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EFFECT OF THE ACCESS OF WORKER HONEYBEES TO THE QUEEN ON THE RESULTS OF INSTRUMENTAL INSEMINATION

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Summary

Altogether 96 queens were inseminated instrumentally with 8 mm^3 of semen, and placed in queenless colonies, protected in various ways: in a screened cage without or with workers; in a cage or isolator provided with queen excluder, permitting free access of workers to the queens. The queens were killed 48 h after insemination, the oviducts were examined for presence of semen, and the number of spermatozoa in the spermatheca counted. The least satisfactory result, with the queen in a screened cage without workers, gave 3·002 million spermatozoa in the spermatheca; the best, when the queen was in a queen-excluder isolator, gave 5·256 million spermatozoa. So the second method gave a 75% increase in the number of spermatozoa that migrated into queen's spermatheca, more than can be gained by a second insemination.

It is recommended that the inseminated queen should be released on a comb under a queen-excluder isolator, which remains in position until the queen starts to lay eggs.

Introduction

The method by which drone semen is instrumentally injected into the oviducts of the queen honeybee is now well established (Mackensen & Roberts, 1948). After a successful natural insemination, a queen contains about 5·34 million spermatozoa in her spermatheca (Woyke, 1960). One drone ejaculates about 11 million, almost twice the number necessary for filling the spermatheca. However, when a queen is instrumentally inseminated with 1 mm^3 of semen, containing 7·0 million spermatozoa, only 1·39 million (20%) enter the spermatheca, compared with 9·6% (Woyke, 1960) or 5·2% (Mackensen, 1964) when the queen is inseminated with 8 mm^3 of semen (56 million spermatozoa).

For genetic and breeding purposes it is desirable to increase the percentage of spermatozoa entering the queen's spermatheca. Some of the factors which can give such an increase have already been investigated (Mackensen, 1955). When these early investigations were conducted, it was thought that the queen mated with only one drone, and therefore only 1-2·5 mm^3 of semen was used for insemination; the average results did not differ significantly. Subsequently it was discovered that multiple mating occurs during one mating flight (Tryasko, 1951; Taber, 1954; Woyke, 1955). In 1960 Woyke proposed a single instrumental insemination with 8 mm^3 of semen or two inseminations each with 4 mm^3. The number of spermatozoa entering the spermatheca depends upon the volume of semen injected (Woyke, 1960; Mackensen, 1964), but naturally mated queens with a larger spermatheca were found to contain more spermatozoa than queens with a smaller one (Woyke, 1966; Foti et al., 1975).

The following conditions and techniques were found to increase the number of spermatozoa penetrating the spermatheca:

1. Grafting the youngest larvae for queen rearing (Woyke, 1967, 1971).
2. Keeping the queen at 34° after insemination, rather than returning her to a nursery cage where temperatures are lower (Woyke & Jasinski, 1973).
3. Inseminating queens when 5-14 days old (Woyke & Jasinski, 1976).
4. Mixing the semen with coconut milk (Camargo, 1975).
5. Race of queens: after Woyke, Jasinski and Smagowska (1974) inseminated queens of different races with equal volumes of semen, statistically significant differences were found in the numbers of spermatozoa which penetrated the spermatheca.

The highest count was recorded for *A. m. mellifera* × *A. m. caucasica* hybrid queens, and the lowest for *A. m. caucasica* × *A. m. carnica* hybrids.

6. If an inseminated queen is allowed to move freely in the mating nucleus her oviducts are cleared of semen, whereas if a queen is kept in a screened cage semen is retained in the oviducts (Veselý, 1970).
7. According to Foti, Grosu and Dragan (1975), a queen caged with 30 workers, both before and after insemination, gave results similar to those obtained when the queen was kept in a nucleus.

Materials and Methods

Investigations were conducted on 96 instrumentally inseminated queens over a period of two years. The new queens emerged into nursery cages provided with queen excluder, in queenless colonies, and were inseminated when 5-14 days old with 8 mm^3 of semen. The semen was collected from free-flying drones caught at the entrance of a hive. The inseminated queens were then distributed among 6 different cages or isolators (Fig. 1):

1. Screened nursery cage without workers.
2. Screened nursery cage with 10 workers.
3. Nursery cage provided with queen excluder.
4. Cage for drones 10 × 6·5 × 2·5 cm with queen excluder on both sides.
5. Small isolator 16 × 10·5 × 4·5 cm containing comb, and with queen excluder on both sides.
6. Large isolator of queen excluder, 26 × 36 cm, covering the whole comb.

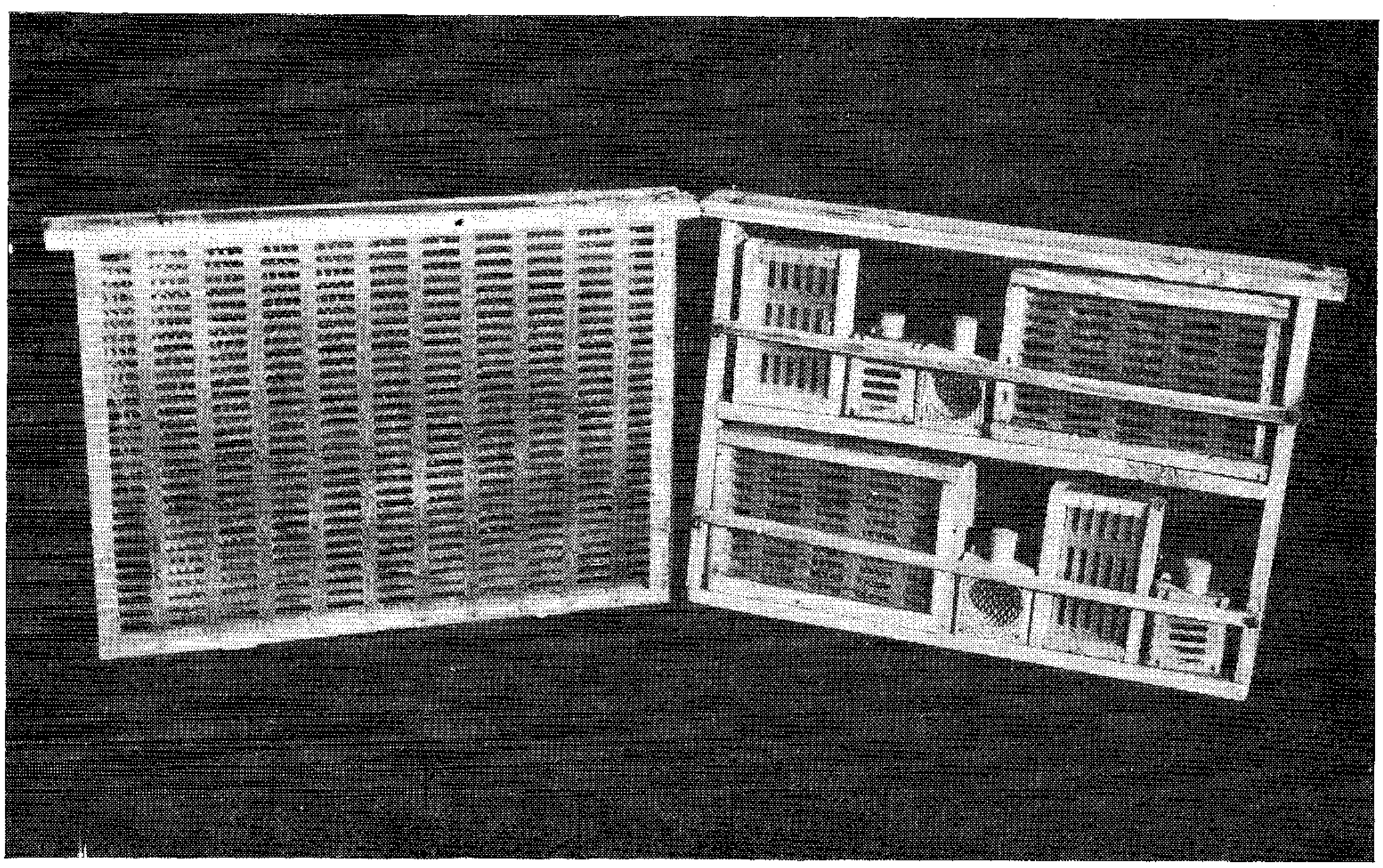

FIG. 1. *left* Queen-excluder isolater used over whole comb.
right Upper row, from left: Queen-excluder drone cage
Queen-excluder nursery cage
Screened nursery cage
Small queen-excluder isolator used over portion of comb

At least one queen in each type of cage or isolator was inseminated each day, and usually several repetitions were carried out. For any one repetition the queens in all types of cages were inseminated using similar drones, whereas queens in subsequent repetitions might be inseminated with drones which differed in age and origin from those used previously. For any one repetition cages and isolators containing the queens were placed within a special storage frame (Fig. 1), which was then put in the centre of the brood nest. The large isolator containing comb was separated from the storage frame by a comb containing brood. The queens were killed 48 h after insemination, and their oviducts checked for retention of semen. Each spermatheca was then removed and separately placed in a drop of 1·5% saline solution, where the tracheal covering was removed. The diameter diagonal to both longest and shorter axes was measured. The spermatheca was then crushed in the saline solution to disperse the spermatozoa, and more saline solution added to bring the total volume up to 1 ml. The resulting suspension was stirred while 9 ml of tap water was added. The spermatozoa in 2 mm^3 of suspension were counted using a Fuchs-Rosenthal counting chamber, and the number in 10 ml of solution was calculated.

Fisher's analysis of variance was applied to the results, and Duncan's new multiple-range test was used to find statistically significant differences between the means. In Tables 1-3, different letters after the standard errors or means indicate that the means differ significantly.

Results

Keeping several queens in queen-excluder cages in one colony

When queens that had emerged in screened cages were put into queen-excluder cages and several placed in the same colony, they were often killed by workers entering the cages. Little improvement was obtained even when the queenless colonies used were composed of workers less than 5 days old. However, the survival rate of the queens was greatly increased when queen cells were transferred directly to nursery queen-excluder cages in the rearing colony, and the queens emerged there.

Number of spermatozoa in the spermatheca of queens kept in different cages and isolators

Table 1 shows that fewest spermatozoa (3 million) entered the spermatheca of queens inseminated with 8 mm^3 of semen and kept alone in a screened cage; this was during the first season. By adding 10 workers with each queen in the screened cage, only 0·241 million more spermatozoa entered the spermatheca, an increase that was not statistically significant. When the screen was replaced by a queen excluder, so giving the workers free access to the queen, the number of spermatozoa per spermatheca increased to 4·514 million, 50% more than the number (3 million) when queens were kept in screened cages without workers. The differences differed statistically, except that the difference of 1·271 million over 3·243 million, the number of spermatozoa in queens kept in a cage with 10 workers, did not differ statistically at the 95% probability level. It differed statistically at $P < 0·05$ if, instead of Duncan's test, individual Student's *t* test was applied.

Table 1 shows that queens released on combs in queen-excluder isolators had still more spermatozoa in the spermatheca, and that the increases over numbers for queens in screened cages without or with workers were statistically significant. The highest number of spermatozoa (5·256 million) was found in the spermatheca of queens released on to comb in a small isolator, and was 2·254 million (75%) above the number in the spermatheca of queens in screened cages without workers.

TABLE 1. Number of spermatozoa (million) in the spermatheca of queens kept in various cages and isolators, permitting different access of workers, after instrumental insemination.

Type of cage or isolator	*First season* 6 *queens per combination*		*Second season* 10 *queens per combination*		*Both seasons* 16 *queens per combination*	
	Range	*Mean ± se*	*Range*	*Mean ± se*	*Mean*	%
1. Screened nursery cage without workers	2·120 –4·375	3·002±0·316*a**	1·370 –3·488	2·475±0·217*a*	2·739	100
2. Screened nursery cage with 10 workers	2·275 –3·940	3·243±0·241*ab*	1·930 –4·085	2·815±0·261*a*	3·029	111
3. Queen-excluder nursery cage	2·620 –5·495	4·514±0·458*bc*	3·140 –5·025	4·036±0·192*b*	4·275	156
4. Queen excluder drone cage	2·425 –6·420	4·417±0·679*bc*	2·995 –6·055	4·222±0·311*b*	4·320	158
5. Small queen-excluder isolator with comb	4·705 –5·980	5·256±0·230*c*	2·235 –5·805	3·943±0·336*b*	4·600	168
6. Queen-excluder isolator on whole comb	2·725 –6·620	5·140±0·580*c*	2·970 –5·485	4·239±0·276*b*	4·690	171
Mean for season	2·120 –6·620	4·262	1·370 –6·055	3·622	3·942	

* Different letters after *se* indicate that the differences between means are significant, $P < 0{\cdot}05$.

Although queens released on combs in queen-excluder isolators had 0·600-0·800 million spermatozoa more than queens in queen-excluder cages without comb, these differences were not significant (Table 1). Thus the greatest differences between numbers of spermatozoa in the spermatheca was that between queens kept in isolators or cages with queen excluder, and queens kept in screened cages without workers. Free access of workers to an instrumentally inseminated queen always resulted in an increase in the number of spermatozoa migrating into the spermatheca.

During the second season 60 queens were inseminated and investigated, 24 more than in the previous season. In general fewer spermatozoa were found in the spermatheca than in the previous year (Table 1). This might have been caused by the different origin of the virgin queens and drones used, as well as by differences in the techniques and conditions used for rearing and maintenance. Nevertheless the relationships between the numbers of spermatozoa in queens kept in the different cages and isolators were very similar to those of the previous year.

The increased number of queens probably led to the statistically significant difference between the number of spermatozoa in queens kept in screened cages with 10 workers and in cages with queen-excluder isolators. The numbers of spermatozoa in queens kept in different isolators or queen-excluder cages did not differ statistically. As in the previous year, the main difference was between queens kept in screened cages and those kept in isolators or cages supplied with queen excluder which gave workers free access to the queen. During the second season the highest number of spermatozoa (4·239 million) was found in queens released on a comb inside a queen-excluder isolator. Here free access of workers to the queens assured a 1·764 million or 71% increase in the number of spermatozoa reaching the spermatheca, over that for queens in screened cages without workers.

Comparing results for both seasons, Table 1 shows that the provision of 10 workers with the queen increased the number of spermatozoa migrating into the spermatheca by 11%. An increase of 56% resulted from replacing the screen cage by a queen-excluder isolator, and with further improvements of conditions the number of spermatozoa rose by 71%.

Woyke (1960, 1962) found 7·0 million spermatozoa per mm^3 of injected semen, so each queen would have been inseminated with 56 million spermatozoa. By releasing the queens into queen-excluder isolators instead of keeping them in screened cages, the efficiency of spermatozoa migration from the oviduct into spermatheca increased during the first season from 5·4% (3·002 million) to 9·4% (5·256 million) and from 4·4% (2·475 million) to 7·6% (4·239 million) during the second season.

Table 2 shows the variation in the mean spermatheca volume of queens from different cages or isolators. Analysis of variance did not show significant differences between mean sizes of the spermatheca of queens kept in different conditions. Most queens reared during the second season had a slightly smaller spermatheca than did those of the first season, which were better developed. Small variations in the size of spermatheca within queens reared in the same season caused some changes in the concentration of spermatozoa in the spermatheca. During the first season, queens in queen-excluder cages had a significantly higher concentration of spermatozoa than those kept in screened cages with 10 workers (Table 2). Differences between absolute numbers of spermatozoa in the spermatheca of these two groups of queens were not found to differ significantly (Table 1). All the other relationships between concentrations of spermatozoa in the spermatheca of queens kept in different cages or isolators were similar to those between the absolute number of spermatozoa.

TABLE 2. Meam volume of spermatheca (mm³), and mean concentration of spermatozoa in the spermatheca (million/mm³), of queens kept in different cages or isolators.

Type of cage or isolator	*Mean volume of spermatheca*		*Mean concentration of spermatozoa in spermatheca*	
	1st season	*2nd season*	*1st season*	*2nd season*
1. Screened nursery cage without workers	0·975 *a**	0·912 *a*	3·078 *a*	2·714 *a*
2. Screened nursery cage with 10 workers	1·011 *a*	0·953 *a*	3·218 *a*	2·954 *a*
3. Queen-excluder nursery cage	0·957 *a*	0 988 *a*	4·767 *b*	4·085 *b*
4. Queen-excluder drone cage	1·025 *a*	0·942 *a*	4·246 *ab*	4·482 *b*
5. Small queen-excluder isolator with comb	0·978 *a*	0·929 *a*	5·427 *b*	4·244 *b*
6. Queen-excluder isolator on whole comb	0·963 *a*	0·983 *a*	5·328 *b*	4·312 *b*
Mean for season	0·985	0·951	4·337	3·799

* Different letters after means indicate that the differences between them are significant, $P < 0 \cdot 05$.

TABLE 3. Retention of semen in oviducts, 48 h after insemination of queens kept in different cages or isolators.

Type of cage or isolator	*First season*: 36 *queens*		*Second season*: 60 *queens*	
	No. queens retaining semen in oviducts	*No. spermatozoa in spermatheca (millions)*	*No. queens retaining semen in oviducts*	*No. spermatozoa in spermatheca (millions)*
Screened nursery cage without workers	1	2·875	1	1·920
Screened nursery cage with 10 workers	—	—	3	1·933 2·120 2·175
Queen excluder nursery cage	—	—	—	—
Queen-excluder drone cage	1	2·730	1	3·435
Small queen-excluder isolator with combs	—	—	—	—
Queen-excluder isolator on whole comb	1	2·725	—	—
Total or average %	3 8·3	2·777	5 8·3	2·317

Retention of semen in the oviducts

Residue of semen was found in the oviducts 48 h after insemination in 8·3% of queens investigated during both seasons (Table 3). Of the 32 queens in screened cages, either without or with workers, residue of semen was found in the oviducts of 5 (15·6%), and of 64 queens kept in queen-excluder cages or isolators, residue was found in only 3 (4·7%). Therefore easy access of workers to the queens considerably decreased the percentage of queens retaining semen in the oviducts. However, not all queens kept in screened cages or queens to which worker bees had easy access retained semen in the oviducts. In these samples retention of semen in the oviducts must be caused by other factors, e.g. infection or spermatozoa killed by water in the insemination needle. Any queen retaining semen in the oviducts 48 h after insemination would probably die. All such queens had fewer spermatozoa in the spermatheca (Table 3) than the average for their respective combination (Table 1).

Conclusion and practical recommendations

Access of workers to the queen during the 48 h after her instrumental insemination considerably increased the number of spermatozoa migrating into the spermatheca. An increase of 2·254 million spermatozoa was reached by changing the conditions during the first season, almost the same as the number of spermatozoa found during the second season in the spermatheca of queens kept in screened cages (2·475 million). The 75% increase of spermatozoa in the spermatheca caused by easy access of workers to the queen is higher than that resulting from a second insemination using an equal volume of semen. A second insemination with 8 mm^3 of semen resulted in a 38% increase (Woyke, 1960) or a 62%-66% increase (Woyke, 1971) in the number of spermatozoa in spermatheca over the number after the first insemination with the same amount of semen. Therefore queens should not be placed in screened cages after instrumental insemination. Also it is recommended that only a few inseminated queens should be kept even in queen-excluder cages in one colony, as the workers eventually neglect them or even attack them. When queens are destined for colonies in other apiaries, they should be kept separately in special boxes or mating nuclei, and prevented from mating naturally by the use of queen excluder. Since double treatment with CO_2 accelerates the start of egg laying, queens should be treated for the first time when they are introduced to the nuclei, and for the second time during insemination.

As some queens were not accepted by the workers when they were returned to normal colonies after insemination, the following procedure was adopted to avoid the loss of queens which could already be laying eggs. When a queen was destined to remain in a normal colony in the apiary in which insemination took place, the marked virgin queen, treated with CO_2, was placed in a cage whose opening had been closed with candy. The cage was then fastened to a comb in a queen-excluder isolator. The isolator was placed in the brood nest of the queenless colony which the queen would finally head. A few days later the isolator was checked to make sure that the queen was free from the cage. All emergency queen cells were destroyed on all combs, and the accepted queen was inseminated. The queen was then returned to the same comb in the isolator, which was retained in the colony until egg laying commenced. It was therefore easy to find the queen at any time e.g. for additional treatment with CO_2, and it was not necessary to close the hive entrance with queen excluder.

After adopting these procedures, losses of inseminated queens were very low, and numbers of spermatozoa in the spermatheca were high.

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