictions that

Table 4 Genotypes (microsatellite allele lengths in base pairs) and tests of the null hypothesis of queen maternity for *A. cerana* drones collected from queenright colonies

Colony	Queen genotype			Observed drone genotypes			Observed frequency of drone genotypes	Expected frequency of drone genotypes under a hypothesis of queen maternity	χ^2	df	P
	A28	B124	Ap43	A28	B124	Ap43					
1	112/117	220/220	130/150	112	220	130	24	24.5	0.12	3	0.99
			112	220	150	24	24.5				
			117	220	130	26	24.5				
			117	220	150	24	24.5				
2	114/117	220/220	154/154	114	220	154	95	96.0	0.02	1	0.88
			117	220	154	97	96.0				
3	112/115	218/220	135/137	112	218	135	26	21.25	6.2	7	0.52
			112	218	137	27	21.25				
			112	220	135	17	21.25				
			112	220	137	23	21.25				
			115	218	135	24	21.25				
			115	218	137	17	21.25				
			115	220	135	16	21.25				
			115	220	137	20	21.25				
4	107/107	220/220	152/154	107	220	152	55	48.0	2.2	3	0.53
			107	220	154	46	48.0				
			117	220	152	41	48.0				
			117	220	154	50	48.0				

worker policing is selected for under conditions of polyandry (Ratnieks 1988). Worker policing via oophagy is also found in the polyandrous wasp *V. vulgaris* (Foster and Ratnieks 2001a, 2001b), and in polyandrous but not monandrous colonies of the hornet *Dolichovespula saxonica* (Foster and Ratnieks 2000, 2001b).

Although experimental conditions vary widely, the data presented here suggest that policing via oophagy of worker-laid eggs is slightly less rapid in *A. cerana* than in *A. florea* or *A. mellifera*. On the first day of the queenless assay, 26.7% of worker-laid eggs remained after 24 h. Such levels of WL egg survival have not been observed in any study of *A. mellifera* (Ratnieks and Visscher 1989) or in *A. florea* (Halling et al. 2001), indicating that *A. cerana* may be more permissive of WL eggs than are other species. However, overall, policing is very effective in *A. cerana*, since we were unable to detect any worker-laid pupae.

Survival of queen-laid eggs was also low in this study, though comparable to other studies of this kind (Oldroyd and Ratnieks 2000; Halling et al. 2001). This may be due to damage during egg manipulation, and to nestmate recognition effects. However, the difference in the survival between (foreign) QL eggs and (foreign) WL eggs is quite clear.

The *A. cerana* colonies available for this study were not strong, forcing us to use whatever eggs were available, rather than age-matched eggs. This introduces two potential biases. First, rates of egg cannibalism may vary with egg age. Second, older eggs are preselected for survival, because unacceptable eggs are removed. Our

methods should not have produced a systematic bias to older or younger eggs in either (QL or WL) treatment. We therefore do not consider it likely that our treatment differences arose from this cause.

An effective worker-policing mechanism, such as we have demonstrated for *A. cerana*, should reduce the benefits an individual worker can gain from laying eggs (Seeley 1985; Keller and Nonacs 1993). However, the level of ovarian development found in *A. cerana* workers (this study; Sakagami 1958) is much higher than that recorded for *A. mellifera* (Ratnieks 1993; Visscher 1996) and *A. florea* (Halling et al. 2001). This suggests that there are still benefits to personal fitness that can arise from worker reproduction. *A. mellifera* workers with activated ovaries are subject to harassment from other workers (Visscher and Dukas 1995). Such aggressive behaviour is perhaps not displayed by *A. cerana*. This possibility should be tested by direct observation using the method described by Visscher and Dukas (1995). The presence of full-sized eggs in the ovarioles of queenright workers implies that some workers lay in queenright colonies, so that actual conflict between workers probably occurs in this species, and this may reduce overall colony fitness.

As in *A. mellifera*, the benefits associated with worker reproduction in queenright colonies appear to be low because few if any worker-laid eggs reach maturity. However, as we only examined about 365 'effective' males, the rate of worker maternity in *A. cerana* may be much higher than the 1.2 in 1,000 males reported by Visscher (1989) from a much larger sample of *A. mellifera*. However, when colonies are queenless, many worker-laid

eggs are reared into viable drones. Because this represents an opportunity for workers to increase their personal fitness, adaptations that enhance a worker's reproductive success when queenless may be expected. If queenlessness is common in *A. cerana*, it may be beneficial for a worker to always have partially or totally activated ovaries, in preparation for queenlessness. Page and Erickson (1988) showed that in queenless colonies of *A. mellifera*, the majority of drones that were reared to maturity derived from those eggs that were laid during the first few days of laying-worker activity. Hence, rapid conversion to oviposition when queenless increases a worker's fitness and partial development of ovaries is expected to reduce the period of latency before a worker can lay. The behaviours of swarming and absconding are a common feature of *A. cerana* biology (Ruttner 1988), and both of these behaviours increase the probability that individual colonies will become queenless.

Rates of worker policing remained high after colonies were made queenless, despite the fact that just 3 days after dequeening, worker-laid eggs were observed in all three discriminator colonies. We suggest that worker-laid eggs vary in their acceptability, and that a very small proportion are tolerated, while the vast majority are policed. Worker-laid eggs were consistently removed in all experiments, and always more rapidly than queen-laid eggs. However, the general acceptability of all eggs (QL and WL) appeared to decline after day 3. This coincided with the period of rapid increase in the proportion of bees with activated ovaries, and the two phenomenon may be causally related. However, the increased defensiveness of the colonies caused by frequent disturbance during these experiments may have reduced their tolerance for all foreign eggs.

We conclude that while worker policing appears to be well-developed in *A. cerana*, rates of worker ovary activation in queenright colonies are much higher than in *A. mellifera* (Ratnieks 1993) or *A. florea* (Halling et al. 2001). *A. cerana* workers most likely lay male eggs at a higher rate than the 7% estimated by Visscher (1996). Thus reproductive conflict may be more overt than in *A. mellifera* or *A. florea*. Careful investigations of the mechanisms of this conflict and its antecedents in *A. cerana*, and possibly in tropical races of *A. mellifera*, are likely to be informative.

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