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INSTRUMENTAL INSEMINATION OF *APIS CERANA INDICA* QUEENS*

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Summary

For breeding purposes, the possibility of instrumental insemination of *Apis cerana indica* was investigated. Altogether 54 queens were inseminated, and 2000 drones were used. Semen, which is difficult to separate from mucus, could be collected from only about one-third of the drones (average 0·16 mm³ semen per drone). This amount resulted only in traces of spermatozoa in the spermatheca of inseminated queens. Increasing the amount injected from 1 mm³ to 4mm³ significantly increased the number of spermatozoa in the spermatheca; increasing the temperature at which the queens were kept after insemination (from 28° to 34°) also significantly increased this number. In some queens inseminated with one large dose of semen, the oviducts were not emptied, in contrast to queens inseminated twice with a smaller amount. Queens inseminated with doses higher than 3 mm³ semen in June and July were still producing worker brood in worker cells at the end of the season.

Insemination of *A. cerana* queens with 2 mm³ *A. mellifera* semen resulted in a considerably larger number of spermatozoa reaching the spermatheca than pure *A. cerana* insemination with the same amount of semen. The reciprocal cross (*A. mellifera* queen × *A. cerana* semen) did not give the same effect. It is deduced that these differing results were due to the greater concentration and activity of the spermatozoa in *A. mellifera* semen. It seems that semen from 40–60 drones is necessary for effective insemination of an *A. cerana* queen.

Introduction

To study the genetics of *Apis cerana indica*, and to breed better strains, control of mating must be assured. No results concerning the instrumental insemination of this species have previously been published, although Ruttner (1969) inseminated *A. mellifera* queens with semen from *A. cerana* drones.

The everted endophallus of *A.c. indica* drones was described by Simpson (1960), and the inverted organ by Bährmann (1961); it lacks hard plates. The anatomy and histology of both male and female reproductive organs were described by Kapil (1962*a*, 1962*b*); he found that the testes, as well as the ovaries, of *A.c. indica* were smaller than those of *A. mellifera*, but that the other parts were similar.

A detailed investigation of the eversion of the endophallus of *A. cerana* was carried out by Ruttner, Woyke and Koeniger (1973). They reported also that *A.c. indica* drones produced 0·20 mm³ semen, with a concentration of 4655 thousand spermatozoa per mm³; the mean number of spermatozoa produced per drone was 1000 – 1500 thousand; *A. mellifera* drones produce about ten times as many.

Sharma (1960), who found two double matings among 9 virgin queens observed, concluded that the mating behaviour of *A.c. indica* was so similar to that of *A. mellifera* that it was doubtful whether they can be regarded as separate species.

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Ruttner, Woyke and Koeniger (1972) observed an *A.c. indica* queen which mated on two different flights, and they also recorded queens mating during one flight with several drones. They found 2·79 mm³ semen in both oviducts of a queen on her return from a mating flight; the spermatheca of a laying queen mated naturally in Pakistan contained 2665 thousand spermatozoa, whereas two *A. cerana* queens mated in Germany had 270 thousand and 610 thousand spermatozoa respectively in the spermatheca. All the *A.c. indica* queens had considerably fewer spermatozoa than was normal for *A. mellifera* queens (about 5300 thousand, Woyke, 1960).

The efficiency of instrumental insemination in *A. mellifera* queens was investigated by Woyke (1960) and Mackensen (1964). Woyke (1963) showed that queens inseminated instrumentally with small doses of semen (up to 4 mm³) flew out of their hives later and mated naturally.

Materials and Methods

The investigation was conducted in the Institut für Bienenkunde in Oberursel in Germany between 15th May and 15th August 1971, using *A.c. indica* bees imported from Pakistan. A total of 2000 *A.c. indica* drones were investigated, and 54 queens (plus 6 *A. mellifera* queens) were instrumentally inseminated during this period.

The drones were caught at hive entrances. Several of them were placed in a small cage in which they were taken to the laboratory. They were excited for a while by releasing them on the window, or by holding one of their legs and leaving the wings free for flight movements. Then the thorax was squeezed and the degree of eversion of the endophallus classified. Semen was collected in the calibrated tip of a syringe for instrumental insemination, and the number of drones required to supply a single dose of semen (1–4 mm³) was recorded. This enabled the average volume of semen taken from one drone to be calculated. The *A.c. indica* workers did not care adequately for queens kept in rearing cages in the colony, so the queens were kept in Foti cages attended by about 40 workers. Insemination dates of queens receiving different amounts of semen was randomized. Queens older than 5 days were inseminated with 1–4 mm³ of semen once or twice, the second insemination being made 4–5 days after the first. Some crosses with *A. mellifera* were also made for comparison. After instrumental insemination some of the cages containing queens were kept at 28°C, and others at 34° (28° is considered as optimal for the adult bees, and 34° is the temperature of the brood nest).

Thirty-three queens were killed 4–5 days after the last insemination, and were dissected in physiological saline solution. The spermatheca was transferred to a new drop of physiological solution and the tracheal covering was removed. The diameter of the spermatheca was measured and the number of spermatozoa in it counted, as described by Woyke (1971). The remaining 21 queens were released in nucleus hives for further observation and investigation. Six queens were left in the colonies for the winter, and their brood production was checked in detail at the end of the season.

Number of drones needed for instrumental insemination

By squeezing the thorax, different degrees of eversion of the endophallus were obtained. Of the 1956 drones examined in 60 tests, 45% did not evert it at all; 25% everted it partly and 11% completely, so that the semen could be collected; 18% were over-excited and the semen was either ejected on to the abdomen (such semen was not collected because of the danger of contamination), or the end of the completely everted

endophallus burst and the haemolymph flowed out and mixed with the semen, rendering it useless for insemination. Thus only about one-third of the drones killed yielded semen.

It was much more difficult to separate the semen from the mucus in *A.c. indica* than in *A. mellifera*. After complete eversion, the sperma was also often attached to the end of the endophallus, while droplets of the brownish fluid from the bulb remained in the region of the sac and bow of the bulb. The sperma alone was then so dense that it was difficult to collect it in the tip of syringe. In cases like this, both components of the semen from the same endophallus were collected together for insemination.

Table 1 shows that variability was considerable, partly because of the number of drones from which semen could not be obtained. On average, the drones from which semen could be collected each yielded 0·16 mm³, and 1 mm³ was obtained on average from 6·5 of these drones. However, the average number which had to be killed to obtain this number of "useful" drones was 16·8.

TABLE 1. Details of the number of *A.c. indica* drones required to provide semen for instrumental insemination (1956 drones used in tests).

	No. tests	*Range*	*Mean ± SE*	*Coefficient of variation*
Mean vol. semen collected per drone (mm³)*	50	0·11–0·25	0·16 ± 0·006	24.3
No. drones needed to provide 1 mm³ semen*	50	4·0 –9·0	6·5 ± 0·20	22·2
No. drones killed in order to collect semen from 1 drone	60	1·0 –7·0	2·7 ± 0·16	45·9
No. drones killed in order to collect 1 mm³ semen	50	4·0 –56·0	16·8 ± 1·53	64·5

* Including only those drones from which semen was successfully collected.

Effect of different amounts of injected semen, and of the temperature at which queens were kept after insemination

Although *A.c. indica* queens are smaller than *A. mellifera* queens, their instrumental insemination is fairly simple because injection of semen into the oviducts is easier in this species than in *A. mellifera*.

Since the volume of semen necessary for insemination was not known, the first queen was inseminated with the semen from 1 drone (0·2 mm³). After four days only a few spermatozoa were found in the spermatheca. The next queen was inseminated with 4 mm³ semen, but the oviducts were still filled with semen four days after insemination. Consequently a systematic investigation was undertaken. Results with different doses and temperatures after insemination are given in Table 2.

Increasing the temperature at which queens were kept after insemination from 28° to 34° resulted in an increase in the number of spermatozoa entering the spermatheca. Similarly, increasing the volume of semen injected from 1 mm³ to 4 mm³ increased the mean number of spermatozoa entering the spermatheca, from 410 thousand to 1221 thousand (at 34°). An analysis of variance showed that both these

TABLE 2. Number of spermatozoa (thousands) in the spermatheca of queens inseminated with different volumes of semen and kept for 5 days after insemination at either 28° or 34°.

Queen / *Semen*	*A.c. indica* / *A.c. indica*				*A. mellifera* / *A.c. indica*	*A.c. indica* / *A. mellifera*	*Overall mean*
Vol. semen injected	*1 mm³*	*2 mm³*	*4 mm³*	*2+2 mm³*	*2 mm³*	*2 mm³*	
			Temperature 28°				
	202·5	145·0	515·0	—	233·0	357·0	
	207·5	475·0	517·0	—	615·0	655·0	
	236·0	895·0†	557·0	—	997·0	1896·0	
Mean[a] ± *SE*	215·3	505·0	529·7	—	615·0	969·7	566·9*
	10·4	±217·2	±13·7	—	±220·2	±471·3	
			Temperature 34°				
	218·0	429·0	758·0†	732·0	575·0	1345·0	
	452·0	790·0	1130·0†	1220·0	837·5	1355·0	
	563·0	899·0	1475·0	1632·5	882·5	2880·0	
Mean[a] ± *SE*	411·0	706·0	1121·0	1194·8[c]	765·0	1860·0	972·6*
	±101·7	±142·0	±207·0	±260·3	±95·9	±510·0	
Overall mean no. spermatozoa[b]	313·2	605·5	825·3	—	690·0	825·3	

* Difference between means for 28° and 34° significant ($P<0·05$)

† Oviducts filled with semen 5 days after insemination

[a] Least significant difference between means ($P<0·05$) = 758

[b] Least significant difference between means ($P<0·05$) = 536

[c] This mean is not included in the overall means given in this Table.

factors influenced the results significantly, but that the interaction between them was not significant.

Table 2 also shows that one *A.c. indica* queen inseminated with 2 mm³ semen and kept at 28°, and two inseminated with 4 mm³ semen and kept at 34°, had oviducts filled with semen 5 days later. From previous observations it is thought that these queens would have died as a result.

Three queens were inseminated twice with doses of 2 mm³ semen and were subsequently kept at 34° for 5 days before examination. The mean number of spermatozoa in the spermatheca (Table 2) was very similar to that of queens inseminated with 4 mm³ semen; however, all these three queens had empty oviducts, whereas two of the queens inseminated with one large dose of semen had filled oviducts.

The results on queens with filled oviducts are not conclusive because of small numbers in each group, but it would appear likely that two inseminations of 2 mm³ each are preferable to one of 4 mm³, if the queens are kept at 34°. One dose of 4 mm³ when the queens were kept at 28° did not result in oviducts remaining filled, but the number of spermatozoa in the spermatheca was much lower than at 34°.

Although increasing the amount of semen injected resulted in an increase in the number and concentration of spermatozoa in the spermatheca, particularly when the queens were kept at 34°, the efficiency of entry of spermatozoa to the spermatheca (no. injected ÷ no. in spermatheca) was less for each consecutive dose except one (Table 3).

TABLE 3. Mean dimensions of spermatheca, and mean concentration of spermatozoa in it, in instrumentally inseminated *A.c. indica* queens (means for 3 queens).

Queen / *Semen*	*A.c. indica* / *A.c. indica*				*A. mellifera* / *A.c. indica*	*A.c. indica* / *A. mellifera*
Vol. semen injected	*1 mm³*	*2 mm³*	*4 mm³*	*2+2 mm³*	*2 mm³*	*2 mm³*
No. spermatozoa injected (thousands)	*4655*	*9310*	*18620*	*18620*	*9310*	*14440*
			Temperature 28°			
Diameter of spermatheca (mm)	1·02	1·04	1·07	—	1·21	0·95
Volume of spermatheca (mm³)	0·557	0·588	0·633	—	0·936	0·449
No. spermatozoa (thousands)	215	505	530	—	615	970
Concentration of spermatozoa (thousands/mm³)	390	830	840	—	658	2000
Efficiency of entry to spermatheca (%)	4·6	5·4	2·8	—	6·6	6·7
			Temperature 34°			
Diameter of spermatheca (mm)	1·02	1·00	1·05	1·03	1·15	1·03
Volume of spermatheca (mm³)	0·556	0·527	0·615	0·582	0·790	0·580
No. spermatozoa (thousands)	411	706	1121	1195	765	1860
Concentration of spermatozoa (thousands/mm³)	752	1311	1904	2022	966	3172
Efficiency of entry to spermatheca (%)	8·8	7·6	6·0	6·4	8·2	12·9

Cross-matings between *A.c. indica* and *A. mellifera*

The number of spermatozoa entering the spermatheca depends to a great extent on its size (Woyke, 1971). The number was found to be lower in *A.c. indica* than in *A. mellifera*, and the mean diameter and mean volume of the spermatheca of queens of the two species showed that *A. cerana* queens had a smaller spermatheca (Table 3).

TABLE 4. Dimensions of spermatheca in 27 *A.c. indica* queens and in 6 *A. mellifera* queens.

	A.c. indica	*A. mellifera*
Diameter (mm)		
Range	0·90–1·10	1·14–1·22
Mean ± SE	1·02 ± 0·01	1·18 ± 0·02
Volume (mm³)		
Range	0·382–0·697	0·776–0·951
Mean ± SE	0·564 ± 0·015	0·863 ± 0·034

This was confirmed (Table 4) by measurements of the spermatheca of 27 *A.c. indica* queens and of 6 *A. mellifera* queens; the ranges of the dimensions of the two groups

showed no overlap. The concentration of spermatozoa in the semen of *A.c. indica* was found to be 4655 thousand/mm³ and in *A. mellifera* 7220 thousand/mm³. Crosses between the two species were made to investigate the effect of these differences, using 2 mm³ semen for insemination. Comparison of results can, therefore, only be made with *A.c. indica* queens inseminated with 2 mm³ semen. Table 3 shows that the spermatheca of *A. mellifera* queens inseminated with semen from *A.c. indica* drones had a slightly higher mean number but a slightly lower mean concentration of spermatozoa than in pure *A.c. indica* matings; the differences were not significant. Temperature had no significant influence on spermathecal number in this cross-mating. However, the reciprocal cross (*A.c. indica* queen inseminated with *A. mellifera* semen) gave a significantly greater overall mean number and mean concentration of spermatozoa in the spermatheca than a pure *A.c. indica* mating. Increased temperature after insemination had a significant effect. The efficiency of insemination of *A. mellifera* queens with *A.c. indica* semen was slightly greater than that achieved in a pure *A. cerana* mating with the same volume of semen (2 mm³), whether the queens were kept subsequently at 28° or 34° (Table 3). When *A.c. indica* queens were inseminated with 2 mm³ *A. mellifera* semen, the efficiency was approximately the same as in the reciprocal mating when the queens were kept at 28°, and it was greater when the queens were kept at 34° (Table 3). This relatively small increase occurred despite the fact that *A.c. indica* spermatozoa entered an *A. mellifera* spermatheca, which was about twice as large as that in an *A.c. indica* queen. The concentration of spermatozoa was 1·6 times as high in the semen collected from *A. mellifera* drones as in that from *A.c. indica* drones. As a result, when *A.c. indica* queens were inseminated with *A. mellifera* semen, number of spermatozoa in the spermatheca was 1·9 times at 28° (and 2·6 times at 34°) as when they were inseminated with *A.c. indica* semen (Table 3). If the concentration of spermatozoa in the spermatheca is similarly compared, *A. mellifera* semen resulted in a concentration of spermatozoa in the spermatheca of *A.c. indica* queens 2·4 times as high (both at 28° and at 34°) as when *A.c. indica* semen was used. Thus the higher number of

TABLE 5. Brood produced at the end of the season by six instrumentally inseminated *A.c. indica* queens.

	Insemination			*Sealed brood in worker combs 13 Aug.*		*Sealed brood in worker combs at end of season*		
Queen no.	*Date*	*No. drones*	*Vol. semen (mm³)*	*No. brood combs*	*Brood*	*Date*	*No. cells*	*% drones*
1	9 June	9	1·0	2	worker + some drone	27 Sept.	not counted	high
17	2 July	14	1·0+2·0	2	worker + some drone	30 Sept.	273	22·0
19	2 July	15	1·0+2·0	2	worker + some drone	30 Sept.	847	9·1
2	14 June	7	1·0	3	worker	27 Sept.	467	0·8
						8 Oct.	476	2·1
9	22 June	23	2·0+1·5	3	worker	27 Sept.	401	0·0
43	24 July	27	2·0+2·2	2	worker	27 Sept.	1748	0·0

spermatozoa in the spermatheca after insemination with *A. mellifera* semen was caused not only by the high concentration of spermatozoa, but also by an additional factor. The results indicate that the female reproductive organs of *A.c. indica* were not less active than those of *A. mellifera* in transferring spermatozoa to the spermatheca, but that the higher concentration, and especially the greater penetration ability, of the spermatozoa in *A. mellifera* semen caused the differences observed in the cross-matings.

Brood from instrumentally inseminated queens

Six instrumentally inseminated *A.c. indica* queens introduced to nucleus colonies all laid fertilized eggs, but among the sealed brood of three of the queens some drones were found in worker cells. Table 5 shows that by the middle of August only three queens (no. 2, 9, 43) had exclusively worker brood in the worker cells. At the end of September only queens no. 9 and no. 43 were still producing only worker brood, and these were the queens which were inseminated with more than 3 mm^3 semen.

Discussion

There are no data concerning the average number of *A. mellifera* drones which have to be killed in order to collect 1 mm^3 semen, but the author's experience suggests that the number is between 1 and 3 compared with about 17 *A.c. indica* drones. On average, an *A. mellifera* drone produces 7·5 times as much semen as an *A.c. indica* drone (Woyke, 1960), with a concentration of spermatozoa 1·5 times as great. The *A. mellifera* drone therefore produces about 11 times as many spermatozoa as the *A.c. indica* drone.

While it was as easy to inject semen into the oviducts of *A.c. indica* queens as into *A. mellifera* queens, after insemination with semen from one drone an *A.c. indica* queen had only traces of spermatozoa in the spermatheca, whereas an *A. mellifera* queen had enough semen to produce worker brood exclusively for one season. In *A. mellifera*, insemination with 2 mm^3 semen resulted in 2400 thousand (Woyke, 1960) or 1900 thousand (Mackensen, 1964) spermatozoa entering the spermatheca. This is about three times the corresponding number found in *A.c. indica* in the present work (505 thousand and 706 thousand when the queens were kept at 28° and 34°, respectively). Presumably due to the lower concentration and smaller penetration ability of *A.c. indica* semen, as well as to a smaller spermatheca (Table 3), the efficiency of entry of spermatozoa to the spermatheca (5·4% and 7·6%) was only about half that in *A. mellifera* (17·3%, 12·4%) found by Woyke (1960) and Mackensen (1964). However, when *A.c. indica* queens were inseminated with 2 mm^3 *A. mellifera* semen (Table 3) the efficiency was 12·9% when the queens were kept at 34°, which was almost the same as that found by Mackensen (1964) for pure *A. mellifera* inseminations.

The spermatheca of *A.c. indica* has been shown here to be smaller than that of *A. mellifera*, and consequently the total number of spermatozoa contained in it may possibly be a less good measure for comparative purposes than the concentration of spermatozoa (no./mm^3). Woyke (1971) showed that the average concentration in *A. mellifera* (after insemination with 1 mm^3 semen) was 1428 thousand/mm^3, which was about double that for *A.c. indica* queens kept at 34°, inseminated with the same volume (752 thousand/mm^3, Table 3).

Naturally inseminated *A.c. indica* queens had up to 2700 thousand spermatozoa in the spermatheca (Ruttner, Woyke & Koeniger, 1973); an average number of 5300 thousand was found in *A. mellifera* (Woyke, 1960). However, in *A.c. indica*,

because of the smaller amount of semen produced by each drone as well as its lower concentration and efficiency of entry to the spermatheca, semen from more drones is required for insemination of queens than in *A. mellifera.* For good insemination it seems to the author that *A. mellifera* queens require semen from about 8 drones, whereas in *A.c. indica,* semen from about 40–60 drones is probably necessary (2–3 inseminations of 3 mm^3 semen appear likely to be optimal). In order to obtain this number of drones from which semen can be collected, 16–24 must be killed in *A. mellifera* and 100–150 in *A.c. indica.*

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