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Cheating honeybee workers produce royal offspring

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The Cape bee (*Apis mellifera capensis*) is unique among honeybees in that workers can lay eggs that instead of developing into males develop into females via thelytokous parthenogenesis. We show that this ability allows workers to compete directly with the queen over the production of new queens. Genetic analyses using microsatellites revealed that 23 out of 39 new queens produced by seven colonies were offspring of workers and not the resident queen. Of these, eight were laid by resident workers, but the majority were offspring of parasitic workers from other colonies. The parasites were derived from several clonal lineages that entered the colonies and successfully targeted queen cells for parasitism. Hence, these parasitic workers had the potential to become genetically reincarnated as queens. Of the daughter queens laid by the resident queen, three were produced asexually, suggesting that queens can 'choose' to produce daughter queens clonally and thus have the potential for genetic immortality.

Keywords: *Apis mellifera capensis*; reproductive parasitism; thelytoky

1. INTRODUCTION

Reproductive cooperation is a defining characteristic of insect societies. However, because individuals within an insect colony are rarely clonal, their interests never overlap completely, leading to reproductive conflicts among colony members (Beekman & Ratnieks 2003). As a result, most insect societies have evolved mechanisms that control selfish individuals in ways analogous to our own bodies curtailing exploitation by malignant cells. In polyandrous honeybees, the most important mechanism for controlling reproduction by selfish workers is worker policing—the selective removal of eggs laid by workers. In arrhenotokous populations, in which if workers do lay eggs they produce males, workers are more related to the sons produced by the queen (relatedness = 0.25) than to the average worker-produced son ($r \sim 0.125$; Ratnieks 1988). As a result, the workers can increase their inclusive fitness (Hamilton 1964) by refraining from individual reproduction (Wenseleers *et al.* 2004) and by removing any eggs laid by workers (Ratnieks & Visscher 1989). In contrast, in populations where workers can produce female offspring via thelytokous parthenogenesis, such as in the Cape honeybee *Apis mellifera capensis* of South Africa (Onions 1912; Anderson 1963), this compromise of effective worker sterility is not evolutionarily stable (Greeff 1996). This is because thelytokously produced offspring of workers are pseudo-clones of their mothers ($r = 1$; Baudry *et al.* 2004). Thus, Cape honeybee workers are predicted to be more tolerant of worker reproduction than workers of other honeybee races because diploid eggs laid by queens or clonally by the queen's workers are genetically equivalent (Hamilton 1972). As it is irrelevant whether an egg is laid by a queen or a worker, worker policing is expected to be reduced or absent in the Cape honeybee (Greeff 1996).

Thelytoky not only alters worker–worker relatedness but also changes relationships between the queen and her workers. Whereas in arrhenotokous subspecies, workers can only compete with the queen and their worker-sisters over the production of males, in *A. m. capensis*, workers can compete with their queen for the production of offspring queens (Beekman & Oldroyd 2008; Boot *et al.* in press). In relatedness terms, a worker that produces the next queen via thelytoky effectively becomes the new queen herself. Hence, the potential fitness payoff for a worker that successfully produces a new queen is enormous. Interestingly, the queen is expected to be largely indifferent to workers producing new queens, because her relatedness to both her own sexually produced daughters and thelytokously produced offspring of daughters is identical ($r = 0.5$; Greeff 1996). However, competition among workers over the production of new queens is predicted to be severe, as each worker can enhance her direct fitness if she or her super-sister (females that share the same father, i.e. are of the same patriline) is the mother of new queens.

Prior to reproductive swarming, a honeybee colony produces 5–10 greatly enlarged brood cells. Eggs are laid in these cells, and the resulting larvae are lavishly fed so

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that they develop as queens (Winston 1987). Here we determine the maternal origin of queen larvae or pupae in *A. m. capensis* using microsatellites and show that, as predicted from the kin structure of *A. m. capensis* colonies, workers contribute significantly to royal offspring.

2. MATERIAL AND METHODS

We encouraged natural swarming in eight colonies of *A. m. capensis* by moving them in early spring to an area in southern South Africa where cultivated canola, *Brassica rapa*, was flowering. Such conditions are highly conducive to population growth and reproductive swarming in honeybee colonies. To further encourage swarming, we constrained the colonies to a single Langstroth box so that they quickly outgrew the space available in their hives. As a result, the bees started to produce queen cells in preparation for reproductive swarming.

The offspring of a queen and the clonal offspring of one of her workers can share the same genotype. Thus, to allow us to distinguish queen- and worker-laid queen cell contents (larvae and pupae; hereafter QCCs), we manipulated the swarming colonies such that each colony's queen was not related to the workers. To do this, we either swapped brood between pairs of colonies every three weeks starting 12 weeks prior to harvesting the first QCC (four colonies) or swapped the queens (four colonies) between pairs of colonies. Swapping brood and queens between colonies is common bee-keeping practice (Morse 1990) and is not known to increase rates of worker reproduction.

We harvested all QCC produced by our colonies during the swarming period. To detect worker reproduction in worker cells, we sampled pre-emergent workers every two weeks throughout the experiment. To monitor the level of ovary activation of resident workers during the swarming period, we dissected approximately 400 adult workers per colony: 200 sampled at the beginning of reproductive swarming and 200 when the colonies were actively producing new queens. To determine the genotype of the resident queen of each colony, we removed a wing for genotyping.

We obtained DNA from tissue using a standard Chelex extraction method (Walsh *et al.* 1991) from wings (queens), hind legs (adult workers and pupae) or the head or abdomen (larvae). All individuals were genotyped at six microsatellite loci: A113, A29, A7, A79, A88 and B124 (Solignac *et al.* 2003). These microsatellite markers were amplified in two triplex polymerase chain reactions (triplex 1: A29/A7/B124 and triplex 2: A113/A79/A88) using standard PCR conditions (Estoup *et al.* 1994). In a few cases where we needed to confirm the sex of an individual, we genotyped it at the locus U351_B, which is tightly linked to the complementary sex-determining locus (Beye *et al.* 2003). Individuals heterozygous at U351_B (and by association the *csd*) are almost certainly female (Beye *et al.* 2003).

PCR products (1.2 µl) from each multiplex reaction were added to 10 µl formamide and 100 nl LIZ DNA size standard (Applied Biosystems). Samples were run on a 3130 xl Genetic Analyser (Applied Biosystems), with capillary length 36 cm and injection time of 15 s at 1200 V, for 41 min. Resultant data files were analysed using GENEMAPPER software (Applied Biosystems) and genotypes for each individual constructed.

We compared QCC genotypes with queen and adult worker genotypes within each colony to determine whether queens, resident workers or foreign workers produced QCC. We also analysed the genotypes of pre-emergent workers. If a QCC is the sexually produced offspring of the resident queen, the two individuals must share at least one allele at each locus. If a QCC is a thelytokous offspring of the resident queen both alleles carried by the QCC at each locus must be present in the resident queen. Individuals were determined to be non-queen laid if they did not share an allele with the resident queen at a locus. QCCs were classed as foreign laid if they did not share alleles with either the resident queen or resident worker consensus genotype at a locus.

3. RESULTS

We first had to confirm that the swaps had been successful. We did this by genotyping a wing from the resident queen and an average of 82 ($\pm$1.92 s.e.m.) adult workers from each colony. In all cases, the workers present in the colonies were not related to the queen at the time the QCCs were collected (table 1). Genotyping workers from the swapped pair colony allowed us to confirm the genotype of queens determined from wings.

We collected a total of 39 QCCs originating from seven colonies (one colony produced no queen cells). Sixteen QCCs from five colonies were offspring of the resident queen (table 1). Twenty-three QCCs from four colonies contained QCC that had genotypes incompatible with having been laid by the resident queen. Of these, eight QCCs shared alleles with the resident workers, while the remaining QCC could not have been produced by either the queen or the resident workers (table 1), and hence were laid by individuals foreign to the sampled colony. We also found a strong patriline bias in queen-laid offspring. For example, in colony 2 five out of seven QCCs were fathered by a single drone (table 1).

Ten QCCs from four colonies were homozygous at all loci tested (table 1), raising the remote possibility that these were haploid males. However, either morphological or genetic analysis of these individuals confirmed that nearly all were diploid and female. Morphological examination of the genital region (Duchateau & van Leeuwen 1990) of QCC 3, 5, 7, and 8 from colony 3, and QCC 7 from colony 7 confirmed that these individuals were female. The sex of three individuals, QCC 2 from colony 2 and QCC 1 and 6 from colony 3 could not be confirmed morphologically because the genital region had been removed for genotyping, but genotyping with microsatellite locus U351_B, confirmed that these individuals were heterozygous at that microsatellite locus and therefore almost certainly females. The sex of two further homozygous individuals (QCC 4 from colony 3 and QCC 1 from colony 5) could not be determined morphologically, and they were homozygous at all loci studied including U351_B. Therefore, these individuals may be diploid or haploid males, or females as they may still have been heterozygous at the *csd*.

An average of 6.86% ($\pm$3.51) of sampled adult workers was drifted foreign workers, though none of these could have produced the observed genotypes of QCC (see table S1 in the electronic supplementary material). We detected a significant increase in workers with active ovaries over the course of queen rearing in colonies 3 and 7 (Fisher's exact test, $n=400$, $p=0.03$ and $n=473$, $p=0.01$, respectively; table S2 in the electronic supplementary material). The number of workers with active ovaries was

Table 1. Genotypes of the resident queen, workers and QCCs for each colony. (The genotype of the resident queen was obtained directly from tissue from her wing, and confirmed by the genotypes of her daughter workers in her swapped colony. The genotype of the mother of the workers in the colony was inferred from the genotype of an average of 82 (±1.92) adult workers in the colony, and confirmed by the genotype of their actual mother in the swapped colony.)

			primer												
colony	swap colony	samples	A113		A29		A7		A79		A88		B124		mother[a]
1	3	resident queen	217	223	136	140	100	108	94	101	140	143	230	230	
		mother of workers	215	223	126	160	110	113	94	99	144	150	215	219	
		no queen cells													
2	4	resident queen	215	215	138	142	107	117	97	119	145	150	228	232	
		mother of workers	223	227	128	132	107	111	97	99	144	150	224	232	
		queen cell 1	215	219	138	138	102	107	97	116	143	145	221	228	q
		queen cell 2	215	215	138	138	107	107	97	97	150	150	232	232	q
		queen cell 3	215	219	138	138	102	117	97	116	143	145	221	228	q
		queen cell 4	215	219	142	142	102	117	97	116	143	145	221	232	q
		queen cell 5	215	219	138	138	102	107	97	116	143	143	221	228	q
		queen cell 6	215	223	132	138	107	107	97	97	150	150	215	228	q
		queen cell 7	223	227	130	132	107	107	99	112	145	150	221	224	w
3	1	resident queen	215	223	126	160	110	113	94	99	144	150	215	219	
		mother of workers	217	223	136	140	100	108	94	101	140	143	230	230	
		queen cell 1	223	223	128	128	111	111	99	99	144	144	232	232	f
		queen cell 2	221	227	128	134	107	114	97	101	139	150	221	224	f
		queen cell 3	227	227	128	128	111	111	97	97	144	144	224	224	f
		queen cell 4	223	223	128	128	107	107	97	97	144	144	232	232	f
		queen cell 5	227	227	128	128	111	111	99	99	144	144	224	224	f
		queen cell 6	230	230	160	160	110	110	94	94	150	150	224	224	f
		queen cell 7	215	215	160	160	110	110	99	99	144	144	215	215	q
		queen cell 8	223	223	160	160	113	113	94	94	150	150	219	219	q
		queen cell 9	215	227	126	130	110	110	94	119	144	150	215	230	q
		queen cell 10	215	225	126	130	105	110	99	101	139	150	215	221	q
		queen cell 11	223	223	138	160	113	113	94	110	143	150	215	219	q
		queen cell 12	223	227	130	132	111	111	99	112	145	150	221	232	f
		queen cell 13	223	227	130	132	113	113	99	114	144	145	221	224	f
		queen cell 14	223	223	128	130	111	111	97	112	144	145	221	232	f
		queen cell 15	223	227	130	132	111	111	99	112	145	150	221	232	f
4	2	colony 4													
		resident queen	223	227	128	132	107	111	97	99	144	150	224	232	
		mother of workers	215	215	138	142	107	117	97	119	145	150	228	232	
		queen cell 1	217	223	128	138	111	111	99	104	144	150	215	232	q
		queen cell 2	223	227	128	128	111	111	97	121	150	150	213	232	q
5	—	resident queen	215	217	132	134	97	104	101	104	150	150	222	224	
		mother of workers	217	225	132	138	111	117	104	106	150	150	215	234	
		queen cell 1	209	209	134	134	111	111	125	125	152	152	215	215	f

(*Continued.*)

Table 1. (*Continued*.)

			primer											
colony	swap colony	samples	A113		A29		A7		A79		A88		B124		mother[a]
6	5	resident queen	217	225	132	138	111	117	104	106	150	150	215	234	
		mother of workers	209	219	130	132	104	111	92	125	147	152	215	219	
		queen cell 1	217	225	132	132	110	117	104	104	140	150	232	234	q
		queen cell 2	215	225	138	138	111	111	99	104	143	150	221	234	q
7	8	resident queen	223	225	132	136	100	104	101	104	150	152	219	232	
		mother of workers	223	223	126	126	96	108	98	100	140	143	219	233	
		queen cell 1	223	227	134	134	96	104	98	101	140	145	219	233	f
		queen cell 2	223	223	126	126	96	105	98	112	143	145	228	233	w
		queen cell 3	223	223	126	126	96	96	96	98	140	140	219	219	w
		queen cell 4	217	225	126	126	96	99	104	104	140	150	217	219	f
		queen cell 5	223	227	126	134	96	105	100	101	140	143	219	219	w
		queen cell 6	219	223	126	134	105	108	98	99	140	150	219	234	w
		queen cell 7	223	223	132	132	108	108	98	98	140	140	219	219	f
		queen cell 8	217	223	126	126	96	99	98	99	140	155	217	219	w
		queen cell 9	223	227	126	134	96	105	100	101	140	143	219	219	w
		queen cell 10	211	223	126	134	105	108	98	106	140	140	219	234	w
		queen cell 11	221	223	132	132	108	108	98	99	143	144	219	233	f
8	7	resident queen	223	223	126	126	96	108	98	100	140	143	219	233	
		mother of workers	223	225	132	136	100	104	101	104	150	152	219	232	
		queen cell 1	211	223	126	134	96	104	99	100	140	143	219	233	q

[a]q, queen laid; f, foreign laid or w, resident worker laid. Shaded QCCs are homozygous at all loci tested.

Table 2. Microsatellite allele lengths (bp) and allele frequencies for QCCs that are potentially daughters of the resident queen and homozygous at all loci. (To avoid biases arising from the social structure of colonies, each worker contributed her paternally derived allele only to the population allele frequency (Queller & Goodnight 1989).)

	colony 2		colony 3			
	QCC 2		QCC 7		QCC 8	
locus	allele	frequency	allele	frequency	allele	frequency
A113	215	0.054	215	0.054	223	0.115
A29	138	0.095	160	0.013	160	0.013
A7	107	0.273	110	0.011	113	0.035
A79	97	0.213	99	0.108	94	0.108
A88	150	0.045	144	0.063	150	0.045
B124	232	0.104	215	0.087	219	0.002[a]

[a] This allele carried by the resident queen of colony 1 was not present in the paternal population, and has been given an arbitrary frequency of 0.002.

particularly high in colony 7 on 22 August 2006, a time when the colony was producing new queens, suggesting that worker reproduction increases when queen cells are present. Nonetheless, none of the workers with active ovaries we detected were responsible for producing QCC (table S3 in the electronic supplementary material). To monitor worker reproduction in worker cells, we genotyped an average of 99 (± 1.41) pre-emergent workers per colony. Six (0.8%) non-queen-laid pre-emergent workers were found, of which four had genotypes consistent with being laid by resident workers, while two were laid by foreign workers (table S4 in the electronic supplementary material).

4. DISCUSSION

Our findings unequivocally demonstrate that in thelytokous *A. m. capensis* both resident queens and workers are responsible for laying eggs in queen cells. Our results also suggest that queen cells are specifically targeted for parasitism by foreign workers. Worker policing evolved to curtail selfish worker reproduction and is highly effective in arrhenotokous *Apis mellifera* where only 0.06% of all males are worker derived (Visscher 1989). This is in contrast with the 0.8% worker-produced offspring we detected in worker-cells (table S4 in the electronic supplementary material), suggesting that worker policing is either absent or reduced in *A. m. capensis*, as predicted based on relatedness grounds (Greeff 1996). Even though *A. m. capensis* patrilines are expected to compete over the production of new queens, nepotistic policing of queen cells could only evolve if honeybee workers can discriminate between eggs laid by their super-sisters and half-sisters. This seems highly unlikely on two grounds. First, successful nepotism removes variance in recognition cues, thereby reducing the ability of workers to discriminate between super- and half-sister larvae (Ratnieks 1990; Ratnieks & Reeve 1991). Second, a hypothesized ability to discriminate between super- and half-sister larvae is inconsistent with our results that show that 59% of QCCs are worker laid, the majority by workers not related to any individual natal to the colony. Clearly, the increased tolerance of worker reproduction in *A. m. capensis* due to thelytoky (Greeff 1996) allows foreign workers to preferentially parasitize queen cells thereby greatly jeopardizing the host colony's fitness. However, increased tolerance of worker reproduction does not explain why the majority of worker-produced queen larvae were offspring of foreign workers and not of natal workers. The most likely explanation is that there are genotypic differences in the tendency of workers to activate their ovaries under queenright conditions and that it is those genotypes that are prone to invading other colonies. Our results indeed show that the number of foreign genotypes represented in queen larvae is rather small (table 1). In addition, genotypic differences in rates of ovary activation have been found in workers of both queenless (Robinson *et al.* 1990; Martin *et al.* 2004) and queenright colonies of *A. mellifera* (Oldroyd *et al.* 1994; Montague & Oldroyd 1998; Châline *et al.* 2002).

Not only do our data provide the first evidence of worker reproductive parasitism of queen cells in queenright honeybee colonies, but also they reveal interesting phenomena about reproduction in *A. m. capensis* queens. In colonies 2 and 3 we observed a total of three individuals homozygous at all loci studied for alleles shared with the resident queen (table 1). If we assume central fusion of meiotic products (Verma & Ruttner 1983; Baudry *et al.* 2004), the probability that a queen heterozygous at five loci (as in colony 3), unlinked to each other or centromeres, could produce a single female offspring homozygous at five independent loci is $0.33^5 = 0.004$ for a single offspring and 7×10^{-8} for three independent offspring. There are four plausible explanations for this unexpected observation: (i) these are male eggs laid arrhentokously by the queen, (ii) these are sexually produced eggs laid by the queen mated to a drone sharing alleles with the queen at each locus studied, (iii) these QCCs were laid by foreign worker(s) that shared a common haplotype with the queen, and (iv) these are eggs laid thelytokously by the queen.

Hypothesis (i) can be discarded because these QCCs were almost certainly female (see above). The likelihood of alternatives (ii)–(iv) can be evaluated by calculating the probability that the observed QCC genotypes could arise under each hypothesis. Table 2 gives the allelic frequencies in the population for the genotypes observed in the three QCCs of interest, calculated from all workers studied ($n = 494$ individuals), and these can be used to calculate the respective probabilities.

Under hypothesis (ii), the resident queen must have mated with a drone carrying one of her alleles at all loci.

This probability is $\prod_j (p_{j1} + p_{j2})$, where p_{j1} and p_{j2} are the frequency of the resident queen's two alleles at the jth locus and is 3×10^{-5} for colony 2 and 4×10^{-6} for colony 3.

Under hypothesis (iii), we evaluate the probability that a random worker in the population could potentially produce an egg thelytokously that had the same genotype as the homozygous QCC and could also have been produced by the resident queen. This probability is $\prod_j p_j$, where p_j is the frequency of the allele carried by the QCC at the jth locus. Thus, the probability that a random worker could be the mother of the QCC of interest is 3×10^{-5} for colony 2 and 2×10^{-7} for colony 3.

Given that hypotheses (i)–(iii) are unlikely, we are left with the final hypothesis—that these QCCs were laid thelytokously by the resident queens as being the most parsimonious. Clonal reproduction of offspring-queens has been previously reported in two species of ant, the little fire ant *Wasmannia auropunctata* (Fournier *et al.* 2005) and *Cataglyphis cursor* (Pearcy *et al.* 2004). In both ant species, queens are produced predominantly asexually while workers are always produced sexually. Interestingly, despite the apparent ability of *A. m. capensis* queens to produce new queens thelytokously, the great majority of queen-laid QCCs were produced sexually (table 1). The paternities of these sexually produced QCCs are not a random sample of the patrilines present in workers, suggesting that some genotypes are more likely to be reared as queens than others. Such patrilinial biases have previously been reported when arrhentokous honeybee colonies replace queens (Tilley & Oldroyd 1997; Osborne & Oldroyd 1999; Châline *et al.* 2003; Moritz *et al.* 2005). We also note that the reduction in heterozygosity which we observed in the three homozygous QCCs is not compatible with the existing model of thelytokous reproduction in Cape honeybee workers (Verma & Ruttner 1983; Baudry *et al.* 2004) in which the probability that a heterozygous locus will become homozygous is one-third per generation (Pearcy *et al.* 2006). This suggests that when queens produce new queens thelytokously they use a mechanism of cell division which is different to that of workers, and which dramatically increases homozygosity yet retains heterozygosity at the *csd*. The reason for this difference remains unexplained, but may possibly arise due to constraints in the kind of meiosis possible in a mated individual.

Thelytokous parthenogenesis with central fusion, as occurs in *A. m. capensis* workers, reduces heterozygosity by up to one-third per generation (Baudry *et al.* 2004), so a tell-tail sign of a clonal lineage is homozygosity at multiple loci in an otherwise highly heterozygous population. Seven QCCs laid by parasites were homozygous at all loci. Thus, these individuals are probably laid by clonal worker lineages similar to the 'pseudo-clone' currently parasitizing *A. m. scutellata* colonies in northern South Africa (Baudry *et al.* 2004). This suggests that the pseudo-clone is not an isolated phenomenon or a rare genotype with unusual characteristics. Rather, we suggest that many *A. m. capensis* workers have the potential to become successfully parasitic and that by specifically targeting queen cells they ensure their genetic immortality.

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