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ORIGINAL RESEARCH ARTICLE

Sustainable multiple queen colonies of honey bees, *Apis mellifera ligustica*

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Summary

Honey bee multiple queen colonies composed of several mated queens able to move around freely were produced by modulating biological factors that evoke fighting and queen elimination within the colony, mainly by ablating mandibles of queens to avoid inter-queen rivalry. Following this method, 128 colonies in eighteen apiaries were set up with multiple queens, all of which were mated and 6-12 months old. One hundred of the colonies (78.1%) retained all introduced queens. In total, 658 out of 733 queens (89.8%) were accepted after their introduction. The majority of these colonies experienced no queen loss for two months and most were still stable after six months. Of 80 colonies, 55 (68.8%) experienced no queen loss over the winter. These results show that our method is viable to produce sustainable multiple queen honey bee colonies for commercial use. In addition this technique will help to increase our understanding of basic questions of the evolution of sociality, such as division of reproduction and the evolution of polygyny.

Colonias sostenibles de abejas *Apis mellifera ligustica* con múltiples reinas

Resumen

Colonias de abejas con reinas múltiples compuestas por varias reinas fecundadas capaces de moverse libremente fueron producidas modulando factores biológicos que provocan la lucha y la eliminación de la reina dentro de la colonia, principalmente quitando las mandíbulas por ablación de reinas para eludir rivalidad de la reina-reina. Siguiendo este método se establecieron 128 colonias con múltiples reinas en dieciocho colmenares, que fueron apareadas con 6-12 meses de edad. Cien de ellas (78,1%) conservaron a todas las reinas introducidas. En total, 658 de las 733 reinas (89,8%) fueron aceptadas después de su introducción. La mayoría de estas colonias no experimentó ninguna pérdida de reinas durante dos meses y la mayoría seguía siendo estable después de seis meses. De 80 colonias, 55 (68,8%) no experimentaron ninguna pérdida de la reina durante el invierno. Estos resultados muestran que nuestro método es viable para producir colonias de abejas sostenibles con múltiples reinas para uso comercial. Además esta técnica ayudará a aumentar nuestra comprensión de las cuestiones básicas de la evolución de la sociabilidad, como la división de reproducción y la evolución de poliginia.

Keywords: Multiple queen colonies, monogyny, polygyny, mandible, young worker

Introduction

The western (*Apis mellifera*) and the eastern (*Apis cerana*) honey bee are rare cases of insects that have been successfully domesticated. Honey bees play an important economic role as a producer of honey, royal jelly, beeswax and propolis, which have been proven to have a variety of biological activities (Krell, 1996) and have an indispensable role in pollination for agricultural and natural eco-systems (Morse and Calderone, 2000).

A large colony population is necessary for a high yield of hive products (Chen, 2001). Because of this, for many decades attempts in

many countries have been conducted in developing methods that increase brood rearing beyond the natural capacity of a normal single queen colony (Kovtun, 1949, 1950; Melnik, 1951; Spoja, 1953; Wallrebenstein, 1958; Haydak and Dietz, 1967). One possibility to achieve this is to create a colony where more than one queen reproduces. Honey bee societies are, however, normally monogynous (Ribbands, 1953). If several queens meet, which may happen because virgin queens emerge simultaneously, or alien queens enter the colony by colony merger (Neumann *et al*., 2001), they typically fight for reproductive supremacy until only one survives and monopolizes reproduction in the colony (Winston, 1987). During the fights, queens grapple together, using their legs and mandibles to position themselves to sting each other. Stinging is the usual cause of death, but is only successful when queens have a good purchase with their mandibles on their opponents and can position themselves suitably (Butz and Dietz, 1994; Gilley, 2001; Dietemann *et al*., 2008). Worker honey bees may also play a role during the elimination of supernumerary queens by destroying queen cells (Tarpy and Fletcher, 1998; Hatch *et al.,* 1999), by affecting queen behaviour through immobilization of queens (Gilley, 2001), by attacking queens with balling behaviour (Robinson, 1984) and by interfering with the outcome of inter queen competition through the "vibration signal" (Allen, 1959; Painter-Kurt and Schneider, 1998) or withholding trophallaxis (Tarpy and Fletcher, 1998).

Polygyny may occur naturally during supersedure and swarming, but is only temporary, with monogyny eventually being reestablished by a variety of means (Gilley and Tarpy, 2005) in a period varying from hours to months (Hepburn and Radloff, 1998). The attempts to artificially produce more productive multiple queen colonies therefore required physical separation of the queens to prevent them fighting (Wallrebenstein, 1958; Haydak and Dietz, 1967). Attempts also have been made to obtain free running multiple queen colonies, but their success was variable and none of the methods was widely accepted. For example, Kovtun (1949, 1950) introduced several queens up to 1.5 years old, with wings clipped, into a hive consisting of combs of emerging brood and honey with the empty cells filled with warm water. It was claimed that there must be no workers in the hive otherwise all the queens will be killed (Kovtun, 1949, 1950). This method is not, however, practical since the comb is easily destroyed by warm water due to the thermoplastic properties of wax (Pirk *et al*., 2004). Secondly, the queens are prone to die due to the lack of care from workers, even if they refrain or survive the fighting. Melnik (1951) introduced three queens sequentially, one queen every two days, into a queenright colony without any treatment. This colony performed less well than single queen colonies for honey production and all the queens were found dead outside the hive when a young queen emerged (Melnik, 1951). Spoja (1953) successfully introduced queens of varying ages with wings clipped by introducing them among worker bees without using cages (Spoja, 1953), but this method did not produce stable multiple queen colonies as many of them did not survive the over wintering period (Spoja, 1953).

Here, we report an efficient method of obtaining stable multiple queen colonies composed of several egg laying and freely moving honey bee queens. Our multiple queen colonies were produced by simultaneously modulating biological factors that normally prevent polygyny in honey bees and providing a suitable social context within the hive. This involved the reduction of the possible sources that evoke fighting and queen elimination within the colony, i.e. removing older intolerant workers (Robinson, 1984) and decreasing the great fighting ability of queens (Dietemann *et al*., 2008).

Materials and methods

The honey bee colonies used were of the Pinghu strain (from Pinghu County, Zhejiang Province, China) derived from the Italian honey bee (*Apis mellifera ligustica*). This strain has been selected by local beekeepers in recent decades for high royal jelly production. Queens were reared from grafted larvae, were allowed to mate freely and to return to their individual colonies until their egg laying ability was established at 6-12 months of age.

The colonies destined to host the multiple queens were prepared as follows: combs of emerging brood were selected and shaken, which triggers flight in the older bees, while young bees tend to remain on the comb (Sigg *et al*., 1997). The combs were then placed in the hive box with the young bees still clinging to them. Young bees were used to avoid workers balling and killing the queens, a behaviour typical for older workers (Robinson, 1984). The host hives were placed at a distance (5-10 m) from their original location to ensure that all remaining foragers (older bees) did not re enter them. One to three day old workers were however preferred to freshly hatched individuals which may not be able to care for the queens efficiently (Lindauer, 1953). The amount of combs and bees to be used in the multiple queen colony depended on the number of queens to be introduced. Four to six combs were used for three to six queen colonies. Additional combs of honey and pollen were added beside the brood combs to provide enough food because the colony was deprived of foragers at the beginning.

Two days after the colonies were prepared, queens were taken out of their original colonies and introduced to different locations in the host hives after a third to half of both mandibles were removed with micro-scissors. The ablation of mandibles reduced their propensity to fight and kill each other (Dietemann et al., 2008). In addition, the large abdomens of the egg laying queens might further reduce their ability to fight (Spiewok, 2006).

In order to test the viability of our method, this protocol was implemented in the seventeen household apiaries in Pinghu, Zhejiang and in our experimental apiary in Hangzhou between 2005 and 2008. In each of these apiaries, one to three multiple queen colonies, each

Table 1. Sustainability of multiple queen colonies created in springs of 2005 to 2008.

Years	Number of colonies	Results	Number of colonies (%)	Kept without queen loss in two months	Kept without queen loss in six months
2005	20	All queens accepted	15 (75.0)		
		1-2 queens eliminated	4 (20.0)	19	16
		All but one eliminated	1 (5.0)		
2006	38	All queens accepted	30 (78.9)		
		1-2 queens eliminated	7 (18.4)	34	30
		All but one eliminated	1 (2.6)		
2007	39	All queens accepted	32 (82.1)		
		1-2 queens eliminated	6 (15.4)	37	31
		All but one eliminated	1 (2.5)		
2008	31	All queens accepted	23 (74.2)		
		1-2 queens eliminated	6 (19.4)	26	20
		All but one eliminated	2 (6.5)		
Total	128	Multiple queen colonies	123 (96.1)	116	97

consisting of four to seven queens were created each spring from March to April to be exploited commercially for royal jelly production. The number of multiple queen colonies varied in each apiary and year due to how many were needed (20, 38, 39 and 31 in the respective years). The acceptances of the queens introduced were recorded, and the sustainability of the colonies successfully established was monitored over six months (Table 1). The monitoring of 7, 10 and 14 multiple queen colonies created in the springs of 2005 to 2007 ended in October each year because of the frequent queen loss caused by robbing by other colonies when tea plantations were in bloom and because of apiaries being displaced to other locations at this period. The remaining 46, and another set of multiple queen colonies (n=5 in 2005, 11 in 2006, 18 in 2007), which were created in the autumn when a strong pollen and nectar flow was present, were allowed to overwinter with four to six frames of workers and ample food supply. Their survival as well as the number of queens over wintering successfully were recorded before colonies were used commercially again in the next spring (Table 2).

Results

The results gathered in the four years from the eighteen apiaries showed the high success of the method. One hundred out of 128 colonies (78.1%) were successfully established without losing one queen (Table 1). In 23 colonies (18.0%), one or two queens were eliminated, and in only five colonies (3.9%) were all but one queen eliminated (Table 1). Of the 123 colonies successfully created, 116 were kept without queen loss for two months and 97 retained their queens for the whole six month period. The majority (55 out of 80) overwintered successfully and no queen loss was recorded the next spring. In fifteen cases, one or two queens per colony were lost during overwintering. In seven cases three or four out of six to eight queens were eliminated. In only three instances (3.8%) were all but one queens eliminated (Table 2). One four queen colony survived for two years without any queen loss. Discussion Multiple queen colonies with two to eight queens cohabiting, free running and laying eggs peacefully (Fig. 1.) have been produced since 1999 using the methods described. We have now empirically demonstrated the efficacy of this protocol, and a success rate of 78.1% was obtained for the creation of these multiple queen colonies.

Discussion

Multiple queen colonies with two to eight queens cohabiting, free running and laying eggs peacefully (Fig. 1.) have been produced since 1999 using the methods described. We have now empirically demonstrated the efficacy of this protocol, and a success rate of 78.1% was obtained for the creation of these multiple queen colonies.

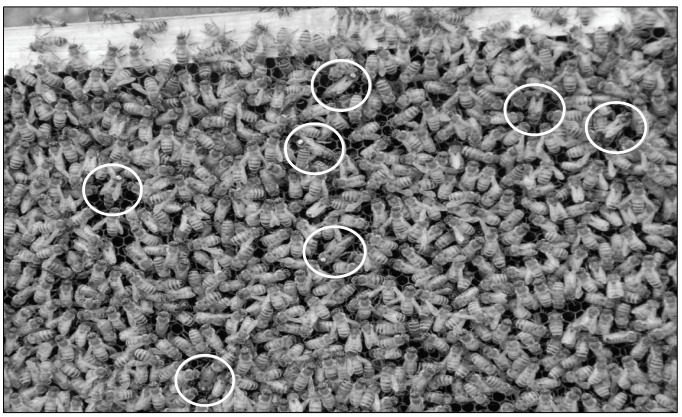

Fig. 1. Seven queens (marked with coloured tags and encircled) coexisting peacefully on one side of a comb.

Table 2. Results of overwintering of multiple queen colonies.

Years	Number of colonies over-wintered	Results	Number of colonies (%)	Possible cause
2005	14	No queen loss	7 (50.0)	/
		1-2 queens eliminated	5 (35.7)	Unknown
		3-4 queens eliminated	2 (14.3)	Other workers drifted in
		All but one eliminated	0 (0)	/
2006	31	No queen loss	21 (67.7)	/
		1-2 queens eliminated	6 (19.4)	unknown
		3-4 queens eliminated	3 (9.7)	unknown
		All but one eliminated	1 (3.2)	inappropriate thermal insulation
2007	35	No queen loss	27 (77.1)	/
		1-2 queens eliminated	4 (11.4)	unknown
		3-4 queens eliminated	2 (5.7)	Robbing and inappropriate mite control
		All but one eliminated	2 (5.7)	unknown
Total	80	No queen loss	55 (68.8)	/
		1-2 queens eliminated	15 (18.8)	/
		3-4 queens eliminated	7 (8.8)	/
		All but one eliminated	3 (3.8)	/

When taking the number of queens accepted *vs.* the number introduced into account, the success ratio reaches 89.8 % (Table 3), which is close to the anticipated success rate of 95-100 % when introducing queens into single queen colonies (Morse, 1979). Provided they are given sufficient food and close attention, these colonies are sustainable enough for long term use (Table 1.) and all queens can survive overwintering (Table 2.). Queens accepted by the colonies laid eggs normally in their polygynous colonies. This method has been accepted by Chinese beekeepers and has been regularly used for several years in hundreds of apiaries in the Zhejiang and Jiangshu provinces.

Table 3. Acceptances of queens introduced into hosting colonies.

Year	Queens introduced	Queens accepted (%)
2005	118	106 (89.8)
2006	226	209 (92.5)
2007	218	192 (88.1)
2008	171	151 (88.3)
Total	733	658 (89.8)

Given that many attempts to create multiple queen colonies have already been made by using both the free-running queen approach (Kovtun, 1949, 1950; Melnik 1951; Spoja, 1953) and the physical separation of queens (Haydak and Dietz, 1967; Wallrebenstein, 1958; Farrar, 1953), it is important to consider why our method is successful. Spoja (1953) suggested that queens need not be of the same age. We would, however, strongly suggest using queens of the same age; more than six months old. Young queens behave more aggressively to their rivals (Spiewok, 2006) and the equality of their status enhances acceptance by workers.

Another key factor is the age of the workers. The sensitivity of young worker honey bees to respond to pheromonal stimuli is very low compared to older workers, both for the perception of queen pheromones and for recruitment for defensive and aggressive behaviour (Robertson, 1984; Free, 1987). Host colonies with young workers for the multiple queens could therefore be described as essentially "neutralized" with respect to queen-worker interactions compared to colonies having a normal age distribution among the workers.

Various methods have been adopted to treat queens to make them cohabit, including using cages (Kovtun, 1949), clipping one or both of the wings (Kovtun, 1949), amputating stings (Lensky and Darchen, 1963) or simply introducing them among bees (Spoja, 1953). Our data showed that mandible ablation is an efficient way to prevent queens from fighting to the death. Queens with ablated mandibles refrain from engaging in lethal contests that typically characterize their reproductive dominance behaviour and coexist peacefully within a colony, while intact queens fight until only one survives (Dietemann *et al.*, 2008).

There are some other factors that enhance the success, such as the season; a strong pollen and / or nectar flow facilitates damping differences in colony odour (Bethe, 1898). In the area of southern China below the Changjiang River multiple queen colonies are mainly created between March and May when rape, the major floral source, is blossoming, and between September and October when the main flow is tea. Spraying some honey water onto the queens and workers before queen introduction triggers grooming behaviour and gives time for the odour of the queen to become acceptable to the workers (Morse, 1979), thereby enhancing the acceptance.

Improving reproductive speed and maintaining strong colonies are preconditions for maximizing colony productivity. The increase in egg laying maintains strong colonies, the development of a large field force possibly improves the productivity as well as enhancing disease resistance in the colony. An increase in the genetic diversity in the colony enhances work efficiency (e.g. Fuchs and Schade, 1994; Jones *et al.*, 2004) as well as resistance against diseases and parasites (Baer and Schmid-Hempel, 1999; Palmer and Oldroyd, 2003; Hughes and Boomsma, 2004, 2006). Mandible ablation has no significant effect on the egg laying ability of queens, and the rate of egg production of three queen colonies and five queen colonies averaged 199 % and 328 % of that of a single queen colony, respectively (Hu and Zheng unpublished). Despite the fact that egg laying rate is less than directly proportional to the number of queens per colony, keeping several of them in a colony is an effective way to improve the egg laying output of a colony (Hu and Zheng unpublished).

Close supervision is however necessary to maintain multiple queen colonies since they are sensitive to internal and external factors. Lack of attention by the beekeeper can result in the loss of one or some queens at any time of the year, but especially over winter. From the point of view of beekeeping practice, we do not recommend keeping a large number of multiple queen colonies in an apiary because of the extra amount of labour they require. It is nevertheless useful to keep a limited number of them as supporting colonies to provide extra brood (the future workforce) or replacement queens to other colonies when needed. Multiple queen colonies can also be used as a source of young workers to produce package bees, of which the demand exceeds the supply in spring in some countries such as the USA due to the increasing demands of pollination and the loss of bees resulting from *Varroa destructor* infestation and associated diseases (Harrison, 2005; Lumpkin, 2005). In addition to their commercial applications, multiple queen colonies are of great interest in theoretical research on reproductive skew, enabling us to deepen our understanding of how reproductive conflicts are resolved in insect societies.

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