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ORIGINAL RESEARCH ARTICLE

Improved technique for introducing four-day old virgin queens to mating hives that uses artificial and natural queen cells for introduction

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Summary

We compared the acceptance of 4-day old virgin queens introduced into mating nucleus hives using natural and artificial queen cells versus a wooden 3-hole mailing cage, a standard introduction method. The queen cell methods gave high acceptance (95% and 93% for natural and artificial, respectively) even though the queen was released from the queen cell approximately 10 minutes after being introduced into the mating hive. By contrast, success using mailing cages was significantly lower (47% and 73%) when the queen was released from her cage after 1 hour or 48 hours, respectively. The equal success rates of the reused and artificial queen cells suggests that high success is not due to chemicals present in natural queen cells transferring to the queens. To further investigate why queen cells give higher introduction success than cages, we introduced virgin queens into queenless observation hives. Workers attacked only 1 of 12 queens leaving a queen cell whereas 5 out of 6 queens leaving a cage were attacked.

Keywords: *Apis mellifera mellifera*, virgin queen, queen acceptance, queen introduction, queen rearing, queen cell, artificial queen cell

Introduction

Introducing queens into queenless hives is a common procedure in modern beekeeping (Laidlaw, 1997). In commercial queen rearing, queenless mating nucleus hives are normally requeened using a ripe queen cell containing a pupa that is due to emerge within a few days. However, there are several advantages to introducing virgin queens rather than cells into mating hives (Moretto *et al*., 2004; Perez-Sato & Ratnieks, 2006). Several methods are used to introduce mated queens, and these can also be used to introduce virgin queens (Snelgrove, 1940). The most common queen introduction method is to use a 3-hole mailing cage and to release the queen from her cage several days after it has been introduced into a queenless hive (review in McCutcheon, 2001). In this way the new queen acquires the odour of the new colony before release and is usually accepted (Snelgrove, 1940; Butler & Free, 1952). However, queen death is common when virgins are introduced. The success obtained for virgin queen introduced using cages is highly variable, ranging from 33% to 80% (Mantilla & Goncalves, 1987; Medina & Goncalves, 2001; Moretto *et al*., 2004; Perez-Sato & Ratnieks, 2006). Clearly, methods that give greater acceptance success would be valuable.

In this study we investigate a novel method of introducing 3–4 day old virgin queens using queen cells, either by reusing an already emerged natural queen cell or by using an artificial plastic queen cell. Both methods gave high rates of acceptance into mating nucleus hives (95%, 93%) even though the queen was released only 10 minutes after being introduced. By contrast, acceptance using wooden 3-hole mailing cages was only 47% and 73% if the queen was released 1 hour or 48 hours after the cage had been introduced. A second experiment using observation hives showed that only a small proportion of queens introduced using queen cells were attacked when they left their cell (1/12, 8%) approximately 10 minutes after being introduced into the queenless hive. However, most (5/6, 83%) queens held in a 3-hole mailing cage were attacked after leaving their cages one hour after being introduced.

Materials and Methods

Experiment 1. Queen introduction into mating nucleus hives

The study was carried out in a queen-mating apiary in Bullock Field, Losehill Hall, Castleton, Derbyshire, England (Ordinance Survey grid reference 154835) during the summer of 2005. The honey bee stocks were a mixture of European subspecies, but predominantly *A. mellifera mellifera*. The mating nucleus hives had 5 medium-depth Langstroth frames (hive volume c. 15 l). Each nucleus had sufficient bees to cover 1–2 frames. They were fed equal amounts of sucrose syrup as needed and throughout the study, both before and after virgins were introduced, using a feeder forming part of the inner cover.

Virgin queens were reared by the Doolittle (grafting) method (Laidlaw & Page, 1997). Ten or eleven days after grafting larvae, the cells were removed from the starter-finisher colony and placed individually in 13 ml glass vials. The vials were held in an incubator at 34°C and 70% relative humidity. Each newly-emerged queen was marked with a numbered disc (Opalithplättchen®). The queens were then transferred to wooden 3-hole mailing cages without attendant workers or candy and held for 3 days at room temperature, 20°C, before being introduced. During this period, they were fed daily by placing a small drop of honey inside one the end holes of the cage after briefly removing the cork. The honey used to feed them came from a single frame taken from a colony in the laboratory's apiary.

After three days in cages, the virgin queens were transported to the field for introduction into the mating hives using four methods:

1. Reused Queen Cell, 1 hour

The virgin queen was held gently in the fingers and transferred into an emerged queen cell. The cell was prepared to accept the virgin queen by cutting an L-shaped slit with a scalpel (Fig. 1a) to make a door through which the queen was inserted (Fig. 1b). The door was then closed (Fig. 1c). The cell tip and the slit were closed with a small amount of a mixture of wax and honey taken from the hive into which she was being introduced (Fig. 1d). The queen cell was then gently pushed into the wax comb in the brood area of the mating hive, and the hive lid was then replaced. From studies carried out in observation hives (Experiment 2) with similar numbers of bees, we know that the workers removed the piece of wax and honey blocking the queen's exit in less than 1 hour and that the queen then left the queen cell. The introduction was made two days after the mating hive had been made queenless by removing the egg-laying queen.

2. Artificial Queen Cell, 1 hour

This method followed method 1 except that the queen was introduced using an artificial queen cell made of plastic. This cell was constructed using a JZ/BZ queen cell protector (Fig. 2a) which was covered with paper and masking tape (Fig. 2b). The hole at the top of the artificial cell was covered using a plastic queen cup (Fig. 2c), and the hole at the tip was sealed with wax/honey paste as in method 1 (Fig. 2d). When the workers removed the wax and honey plug the virgin queen could leave the artificial queen cell.

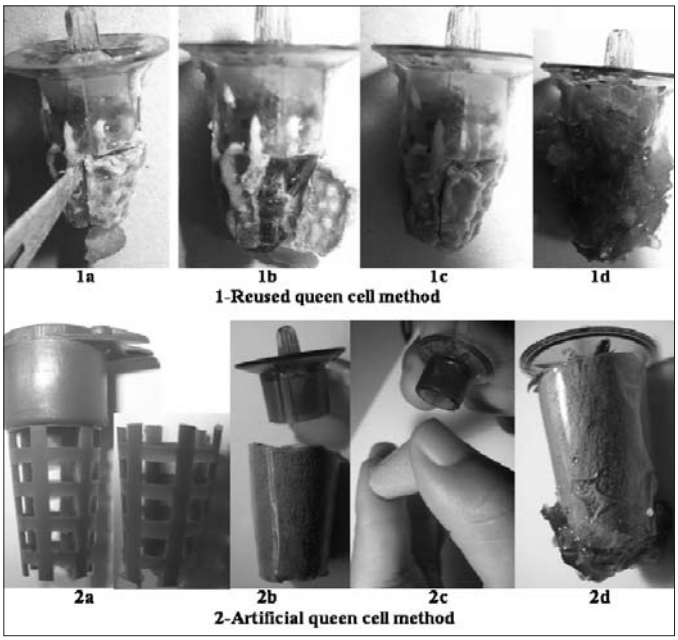

Fig. 1 Procedures used to introduce a four day old virgin queen using natural (method 1) and artificial queen cells (method 2). **1. Reused natural queen cell**: a scalpel is used to cut an L-shaped slit in the cell (1a), a door is opened and the virgin queen is placed inside the cell (1b), the door is closed (1c), the queen cell is then sealed with wax and honey ready to be introduced (1d). **2. Artificial plastic queen cell**: a plastic JZ/BZ queen cell protector is cut in two pieces with the right part being used to make the queen cell (2a). The plastic cell is covered with paper and masking tape and its top closed with a plastic queen cup (2b). The queen is introduced into the plastic cell, which is closed at the top with the plastic queen cup (2c), and then sealed with wax and honey ready for introduction (2d).

3. Mailing cage, 1 hour

The queen was introduced using the same 3-hole mailing cage in which she had been held in the laboratory. The cage did not contain attendant worker bees. During introduction, the cork at one end of the cage was removed and the hole was temporarily blocked using wax and honey as in methods 1 and 2.

4. Mailing cage, 48 hours

This method followed method 3 except the cage was introduced into the mating hive without removing the cork. Two days later the cork was removed and replaced with wax and honey. The initial introduction was made a few hours after the mating hive had been dequeened, following normal beekeeping practice. The release of the virgin queen took place two days after dequeening the queen-mating hive, as in the other treatments.

One day after each virgin queen had been released we inspected the mating hive to determine whether the queen had been accepted. Queens that were found alive and uninjured on the combs were considered to have been accepted. Queens found being 'balled', with injured legs, or dead outside the hive on the next day were considered to have been rejected.

Queens were introduced in two trials during the summer 2005 (July-August). Twenty virgin queens were introduced per method in each trial, to give a total of 40 per method.

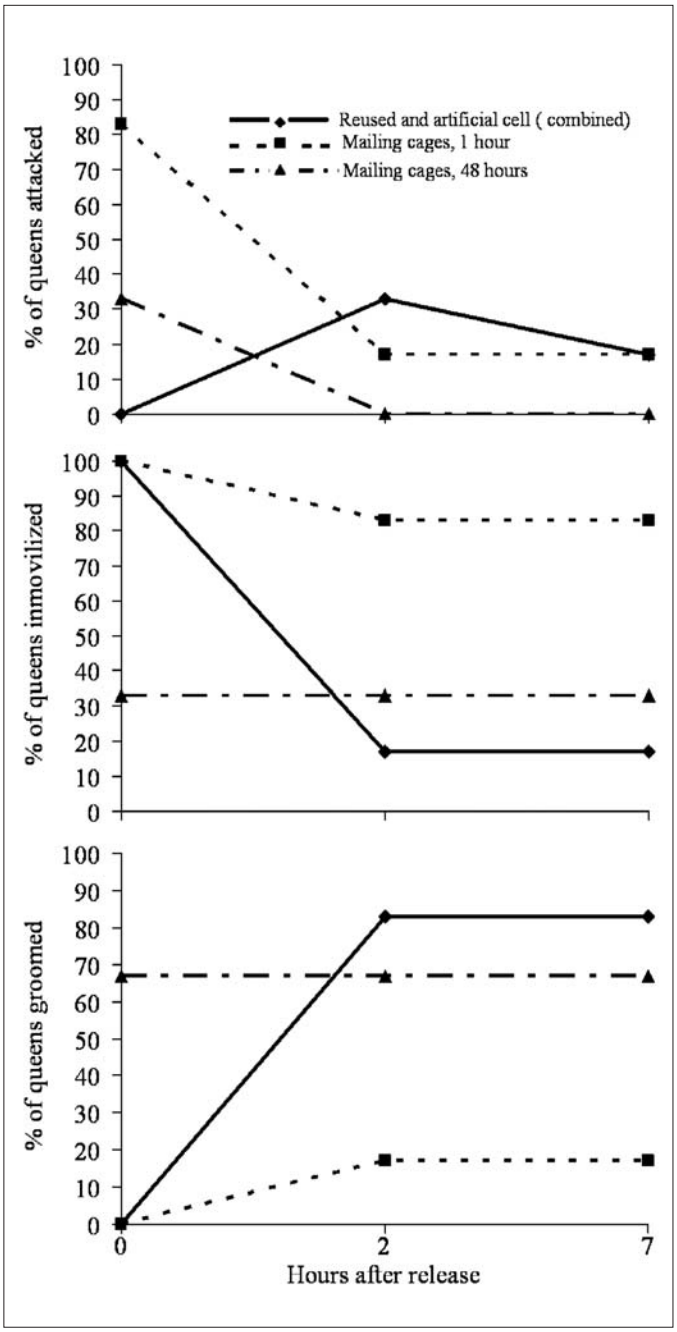

Fig. 2 Proportions of virgin queens that were attacked, immobilized and groomed by workers on leaving their cells or cages on release and after 2 and 7 hours (Experiment 2).

Experiment 2. Queen introduction into laboratory observation hives

4 colonies were set up in observation hives with 2 deep Langstroth frames at the Laboratory of Apiculture and Social Insects, University of Sheffield. 6 virgin queens were introduced into these observation hives using each of the four methods described above. After a virgin queen had been introduced, she was removed and the colony's own mother queen was reintroduced. The colony was then left undisturbed for 5 days, during which time the mother queen resumed normal egg-laying activities. The mother queen was then removed again, so that colony could be used to introduce another virgin queen. Each observation hive received one or two queens per introduction method.

The behaviour of each introduced queen and the workers contacting her was observed for 10 minutes three times after the queen emerged from her cell or cage. The observation periods were when the virgin left her cell or cage, and two and seven hours after leaving. The behaviour of the queen and workers were classified according to the categories used by Gilley (2001), as chase (a worker pursued a queen closely as she moved rapidly away), clamp (a worker closed her mandibles on a queen's legs or wing bases), groom (a worker antennated and licked a queen abdomen or mandibulated her wings and thorax) and immobilized (many workers clamped the queen, effectively halting all movement). If workers both chased and clamped the queen, she was considered to be attacked.

Statistical analyses

A 4x2 chi-square test was used to compare the numbers of virgin queens accepted among the four methods (Experiment 1). We used further 2x2 chi-square tests to investigate differences between the standard method (mailing cage 48 hours) and each of the other methods. Fisher's Exact Tests was used as an alternative to chi-square when individual cell numbers fell below five (Experiment 2).

Results

Experiment 1

Table 1 shows that the proportions of introductions that resulted in queen acceptance were 95%, 93%, 47%, 73% for virgin queens introduced using methods 1–4 (reused natural queen cell 1 hour, artificial queen cell 1 hour, mailing cage 1 hour, mailing cage 48 hours). These success rates differ significantly (4x2 test; χ^2 = 40.33, $P \leq 0.0001$). Additional 2x2 χ^2 tests show that the success rate of the standard method, mailing cage 48 hours, was significantly lower than either queen cell method (reused cell: χ^2 = 7.44, $P \leq 0.006$; artificial cell: χ^2 =5.54, P 0.019) or both queen cell methods combined (χ^2 = 10.42, P = 0.0012), and was significantly higher than the mailing cage 1 hour method (χ^2 = 5.34, $P \leq 0.02$). There was no difference in the success rates of reused and artificial queen cells (χ^2 =0.213, P=0.644).

Experiment 2

The proportions of virgin queens accepted into the observation hives were 100% (6/6), 83% (5/6), 67% (4/6), and 17% (1/6) for methods 1–4 respectively (Table 2). These proportions differ significantly (P=0.022, Fisher's exact test) and follow the same trend seen in Experiment 1 with queen cells giving the highest acceptance. As the results of the two cell methods were similar and did not differ significantly from each other (P=0.5), they were combined. The introduction success of the cell methods combined did not differ significantly from the mailing cage 48 hours method (P=0.2451, Fisher's exact test), but was significantly more successful than the mailing cage 1 hour method (P=0.0039, Fisher's exact test).

All of the virgin queens introduced in cells or using the mailing cage 1 hour method were immobilized within a few

Table 1. Numbers of 4-day old virgin queens introduced into mating nucleus hives by each introduction method, and accepted after 24 hours. Chi-square tests compare methods 1–3 to the standard method, method 4.

	Virgin Queens		
Introduction method	**Introduced, n (%)**	**Accepted, n**	**P**
Mailing cage 48 hours (method 4)	40	29 (73%)	
Reused queen cell 1 hour (method 1)	40	38 (95%)	0.006
Artificial queen cell 1 hour (method 2)	40	37 (93%)	0.019
Mailing cage 1 hour (method 3)	40	19 (47%)	0.023

Table 2. Numbers of 4-day old virgin queens introduced and accepted in queenless observation hives by each introduction method. Fisher's tests compare reused and artificial cell combined versus methods 3 and 4.

	Virgin Queens		
Introduction method	**Introduced, n (%)**	**Accepted, n**	**P**
Reused and artificial cell combined (methods 1&2)	12	11 (92%)	
Mailing cage 1 hour (method 3)	6	1 (17%)	0.0039
Mailing cage 48 hour (method 4)	6	4 (67%)	0.2451

minutes of being released (Fig. 2). However, whereas none of the queens introduced in cells were attacked before being immobilized, five out of six of the queens introduced using the mailing cage 1 hour method were attacked (P=0.0007, Fisher's exact test). Also, two hours after release, the proportion of queens being groomed by workers was significantly higher for queens introduced in cells than in cages (P=0.0039, Fisher's exact test). The reaction of workers towards queens introduced in cages and released after 48 h was intermediate. Two of the six queens were attacked within ten minutes of release, with the other four being groomed during this period, which was significantly more than in this observation period than for either the queens released from cells (P=0.0049, Fisher's exact test) or using the cage 1 hour method (P=0.0303, Fisher's exact test). There were clear relationships between the behaviours of the workers and whether or not queens were subsequently accepted. Of the queens released within 1 hour and subsequently rejected, 5 of the 7 were attacked soon after release and all were still immobilised at the later observations. Only three of the eighteen accepted queens were sporadically attacked and all the accepted queens were being groomed from two hours after release.

Discussion

Experiment 1 shows that introducing 4-day old virgin queens using both natural and artificial queen cells gave high acceptance rates (95% and 93%, respectively). The similarity shows that the high success rate was not due to any special feature of natural queen cells not also possessed by the similarly shaped plastic cells. The similarity also suggests that the high success rate of reused natural queen cells was unlikely to have been due to some chemical present on or inside the natural queen cell as this would not have been present in the plastic queen cell. The high success of the queen cell methods is even more remarkable when it is considered that queens were released into their new hive within an hour of being introduced. (In fact, Experiment 2 showed that queens were released on average within only 10 minutes). Recommendations for queen introduction normally suggest releasing queens after several days in a cage (Snelgrove, 1940; review in McCutcheon, 2001). When queens were released as rapidly from a 3-hole mailing cage only 47% were accepted. This increased, but only to 73%, for virgin queens released after 48 hours in the new colony. Thus, even queens released from a mailing cage after 48 hours had significantly lower acceptance

than queens released from a queen cell after less than 1 hour. The success rate of the queen cell methods is comparable with the success rate of 100% for queens that emerge naturally from their own cell (Perez-Sato & Ratnieks 2006). However, the overall success rate of requeening a mating hive with a ripe queen cell is actually much lower (around 70%) because a proportion of the cells do not emerge (Szabo, 1982; Perez-Sato & Ratnieks, 2006).

Experiment 2 provides some insight into why the queen cell methods gave higher introduction success. In particular, queens leaving both natural reused and artificial queens cells were immobilized but not attacked immediately after release. In contrast, most of the queens released from a mailing cage after 1 hour were attacked, as were some of those released from a mailing cage after 48 hours. This may be due to the behaviour of queens when released. Queens introduced using cages and released after 1 hour appeared nervous, frequently ran over the combs, and resisted being immobilised by the workers. In contrast, queens released from cells were more passive and readily acquiesced to being immobilised by workers. It has been found that foreign queens are balled by a mix of aggressive and non-aggressive workers (Robinson, 1984). Possibly the greater nervousness and resistance of queens released from cages results in more workers releasing alarm pheromone (Winston, 1987). As a consequence workers that immobilise the queen may be more aggressive which would reduce the probability of the queen surviving (Robinson, 1984).

There are several other possible reasons for the higher success rate using cells. Virgin queens are not easily contacted by the workers in the new hive before being released. These could affect the behaviour of the queen on being released. In addition, the physical shape of the queen cell and the time that the queen remains inside the cell before being released may also influence her behaviour on being released. Finally, workers have no opportunity to attack a queen before she is released. They cannot enter the cage or be aggressive through the wire mesh, so they will be less likely to mark queens with alarm pheromone (Yadava & Smith, 1971). During Experiment 2, caged queens were attacked (stung) by workers inside their cages. This suggests that queens can be marked with the alarm pheromone before they leave their cages resulting in rejection when they are released.

Experiment 1 shows that holding virgin queens in a mailing cage for 48 hours increases acceptance, but Experiment 2 suggests that the mechanism for this greater acceptance is not the same as for the high acceptance of queens from queen cells. Thus, four of the six queens held in a mailing cage for 48 h were groomed immediately after release whereas none of the queens from cells were groomed this quickly. Being held in a cage for several days before release is thought to make queens acquire the odour of their new colony before they are released, so making them more likely to be accepted (Snelgrove, 1940; review in McCutcheon, 2001). Our results support this idea, but the results also show that acquiring the odour of the new colony is not necessary for acceptance. The results also show that even after being held in a cage for two days, queens may still be attacked.

Overall, our results suggest that queen introduction via queen cells is a superior method for requeening queen mating hives. It is quicker and has a higher success rate than using cages. It also gives a higher overall success rate than using ripe queen cells, the normal method used in commercial queen rearing, because a significant proportion of the queen cells fail to emerge. Because artificial queen cells can be used, and can in fact be made by simple modifications to existing equipment such as queen cell protectors, the queen cell method is also practical. Further research is needed to determine if these queen cell introduction methods also gives high success in requeening normal honey production hives with mated queens.

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