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ORIGINAL ARTICLE

Genetic reincarnation of workers as queens in the Eastern honeybee *Apis cerana*

MJ Holmes[1], K Tan[2,3], Z Wang[3], BP Oldroyd[1] and M Beekman[1]

Thelytokous parthenogenesis, or the asexual production of female offspring, is rare in the animal kingdom, but relatively common in social Hymenoptera. However, in honeybees, it is only known to be ubiquitous in one subspecies of *Apis mellifera*, the Cape honeybee, *A. mellifera capensis*. Here we report the appearance of queen cells in two colonies of the Eastern honeybee *Apis cerana* that no longer contained a queen or queen-produced brood to rear queens from. A combination of microsatellite genotyping and the timing of the appearance of these individuals excluded the possibility that they had been laid by the original queen. Based on the genotypes of these individuals, thelytokous production by natal workers is the most parsimonious explanation for their existence. Thus, we present the first example of thelytoky in a honeybee outside *A. mellifera*. We discuss the evolutionary and ecological consequences of thelytoky in *A. cerana*, in particular the role thelytoky may play in the recent invasions by populations of this species.
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INTRODUCTION

Thelytokous parthenogenesis, or the ability to produce female offspring without mating, is rare in animals (Suomalainen *et al.*, 1987). However, in the Hymenopteran insects, all of which are haplo-diploid, thelytoky is widespread, having evolved at least 255 times (Normark, 2003). It is particularly common in eusocial species, occurring in at least 51 extant species (Rabeling and Kronauer, 2013). Among the honeybees (*Apis* spp.), thelytoky is ubiquitous only in a subspecies of *Apis mellifera*, the Cape honeybee *A. m. capensis* (Rabeling and Kronauer, 2013). Thelytoky is either absent or very rare in all other honeybees (Mackensen, 1943; Goudie and Oldroyd, 2014). This absence is quite surprising because a worker that reproduces thelytokously has the potential to enormously raise her personal fitness by laying an egg in a queen cell. In honeybees, queens and workers are genetically identical and caste differentiation is triggered by differential feeding of very young larvae (de Wilde and Beetsma, 1982; Kucharski *et al.*, 2008), so any diploid egg laid in a queen cell can develop into a queen. A thelytokous worker can therefore be genetically reincarnated as a queen if she lays her egg in a queen cell (Goudie and Oldroyd, 2014). Thelytoky has selected for highly competitive workers in *A. m. capensis* (reviewed in Beekman and Oldroyd, 2008); workers in colonies with a queen typically have much higher rates of ovary activation than other (sub)species, and workers specifically target queen cells for oviposition (reviewed in Goudie and Oldroyd, 2014).

Although thelytoky occurs regularly in *A. m. capensis*, there is evidence that it occasionally occurs in other (sub)species of honeybee. When virgin queens of commercial strains of *A. mellifera* are induced to lay under CO_2 narcosis, ~1% of the eggs they produce develop into females (Mackensen, 1943). Although this may be the result of developmental errors during arrhenotoky (the process by which a haploid egg develops into a male) (Goudie and Oldroyd, 2014), it is suggestive that thelytoky is a rare or perhaps latent trait in honeybees that appears and is selected for under certain environmental conditions.

The Eastern honeybee *A. cerana* is native to and endemic throughout Asia, and is a sister species of *A. mellifera* (Lo *et al.*, 2010). Its workers have unusually high rates of ovary activation even when a queen is present (Oldroyd *et al.*, 2001; Nanork *et al.*, 2007; Holmes *et al.*, 2014), not unlike *A. m. capensis*. However, the reasons behind these unusually high rates of ovary activation are unclear (Holmes *et al.*, 2014).

However, working with *A. cerana* in China, we observed anomalous appearances of three adult queens roaming in two colonies from which we had previously removed the queen and that no longer contained queen-laid brood from which new queens could be raised. Normally, the workers would raise new queens from eggs or very young larvae (<3 days old) to replace the old queen, but such queen cells were removed during the course of an experiment. The development time from an egg to adult queen in *A. cerana* is 14–16 days (Oldroyd and Wongsiri, 2006). Thus the three young queens found in our colonies, 22–48 days after removal of the queen, could not have been reared from the original queen's brood. Were these the thelytokous daughters of workers?

Here, we investigate experimentally if workers of *A. cerana* are capable of thelytoky and use this ability to lay eggs in queen cells

[1]Behaviour and Genetics of Social Insects Laboratory, School of Biological Sciences, University of Sydney, Sydney, New South Wales, Australia; [2]Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Science, Kunming, Yunnan Province, People's Republic of China and [3]Eastern Bee Research Institute of Yunnan Agricultural University, Heilongtan, Kunming, People's Republic of China
Correspondence: MJ Holmes, Behaviour and Genetics of Social Insects Laboratory, School of Biological Sciences, University of Sydney, Macleay Building A12, Science Rd, University of Sydney, Sydney, New South Wales 2006, Australia.
E-mail: michael.holmes@sydney.edu.au
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when the colony's queen has been removed. We sampled queen cells from colonies without a queen and genotyped the contents to determine the maternity of the pupae or larvae present in those cells.

MATERIALS AND METHODS

Collection of samples

All sampling was conducted on the campus of Yunnan Agricultural University, Kunming, China. On 16 June 2011, a queen cell was observed in a queenless colony (colony 1). This colony had been queenless since 16 May of that year and therefore no longer contained brood laid by the original queen from which new queens could be raised. However, a virgin queen was observed on 24 May, she apparently never returned from her mating flight, as she was never seen again and no queen-laid eggs were ever observed after 16 May. The queen cell harvested on 16 June contained a fully developed pupa that was morphologically female. The pupa was stored in 70% ethanol for subsequent analysis.

On 4 August, another queen cell was harvested from colony 1. This colony was still in a queenless state and did not contain brood. Once again, the queen cell contained a fully developed pupa that was morphologically female. The pupa was stored in 70% ethanol for subsequent analysis.

On 5 May 2013, we experimentally dequeened four *A. cerana* colonies and regularly inspected and harvested queen cells. Queens from all four colonies were stored in 70% ethanol for analysis. Queen cells were harvested every second day for the first 2 weeks, and at weekly intervals thereafter until 5 July 2013, after which time no more queen cells were produced. All queen cell contents (QCCs) were stored in 70% ethanol for subsequent analysis.

Genetic analysis

DNA from adult queens and workers was extracted using a Chelex extraction method (Walsh *et al.*, 1991). For larvae and pupae from inside queen cells, a cleaner preparation of DNA was required, so we used a high-salt extraction method (Aljanabi and Martinez, 1997). All samples were genotyped at nine polymorphic microsatellite loci. Six of these loci were specific to *Apis cerana* (Takahashi *et al.*, 2009) and three were specific to *A. mellifera* (Solignac *et al.*, 2003). DNA was amplified using standard PCR conditions (Estoup *et al.*, 1994) and genotypes were assigned as in Holmes *et al.* (2010).

Where possible, the original queens were genotyped directly. The original queen was not available for colony 1 as this colony lost its queen by chance. However, we had previously collected a sample of 24 worker pupae from this colony for another experiment; the genotype of the original queen was inferred from this sample (Oldroyd *et al.*, 2000).

Determining maternity of QCCs

We compared the genotypes of QCCs with that of the original queens from their colonies in order to determine whether they were the offspring of queens or workers. If the queen pupae were daughters of the original queens, they would share an allele with the original queen at all nine loci; this would not necessarily be the case if they were produced by workers. We also noted the timing of the appearance of the queen cells. Queen cells that appeared >16 days after a colony lost its original queen could not possibly contain brood laid by this queen (Oldroyd and Wongsiri, 2006).

We used the procedure employed by Holmes *et al.* (2010) to distinguish whether the pupae in queen cells were worker- or queen-laid. Briefly, during thelytoky via central fusion there is a one-third chance that an allele present in the mother at a particular locus will become homozygous in her daughter (Baudry *et al.*, 2004). Additionally, there is a 50% chance that the homozygous allele will be from the father (Allsopp *et al.*, 2010). Thus there is a one-sixth of chance that a worker's offspring will be homozygous for a paternal allele at a locus. Therefore, when a QCC is homozygous at at least one locus for an allele not carried by the queen, it must have been thelytokously produced by a worker (Allsopp *et al.*, 2010).

Misclassification error

A QCC also has a one-sixth of chance of becoming homozygous for an allele carried by the queen, so even if it shares an allele with the original queen at all loci, but is homozygous for a queen allele at at least one locus, it may be the thelytokous daughter of a worker (Holmes *et al.*, 2010). Alternatively, the queen could have mated with a male carrying the same allele at that locus.

We calculated the probability of misclassifying offspring homozygous for a queen allele at a locus as being worker-laid as in Holmes *et al.* (2010). First, we calculated the average frequency of the two alleles carried by the original queens of each colony as $(p_{ik} + p_{jk})/2$, where p_{ik} is the population frequency of the first queen allele at the kth locus and p_{jk} is the frequency of the second queen allele at the kth locus (Holmes *et al.*, 2010). We then calculated α, the average of these average frequencies over the i loci (Holmes *et al.*, 2010). We then determined the number of loci, n, which were homozygous for a queen allele for each of our anomalous queens and QCCs. The probability that any of our anomalous queens or QCCs was homozygous for a queen allele due to the original queen mating with a male that shared her alleles was then estimated as α^n (Holmes *et al.*, 2010).

We obtained population allele frequencies by genotyping 107–135 workers at each locus from six *A. cerana* colonies within our population.

RESULTS

The two QCCs sampled from colony 1 were morphologically female. Based on the lag between the removal of the original queen and the appearance of the queen cells, these young queens could not have been laid by the original queen. Genotyping confirmed that they were not the daughters of the queen (Table 1).

We harvested a total of 37 QCCs from the 2013 colonies, of which 4 (10.8%), from a single colony, appeared to be worker-laid queens (colony 2 in Table 1). Of the remaining 33 QCCs, 23 (62.2%) had

Table 1 Genotypes and sampling dates of original queens and potentially worker-laid queen-cell contents (QCCs) harvested in 2011 and 2013

Date[a]	Colony	Sample	Days[b]	Ac1	Ac2	Ac3	Ac5	Ac30	Ac35	A107	Ap43	B124
16 May 2011	1	Original queen[c]		200/202	132/136	318/318	166/166	226/226	126/128	159/160	130/150	217/219
16 June 2011	1	QCC	31	200/202	**132/132**	318/320	*165/165*	**226/226**	126/130	*157/157*	128/130	217/219
04 August 2011	1	QCC	80	200/202	132/134	318/322	*165/165*	**226/226**	126/128	*162/162*	*148/165*	217/219
05 May 2013	2	Original queen		202/202	132/134	322/322	163/163	232/232	128/130	165/166	130/132	215/219
10 May 2013	2	QCC	5	**202/202**	**134/134**	318/322	163/165	**232/232**	120/128	*168/168*	130/145	**219/219**
14 May 2013	2	QCC	9	**202/202**	132/140	318/322	163/165	229/232	**128/128**	*156/156*	132/152	217/219
14 May 2013	2	QCC	9	**202/202**	**134/134**	**322/322**	163/165	**232/232**	**128/128**	*168/168*	**130/130**	215/219
14 May 2013	2	QCC	9	**202/202**	**134/134**	312/322	**163/163**	229/232	128/130	*168/168*	128/132	**219/219**

Genotypes highlighted in italics indicate a locus where the individual did not share an allele with the original queen. Genotypes in bold indicate a locus where the offspring became homozygous for a queen allele.
[a]Date the colony was either dequeened or first noted as queenless (colony 1).
[b]The number of days between the onset of queenlessness and the sampling of anomalous queen cells. Note that the developmental time of an *A. cerana* queen is 14–16 days (Oldroyd and Wongsiri, 2006).
[c]Queen was lost accidentally and so was not stored; her genotype was inferred from a sample of workers collected from this colony on 14 March 2011. As the original queen was paint-marked, we are confident that she was the only queen that had been in this colony until she disappeared in May 2011.

genotypes compatible with being queens raised from the queen brood, whereas 8 (21.6%) appeared to be worker-laid males and 2 (5.4%) appeared to be queen-laid males.

Whenever we assigned a QCC as being worker-laid (Table 1), α^n was on average 0.051. This suggests that there was a 5.1% chance that these queens were the offspring of the queen rather than a worker (Holmes *et al.*, 2010).

The estimation of misclassification error is far more important for the QCCs that appeared within 14–16 days of removal of the queen, as we cannot exclude these being queen-laid due to timing. The four individuals in question are those from colony 2, which were QCCs collected between 5 and 9 days after removal of the queen (Table 1). If we only consider these individuals in the calculation, α^n is on average 0.012. Thus, for these samples there is only a 1.2% chance that they were laid by the queen rather than a worker.

DISCUSSION

When a honeybee worker reproduces thelytokously, recombination will result in a loss of heterozygosity in one-third of meioses (Baudry *et al.*, 2004). This means that a worker's thelytokously produced daughters can be definitively identified by homozygosity for a paternal allele at one or more loci (Allsopp *et al.*, 2010). Across our 2011 and 2013 samples, we positively identified six female progeny as being worker-laid because they lacked queen alleles at one or more loci (Table 1). In all but one case, these individuals were homozygous for a non-queen allele (Table 1). This is proof that these individuals are the thelytokous daughters of workers (Allsopp *et al.*, 2010).

One of our 2011 QCCs lacked queen alleles at three loci but only became homozygous at two of these (second QCC colony 1; Table 1). The presence of two non-queen alleles at a single locus shows that this individual had not been laid by a natal worker (Allsopp *et al.*, 2010), unless a mutation occurred. This suggests that, as occurs regularly in *A. m. capensis* (Jordan *et al.*, 2008; Allsopp *et al.*, 2010; Holmes *et al.*, 2010), a worker from another colony entered and parasitised the queen cell.

This is the first evidence of thelytoky in *A. cerana*. *A. cerana*'s high levels of ovary activation are paradoxical, as most haploid worker-laid eggs are rapidly removed by other workers (Oldroyd *et al.*, 2001; Holmes *et al.*, 2014). Such efficient policing is predicted to lead to low levels of ovary activation (Wenseleers and Ratnieks, 2006; Holmes *et al.*, 2014). The ability to lay diploid eggs via thelytoky changes the kin structure of a honeybee colony (Greeff, 1996). When workers reproduce thelytokously, they are related to their own daughters by unity and are therefore far less likely to refrain from personal reproduction (Greeff, 1996). More importantly, thelytoky opens the possibility for workers to be reincarnated as queens if their daughter is raised as the next queen (Jordan *et al.*, 2008; Goudie and Oldroyd, 2014). We therefore expect workers to compete strongly with their sisters, resulting in high levels of ovary activation (Greeff, 1996). Our work suggests that the so far unexplained high ovary activation rates found in *A. cerana* (Oldroyd *et al.*, 2001; Nanork *et al.*, 2007; Holmes *et al.*, 2014) result from the changes in colony kin structure due to thelytokous reproduction by its workers.

The ability of *A. cerana* workers to reproduce thelytokously could also explain the rapid expansion of a single *A. cerana* swarm introduced into Australia in 2007 into over 800 colonies by 2013 (Koetz, 2013). Thelytoky impacts invasion potential of invasive species in general for three main reasons (Rabeling and Kronauer, 2013; Goudie and Oldroyd, 2014). First, thelytoky allows invasive species to overcome the problem of locating mates in initially low-density populations. Second, thelytoky can cause socially parasitic lineages to arise, wherein females infiltrate the colonies of their own or related species and lay eggs. Finally, thelytoky ensures that the 'invasive genotype' of the founder population is preserved. So far, thelytoky is known to have assisted the spread of six invasive social Hymenoptera (Rabeling and Kronauer, 2013). In addition, at least three species, though not currently classified as invasive, thrive in anthropogenically modified habitats (Kronauer *et al.*, 2012).

Thelytoky has already led to the evolution of specialised social parasites as in the Cape honeybee *A. m. capensis* (Goudie and Oldroyd, 2014) and the ant *Pristomyrmex punctatus* (Dobata *et al.*, 2009; Dobata *et al.*, 2011). In the Cape honeybee the consequences of the parasitic lineage are devastating. After its first occurrence in 1990 this lineage has been responsible for the death of hundreds of thousands of commercial honeybee colonies, nearly wiping out the South African beekeeping industry (Allsopp, 1992; Beekman *et al.*, 2008). Given the potential that thelytoky provides insect workers, it is essential to further investigate the prevalence of thelytokous reproduction in *A. cerana*, the conditions under which such reproduction occurs and the likelihood of a new parasitic lineage to evolve.

DATA ARCHIVING

Microsatellite genotype data for queens, QCCs and workers used to infer the genotype of the original queen of colony 1, and workers used to estimate allele frequencies are available for download from http://hdl.handle.net/2123/10070.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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