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Biol. Lett. 2005 **1**, 129-132
doi: 10.1098/rsbl.2005.0294

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doi:10.1098/rsbl.2005.0294
Published online 16 May 2005

Honeybee workers use cues other than egg viability for policing

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Worker policing, wherein social insect workers prevent their sisters from reproducing by eating worker-laid eggs, is recognized as a textbook example of kin selection in action. However, the evolutionary basis of policing was recently challenged in a study that suggested that police-workers remove worker-laid eggs not because rearing workers' sons reduces worker fitness, but merely because worker-laid eggs have low viability. Here, we refute Pirk *et al.*'s conclusions. First, we confirm earlier work that showed equal viability of eggs laid by queens and workers. Second, a statistical analysis of the data of Pirk *et al.* reveals that their own data do not support the conclusion that worker-laid eggs are policed merely because of their low viability. Third, we present data that unequivocally show that police-workers cannot discriminate between dead and live eggs. Hence, our study seriously weakens the challenge to the kin-selected basis of policing in honeybees.

Keywords: *Apis mellifera*; worker policing; egg marking; kin selection; inclusive fitness; egg viability

1. INTRODUCTION

Kin-selection theory suggests that policing of selfish reproductive workers is important for social cohesion in insect societies. Without some method of policing, such as removal of worker-laid eggs, genotypes that promote worker reproduction will spread in populations, reducing colony-level fitness or the inclusive fitness of non-reproductive workers (Ratnieks 1988). The mechanism that allows workers to distinguish and remove worker-laid eggs while retaining queen-laid ones is thought to be mediated by an egg-marking pheromone placed on eggs by queens, but which is absent from worker-laid eggs (Martin *et al.* 2002*b*; Katzav-Gozansky *et al.* 2003).

Despite the strong theoretical reasons and ample empirical support for worker policing, Pirk *et al.* (2004) have recently argued that the low levels of workers' sons that are reared in honeybee colonies that contain a queen (less than 0.1%; Visscher 1989) are not due to policing of worker-laid eggs that do not possess a queen signal. Rather, Pirk *et al.* (2004) state that 'to achieve an efficient removal of worker-laid eggs in a honeybee colony, it appears to be sufficient for a honeybee worker to simply discriminate between dead and live eggs' (p. 8650). This implies that experimental demonstrations of worker policing (of which there are now about 100 published papers) are potentially in error. Pirk *et al.* (2004) argue that low rates of reproduction in honeybee colonies are not the result of adaptive worker policing based on recognition of queen-laid eggs, but of the low viability of worker-laid eggs. If they are correct, our most important example of kin selection in action (e.g. Dugatkin 1997, 2004; Whitfield 2002) is seriously challenged (Gadagkar 2004; Sugden 2004).

Here, we show that, in fact, honeybee workers can and do assess the maternity of eggs without reference to their viability. We compare the policing rates of queen- and worker-laid eggs that had been killed by carbon dioxide, and compare these with the policing rates of unkilled eggs. If, as Pirk *et al.* (2004) argue, police workers only use viability to distinguish queen-laid eggs from worker-laid eggs, then dead eggs should be removed more quickly than live ones. If, on the other hand, they use cues that are independent of viability, then they should remove live queen-laid eggs at the same low rate as dead queen-laid eggs.

2. MATERIAL AND METHODS

We obtained combs containing several hundred eggs laid by queens (from queen-right colonies) and workers (from laying-worker colonies without a queen) of the European honeybee *Apis mellifera*. We then divided each comb into halves. One half was placed in a zip-lock plastic bag, which we then filled with carbon dioxide before sealing the bag. The other was placed in a bag but not fumigated with carbon dioxide. The comb sections were then placed in an incubator at 35 °C and high humidity for 24 h.

The following day we conducted standard policing bioassays (Ratnieks & Visscher 1989; Oldroyd & Ratnieks 2000; Martin *et al.* 2002*a*) on the treated and untreated eggs. Briefly, we transferred rows of 20 treated (dead) and untreated (live) eggs from both sources (queen-laid and worker-laid) into drone combs (treated and untreated eggs of both egg sources were placed in the same drone comb) and sandwiched these combs between brood combs in populous discriminator colonies. The queens of our test colonies could not access the combs, and so could not interfere with our experiment by laying additional eggs. We checked the test combs for the presence of transferred eggs at regular intervals for 6 hours. We repeated the entire study on four separate days using different egg-source colonies where possible, and two or three discriminator colonies per day.

To confirm that the viability of eggs that we treated with carbon dioxide was zero, and that the untreated eggs were viable, we randomly selected rows of 20–30 eggs not used in the policing trial and marked them with coloured pins. We then returned the comb with the marked rows of eggs to the incubator and checked the eggs at irregular intervals. Larvae were removed as soon as they were observed to avoid underestimating hatch rates owing to desiccation of the larvae. Eggs were classified as non-viable if they failed to hatch within 4 days, and viable if they did hatch. We checked the viability of eggs for each policing trial.

3. RESULTS

None of the eggs treated with carbon dioxide and kept in the incubator hatched (in total 105 worker- and 113 queen-laid eggs), showing that the treatment killed them. The hatch rates of control queen-laid and worker-laid eggs were marginally statistically different (*G* of totals, $p=0.042$; table 1), with the overall hatch rate of worker-laid eggs (78%) slightly higher than that of queen-laid eggs (68%). Heterogeneity analysis showed a statistically significant effect of day (table 1).

Police workers removed dead and live worker-laid eggs rapidly, whereas both dead and live queen-laid eggs were removed at a much slower rate (figure 1). A Cox regression analysis (Collett 1994; table 2)

Received 11 October 2004
Accepted 10 January 2005

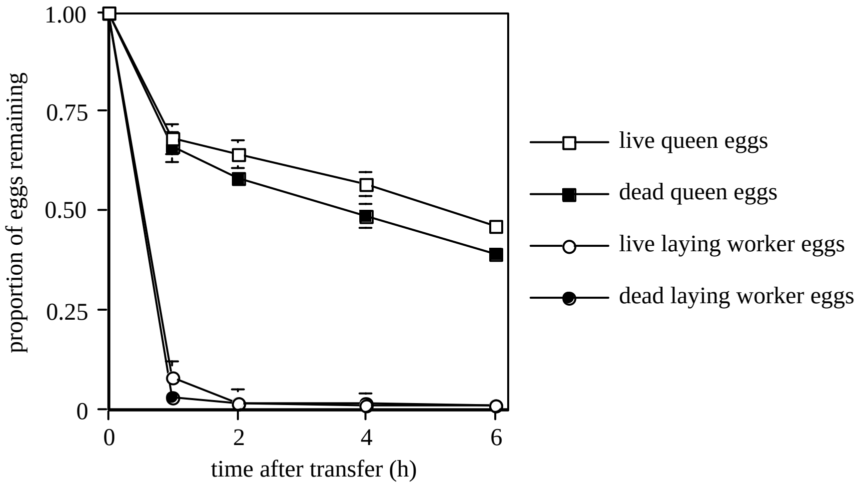

Figure 1. Removal rates of dead (treated with carbon dioxide) and live (untreated) queen- and worker-laid eggs ($N=200$ for each egg-source). Values are the mean of all discriminator colonies (n=2 or 3) over all days (n=4). The bars represent the standard errors of the means.

Table 1. Contingency analysis of the hatch rates of queen- and worker-laid eggs. Eggs were kept in an incubator and checked at irregular intervals. Eggs that had not hatched after 4 days were presumed dead. 'Trial' refers to the policing trial hence for each policing experiment we checked the viability of the eggs used.

trial	egg source	hatched	not hatched	*G*	d.f.	*p*
1	queen	11	12	1.423	1	0.23
	worker	15	8			
2	queen	7	23	15.94	1	<0.01
	worker	27	11			
3	queen	30	0	5.06	1	0.02
	worker	32	4			
4	queen	43	4	1.180	1	0.28
	worker	37	7			
G of totals				4.133	1	0.04
total of *G*s				23.60	4	0.01
heterogeneity				19.47	3	<0.001

shows that there were statistically significant differences in removal rates for worker-laid and queen-laid eggs (table 2; $p<0.001$) but that the removal rates of dead and live queen-laid eggs were not significantly different (table 2; $p=0.26$). Similarly, dead and live worker-laid eggs were removed at the same rate (table 2; $p=0.88$). Neither the day of the trial nor the discriminator colony in which the assay was performed had a significant effect on removal rates (table 2).

4. DISCUSSION

Our results clearly show that honeybee police workers do not use egg viability as a marker for removing worker-laid eggs. There is no statistical difference in removal rates of killed and non-killed eggs, whereas experiments presented here for honeybees and across a wide range of other species (Ratnieks & Visscher 1989; Gobin *et al.* 1999; Liebig *et al.* 1999; Foster & Ratnieks 2001; Halling *et al.* 2001; Oldroyd *et al.* 2001; Foster *et al.* 2002; Martin *et al.* 2002*a*; Hartmann *et al.* 2003; Pirk *et al.* 2003; Endler *et al.* 2004) have repeatedly shown that worker-laid eggs are actively policed.

Our conclusions are in stark contrast to those of Pirk *et al.* (2004). In summary, Pirk *et al.* (2004) present two observations. First, the viability (measured as the proportion of the eggs that hatched into larvae) of the worker-laid eggs they used (22.8%) was about one-quarter of that of the queen-laid eggs they used (81%). Second, as with all published studies of worker policing in honeybees, about half of the queen-laid eggs were retained by the discriminator colonies, whereas all worker-laid eggs were destroyed within a few hours.

Pirk *et al.* (2004) interpret this apparent correlation as evidence that it is unnecessary to invoke adaptive policing mechanisms based on recognition of a queen's egg-marking signal to explain the very low levels of worker reproduction in queen-right colonies of honeybees. Pirk *et al.* (2004) argue that control of successful worker reproduction does not require active discrimination of worker-laid eggs, merely the removal of dead eggs and dead larvae. Clearly, their data do not support this assertion, because viable worker-laid eggs, which comprised at least 20% of their sample, were removed by their discriminator colonies. Their data actually show that there is a significant difference in

Table 2. Likelihood ratios comparing the survival of dead and live queen- and worker-laid eggs in worker policing bioassays ($n=200$ for each egg source).
(The likelihood ratios compare the survival of eggs of different sources (dead queen-laid eggs, live queen-laid eggs, dead worker-laid eggs and live worker-laid eggs). The survival function is first modelled without ('null') and then with ('overall') egg type, day and discriminator colony as factors, and the improvement of the ability of the model to describe the data tested with a χ^2-test. The procedure then tests the effect of adding 'source of eggs', 'day' or 'discriminator' as factors. Lastly, we present a pairwise comparison between the survival of worker-laid and virgin-laid eggs with eggs laid by mated queens. See figure 1 for a graphical presentation of these data.)

term	−2 log likelihood	χ^2	d.f.	p
null	8091.5			
overall	7945.3	141.5	5	<0.001
source of eggs		128.6	3	<0.001
day of trial		1.1	1	0.30
discriminator		0.6	1	0.42
pairwise comparisons:				
dead versus live queen eggs		1.3	1	0.26
dead versus live worker eggs		0.02	1	0.88

removal rates between queen- and worker-laid eggs after correcting for the difference in viability between the two egg types. Pirk *et al.* (2004) used a total of 60 eggs of each egg source in their policing assays. Given the viability rates of queen- and worker-laid eggs, this means that the number of viable eggs used was 48.6 and 13.7 viable queen- and worker-laid eggs, respectively. After 24 h, 33 queen-laid eggs (55% of a total of 60 eggs used; see Pirk *et al.*'s (2004) fig. 2) and no worker-laid eggs remained. A χ^2_1 goodness of fit shows that this difference is statistically significant ($\chi^2_1=18.7$, $p<0.001$). Thus, police workers in their discriminator colonies must have used an additional or alternative cue to promptly remove live worker-laid eggs.

Pirk *et al.* (2004) argue that 'caste differences between workers and queens may underlie the obvious differences in egg viability' (p. 8650). We have shown that such differences in egg viability do not in fact exist, supporting earlier work (Ratnieks & Visscher 1989). Eggs from weak-laying worker colonies that have been queenless for a long time may have low hatchability. However, worker-laid eggs in one of our trials had hatchability approaching 100% (trial 3; table 1), whereas one sample of eggs from a queen from a weak colony had hatchability of around 50% (trial 2; table 1). Our data clearly show that worker policing cannot be explained away as consequence of low viability of worker-laid eggs.

Kin-selection theory makes clear predictions that worker policing should occur in *A. mellifera* and other polyandrous social insect species (Ratnieks 1988; Barron *et al.* 2001). Where policing mechanisms break down in *A. mellifera* as a result of occasional unusual workers that lay acceptable eggs, the resulting colony-level phenotype is lethal as soon as the number of egg-laying workers becomes very high (Barron *et al.* 2001) owing to workers laying masses of viable eggs. Pirk *et al.* (2004) argue that 'factors other than colony kin structure can govern worker reproduction in a wide variety of social insect species' (p. 8650). Although we do not dispute the existence of factors other than relatedness, we have shown that workers distinguish queen-laid eggs from worker-laid eggs based on cues that are unrelated to egg viability. Hence, the challenge to the honeybee's kin-selected ability to distinguish queen- and worker-laid eggs is seriously weakened.

Our work is funded by the Australian Research Council. We are grateful to Michael Duncan for suggesting carbon dioxide as an efficient non-invasive way to kill eggs, and for beekeeping support. We also thank Francis Ratnieks and one of the reviewers who made some valuable suggestions that improved the manuscript.

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