REPRODUCTION AND ARTIFICIAL INSEMINATION OF *APIS CERANA INDICA FABR*

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Abstract :

Investigations on the reproduction and artificial insemination of the hill variety of Apis cerana indica were conducted in West Germany during 1971. The drones of this species produce 0·2 mm3 of semen. One of the queens returning from a second mating flight had 2·8 mm3 of semen in her oviducts. She mated probably with 30 drones. Naturally mated queens had from 0·270 million to 2·665 million spermatozoa in their spermatheca. Artificial insemination of A. c. indica queens with 1 mm3 to 4 mm3 of semen resulted in 411 thousands to 1,121 thousands of spermatozoa in their spermatheca. Queens inseminated with doses higher than 3mm3 of semen produced at the end of the season exclusively worker brood. Two or three inseminations with 3 mm3 of semen each are recommended. For this purpose 100 to 150 drones should be tried, out of wnich 40 to 60 may give the required quantity of semen.

Introduction :

Improvement in the breed of *Apis cerana indica* is possible only after a study of its genetics and controlled mating. No results concerning the artificial inseminations of this bee have been published before the present investigation was undertaken.

Data concerning the anatomy and physiology of the male and female reproductive organs as well as the natural mating are necessary to assure success in the artificial insemination of *A. c. indica.*

The anatomy and histology of the male and female reproductive organs of this bee were described by Kapil (1962 a, b). Baehrmann (1961) and Simpson (1960) described uneverted and everted endophallus of this bee, respectively. Sharma (1960) found repeated matings in two out of the nine virgin queens observed by him.

Various methods of artificial insemination of *Apis mellifera* queens were tried and comparable results of such trials were given by Mackensen (1955, 1964), Woyke (1960, 1963, 1971) and Woyke and Jasinski (1973).

Material and Methods :

The investigations were conducted on the hill variety of *Apis cerana indica* Fabr. imported from Pakistan to west Germany in 1971. The experiments were carried during the whole season, from May to September 1971, in the Institut fur Bienenkunde, Oberursel, headed by Prof. Dr. F. Ruttner. The research was supported by Deutsche Forschungsgemeinschaft, and Deutsche Akademische Austauschdienst. The results were worked out in Poland partly with the support from USDA PL 480. Detailed data of this and other investigations concerning this problem are

given by Ruttner, Woyke and Koeniger (1972, 1973) and by Woyke (1973).

Results

Eversion of endophallus of the drones

When the thorax of a sexually mature *A. c. indica* drone is squeezed, it everts its endophallus. At first a stage referred to as ' partly everted endophallus ' is reached (Fig. 1). At this stage, the three upper cornua (UC) as well as the bursal cornua (BC) are everted. The end of endophallus is formed by cervix (CE) and no semen is ejaculated from the endophallus. Sexually mature and well excited drones contract their abdominal muscles which results in an increase of pressure in the abdomen and endophallus. With other drones, this can be made artificially by squeezing the abdomen of the drones. Increase of pressure causes further eversion of endophallus. After the cervix is completely everted, and as the bulb starts to evert, a drop of semen appears at the end of endophallus (Fig.2).

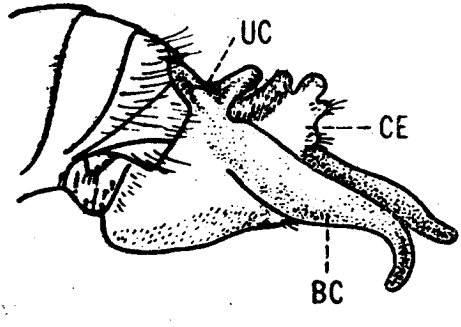

Fig. 1. Partly everted endophallus of *Apis cerana indica* drone. BC : bursal cornua, CE : cervix, UC : upper cornua.

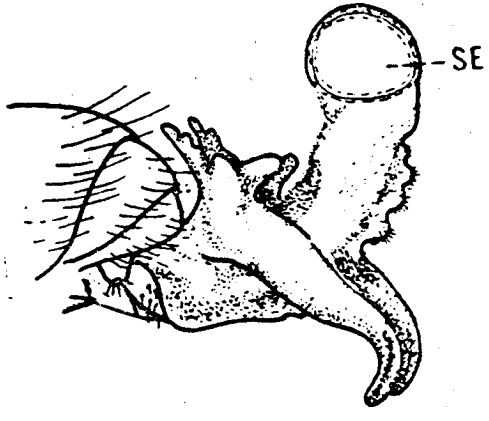

Fig. 2. Stage of eversion of the endophallus in which the semen should be collected for artificial insemination. SE semen.

The semen consists of two main parts : the sperma containing the spermatozoa, which is stored until the time of ejaculation in the *vesicule seminales*, and the brownish fluid which is stored till the time of ejaculation in the bulb of endophallus. The sperma alone is very dense, dried quickly in air and is difficult to collect into the syringe for artificial insemination. After the two components are mixed, the semen gets less viscous and can be easily collected into the syringe for artificial insemination. Nevertheless, even well mixed semen of *A. c. indica* drones is more viscous than that of *A. mellifera*. The semen is pushed out of the endophallus by mucus produced by

mucus glands. Only the semen without mucus should be collected for artificial insemination in the tip of syringe. It is more difficult to separate the semen from the mucus in *A. c. indica* than it is in *A. mellifera*.

Further increase of pressure inside the copulatory apparatus causes complete eversion of endophallus (Fig. 3). In well excited drones this stage of eversion is reached before the semen is ejaculated (Fig. 2). As a result, the two components of the semen are not mixed Droplets of the brownish fluid from the bulb can be found in the proximal part of bulb near the cervix. The dense sperma can be found in different places. When the semen appears on the distal end of the unruptured endophallus, both components can be collected together. To avoid contamination the semen if ejected on to the tergites of abdomen or mixed

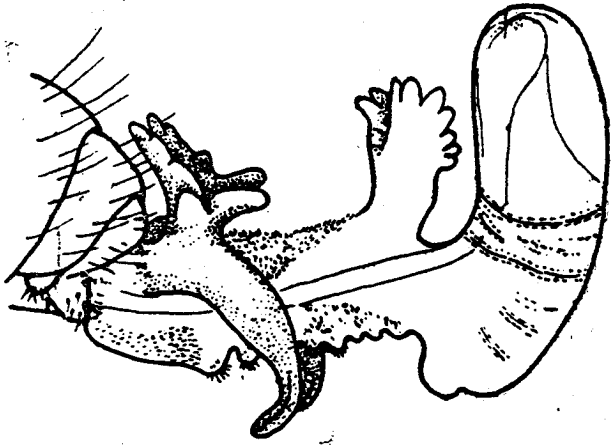

Fig. 3. Completely everted endophallus of *Apis cerana indica* drone.

with the haemolymph when the endophallus bursts, should not be used for artificial insemination.

During the investigation conducted throughout the whole season, it was observed that by squeezing the thorax of 1956 drones captured at the hive entrance, almost one half of them (45%) did not evert the endophallus. The semen could be taken from 36.6% of the drones of which 25·5% everted the endophallus partly and 11·0% completely. Almost 20% of the drones were overexcited and the semen was either ejaculated on to the abdomen, or was mixed with the haemolymph from the ruptured endophallus.

Production of semen :

The average volume of semen produced by one drone was calculated from 15 sam-

ples of 1 mm^3 to 4 mm^3 of semen collected in the syringe. Altogether 27·5 mm^3 of semen was collected from 137 drones. The volume of semen produced by one drone ranged from 0·18 mm^3 to 0·25 mm^3. The overall mean was 0·20 mm^3.

Since an *A. mellifera* drone produces about 1·5 mm^3 of semen, it turns out that the *A. c. indica* drone produces an average of about 7·5 times less semen than that of the *A. mellifera* drone.

It is not possible to collect in a routine work of artificial insemination, all the semen produced by a drone. Table 1 shows that an average of only 0·16 mm^3 of semen can be collected from one drone. Only one third of the drones tried yielded semen for artificial insemination. Thus 1 mm^3 of semen was collected on an average from 6·5 drones for which 17 of them were killed. The variation here was extremely high. It happened that only 4 drones had to be killed in one case, but in another case as many as 56 of them had to be killed to collect the same amount (1 mm^3) of semen.

In the 5 samples of 1 mm^3 of semen collected from *A. c. indica* drones, the concentration of spermatozoa ranged from

TABLE 1

Characteristics of usefulness of *A. c. indica* drones for artificial insemination (1956 drones investigated)

	No. of tests	Range	Mean ± S. E.
Mean mm^3 of semen collected from 1 drone	50	0·11–0·25	0·16±0·006
No. of drones killed to collect semen from 1 drone	60	1·0–7·0	2·7±0·16
No. of drones from which 1 mm^3 of semen was collected	50	4·0–9·0	6·5±0·20
No. of drones killed to collect 1 mm^3 of semen	50	4·0–56·0	16·8±1·53

3·475 million to 5·625 million in 1 mm^3 of semen. The mean was 4·655 million. The average concentration in the semen of *A. mellifera carnica* drones investigated at the same time was 7·220 million spermatozoa per mm^3 of semen. Thus the *A. c. indica* drones have more than 1·5 times lower concentration of spermatozoa in semen than the *A. mellifera* drones.

Multiplying the number of spermatozoa in 1 mm^3 (4·655 million) by the volume

of semen produced by one drone (0·20 mm^3) it appears that an *A. indica* drone produces on an average 930 thousand, roughly 1 million of spermatozoa.

Ruttner (1969) found in the drones of *A. cerana* from Peking 1·5 million of spermatozoa in the both *vesiculae seminales*. Since the *A. mellifera* drones have 11 million spermatozoa, the *A. c. indica* drones produce only 9–14% of this number (7·3 to 11·0 times less).

Queens mated naturally :

We have still insufficient data on natural mating and reproduction of *A. c. indica* queens which would serve as a base for artificial insemination. Repeated matings, i. e., matings on two different flights, were found in *A. c. indica* queens.

A queen mated already in one of her previous flights was killed and dissected immediately after returning from her second successful flight. It was found that one of the oviducts had 1·83mm ^{3} of semen, and the other 0·96 mm^3. Thus the queen had received 2·79 mm^3 of semen in one mating flight. Since one drone produces about 0·20 mm^3 of semen, the queen mated probably with 14 drones. The spermatheca of this queen was filled with spermatozoa from the previous flight. But the number could not be counted as the queen had been fixed. Several other queens returned also twice with a mating sign. Therefore it is not improbable, that some queens mated with as many as 30 drones.

The knowledge of the number of spermatozoa in the spermatheca of naturally inseminated queens is very important for a successful artificial insemination. Investigation of a queen mated naturally in Pakistan, and laying eggs for one season showed 2·665 million spermatozoa in her spermatheca. Two other queens mated in Germany in a mating yard with *A. c. indica* drones, had 270 thousand and 610 thousand spermatozoa in their spermathecae. All the figures are few times lower than the average of 5 million of spermatozoa found in the spermatheca of naturally mated *A. mellifera* queens.

Artificial insemination :

Data described in this paper concern the artificial insemination of 62 queens of which 54 were of *Apis cerana indica*. The *A. c. indica* workers did not care properly for queens kept in rearing cages in a colony. Therefore, the queens were kept in Foti cages, attended by ca.40 workers and supplied with honey. The cages were put in an incubator. Queens older than 5 days were inseminated with 1 to 4 mm^3 of semen once or twice. The second insemination was made 4–5 days after the first. Some crosses with *A. mellifera* were made for comparison. After the artificial insemination, some queens in cages were kept at 28°C, and the others at 34°C. The 28°C temperature is considered as the optimal for the longevity of the life of bees, and 34°C is the temperature of the bee colony. Some queens were released into nuclei for further observation or were used for other investigations. The remaining 33 queens were killed 4 to 5 days after the last insemination. The queens were dissected, their oviducts examined, the spermathecae

deprived of the tracheal cover were measured and the number of spermatozoa in each counted.

A. c. indica queens are smaller than *Apis mellifera* queens. The instruments (hooks and tip of syringe) look huge compared with the size of the reproductive organs of *A. c. indica* queens. Nevertheless, the insertion of the tip of syringe in the orifice of vagina and injection of the semen in the oviducts is even easier than in *A. mellifera* queens.

Single insemination :

Since the volume of semen to be used for artificial insemination was not known, the first queen was inseminated with the semen from 1 drone (0.2 mm^3). After four days only a few spermatozoa were found in the spermatheca. The next queen was inseminated with 4 mm^3 of semen but the oviducts were four days after insemination still filled with semen. A systematic investigation was therefore undertaken. Results of insemination with different doses and post-insemination temperatures are presented in Table 2.

Spermatozoa were found in the spermatheca of all inseminated queens. The increase from 1 mm^3 to 4 mm^3 of the volume of *A. c. indica* semen injected into the oviducts, increased the number of spermatozoa in the spermatheca of queens kept at 28°C from 215 thousand to 530 thousand and in those kept at 34°C from 411 thousand to 1,121 thousand. Thus

TABLE 2

Number of spermatozoa (in thousands) in the spermatheca of queens inseminated with different volumes of semen and kept afterwards at different temperatures.

A. c. indica × *A. c. indica*				*A. mellifera* × *A. c. indica*	*A. c. indica* × *A. mellifera*
1 mm^3	2 mm^3	4 mm^3	2×2 mm^3	2 mm^3	2 mm^3
			Queens kept at 28°C		
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	1,896.0
Mean 215·3	505·0	529·7	–	615·0	969·7
			Queens kept at 34°C		
218·0	429·0	758·0*	732·0	575·0	1,345·0
452·0	790·0	1,130 0*	1220·0	837.5	1,355·0
563·0	899·0	1,475 0	1,632·5	882·5	2,380·0
Mean 411·0	706·0	1,121·0	1,194·8	765·0	1,860·0

* Oviducts filled with semen 5 days after insemination

increasing the volume of semen injected resulted in a significant increase of the number of spermatozoa entering the spermatheca. The increase of temperature at which the queens were kept after artificial insemination from 28°C to 34°C increased the number of spermatozoa entering the spermatheca by 196 thousands to 591 thousands depending upon the dose in *A. c. indica*. The increase was even higher (890 thousands) when *A. c. indica* queens were insemin: ted with *A. mellifera* semen.

Table 2 shows that two queens ins minated with 4 mm^3 of semen and kept afterwards at 34°C, had 5 days after insemination, the oviducts still filled with semen. Among the queens kept at 28°C a similar situation was found in one queen which had a higher number of spermatozoa in the spermatheca (This queen was inseminated with only 2 mm^3 of semen but the concentration of the injected semen could be very high). All the queens with filled oviducts would die.

Double insemination :

Comparison of the results of one insemination (4 mm^3) with two inseminations (2 X2 mm^3) of the same amount of semen (Table 2) show that in the latter, there was an increase in the number of spermatozoa in the spermatheca from 1,121 thousands to 1,194 thousands. The difference in number was, however, very small and was not found to be statistically significant. The important difference was that all the queens inseminated twice with smaller volumes of semen emptied the oviducts whereas all those inseminated with one big dose did not do this.

Cross insemination between A. c. indica and A. mellifera :

The number of spermatozoa entering the spermatheca after the artificial insemination is lower in *A. c. indica* than that in *A. mellifera* bees. To find the cause, crosses between the two species were made Table 2 shows that sp rmatozoa of one species injected into the oviducts of the other, entered the spermatheca. Insemination of *A. mellifera* queens with the semen of *A. c. indica* drones resulted in an increase in the number of spermatozoa in the spermatheca over pure *A. c. indica* mating. Thus the mean number of spermatozoa in queens inseminated with 2 mm^3 of semen and kept at 34°C increased from 706·0 thousand to 765.0 thousand. The increase was not high and was not found to be statistically significant. But these data suggest a tendency of higher efficiency of *A. mellifera* female reproductive organs over those of *A. c. indica*.

The reciprocal cross, i. e., insemination of *A. c. indica* queens with the semen of *A. mellifera* drones resulted in an enormous increase of the number of spermatozoa in the spermatheca of queens kept at both the temperatures. The mean for queens inseminated with 2 mm^3 of semen and kept at 34°C was in this cross 1,860·0 thousand against 706·0 thousand in pure *A. c. indica* mating. The difference was statistically significant. Thus the higher efficiency of artificial insemination of *A. mellifera* was mostly caused by the semen.

Efficiency of insemination :

The increase in the doses of *A. c. indica* semen from 1 mm^3 to 4 mm^3 (or 2 mm^3

+2 mm^3) did not increase the number of spermatozoa in the spermatheca 4 times but only 2·7 or 2.9 times. Thus the efficiency of entry of spermatozoa in the spermatheca (the percentage of spermatozoa entering the spermatheca out of the total injected into the oviducts) decreased from 8·8% to 6·0% (single insemination) or 6·4% (double insemination.)

Insemination of *A. c. indica* queens with the semen of *A. mellifera* drones caused great changes. The concentration of spermatozoa in the semen of *A. mellifera* was found in this investigation to be 7·220 millions per mm^3 which is much higher than that in *A. c. indica* (4·665 million/ mm^3). Thus *A. c. indica* queens inseminated with 2 mm^3 of *A. mellifera* semen received 14·440 millions of spermatozoa in the oviducts instead of 9·310 millions of spermotozoa present in 2 mm^3 of *A. c. indica* semen. An increase of 1·55 times in the number of spermatozoa in *A. mellifera* semen resulted in (1·860 millions) 2·63 times increase in the number of spermatozoa in the spermatheca of *A. c. indica* queens, as compared with pure *A. c. indica* insemination (0·706 millions). The enormous increase of the number of spermatozoa in the spermatheca of *A. c. indica* queens inseminated with *A. mellifera* semen, was thus not only caused by higher efficiency of penetration of the *A. mellifera* spermatozoa but also by higher concentration of spermatozoa in the latter.

Size of spermatheca and concentration of spermatozoa in it :

The number of spermatozoa entering the permatheca depends to a great extent on its size (Woyke, 1971). Therefore the concentration of spermatozoa in the spermatheca characterizes the efficiency of insemination very well.

The diameter of spermatheca of 27 *A. c. indica* queens ranged from 0·90 mm to 1·10 mm and the overall mean was 1·02±0·010 mm. The volume ranged from 0·382 mm^3 to 0·697 mm^3 and the overall mean was 0·564 ± 0·015 mm^3. The diameter of spermatheca of 6 *A. mellifera* queens investigated here ranged from 1·14 mm to 1·22 mm and the overall mean was 1·18± 0·015 mm^3 and the volume ranged from 0·776 mm^3 to 0·951 mm^3 and the overall mean was 0·863±0·034 mm^3. Thus the biggest spermatheca of *A. c. indica* queens were smaller than the smallest spermatheca of *A. mellifera* queens.

The average concentrations of spermatozoa in the spermatheca of *A. c. indica* queens inseminated with 2 mm^3 of semen and kept afterwards at 28°C and 34°C were 830 thousands and 1.311 thousands in 1 mm^3, respectively. The concentrations in *A. mellifera* queens inseminated with the same volume of *A. c. indica* semen and were kept afterwards at 28°C and 34°C were 658 thousands and 966 thousands spermatozoa, respectively, which are less than those in pure *A. c. indica* inseminations. So the higher absolute number of spermatozoa found in spermatheca of *A. mellifera* queens inseminated with *A c. indica* semen was mostly caused by bigger spermatheca of *A. mellifera* queens. Thus the activity of female reproductive organs in transferring the spermatozoa into the spermatheca, was in *A. c. indica* queens not lower, but perhaps even higher, than that in *A. mellifera.*

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Concentration of spermatozoa in the spermatheca of *A. c. indica* queens inseminated with 2 mm^3 of *A. mellifera* semen and kept at 28°C and 34°C was 2,000 thousand/mm^3 and 3,172 thousand/mm^3, respectively, which is 2·4 times more than in pure *A. c. indica* insemination. The penetration ability of *A. mellifera* spermatozoa into the spermatheca is much higher than that of *A. c. indica*.

Brood production of artificially inseminated queens :

All six artificially inseminated *A. c. indica* queens introduced to the colonies started to lay eggs. All of them laid fertilized eggs but among the sealed brood of some queens, some drones were found in worker cells. Table 3 shows that by the middle of August, only three queens produced exclusively worker brood in the worker cells. Of those queens one was inseminated once with 1 mm^3 of semen and the two others twice with 2·0 mm^3 + 1·5 mm^3 and 2·0 mm^3 + 2·2 mm^3 of semen. At the end of September only queens inseminated with more than 3 mm^3 semen still produced exclusively worker brood.

The previous results showed that queens inseminated with larger doses of semen did not empty their oviducts which led to their death. Therefore, few inseminations with smaller doses, e. g., twice or thrice with 3 mm^3 are recommended.

The above results show that *A. c. indica* queens can be inseminated artificially so that they produce exclusively worker brood.

TABLE 3

Brood produced at the end of the season by artificially inseminated *Apis cerana indica* queens

		Insemination		Sealed brood in worker combs 13 Aug.		Worker cells with sealed brood at the end of season		
Queen No.	Date	No. of drones	mm^3 of semen	No. of brood combs	Kind of brood	Date	No. of cells	% of drones
1	9·6·71	9	1·0	2	Workers + some drones	27·9·71	Not counted	High
2	14·6·71	7	1·0	3	Workers	27·9·71 8·10·71	465 476	0·8 2·1
9	22·6·71	23	2·0+1·5	3	Workers	27·9·71	401	0·0
17	2·7·71	14	1·0+2·0	2	Workers +some drones	30·9·71	273	22·0
19	2·7·71	15	1·0+2·0	2	Workers +some drones	30·9·71	847	9·1
43	24·7·71	27	2·0+2·2	2	Workers	27·9·71	1748	0·0

Discussion and Conclusions :

A comparison of the results presented, with the data published earlier for *Apis mellifera* (Woyke, 1960), show that *A. c. indica* drones produce 8 times lower volume of semen (0·20 mm^3), than *A. mellifera* drones (1·60 mm^3). The concentration of spermatozoa in the semen is 62% (4·655 million/mm^3) of that found in *A. mellifera* (11 million). The penetration ability of *A. c. indica* spermatozoa into spermatheca is also lower than that of *A. mellifera*. Naturally mated *A. c. indica* queens had lower number of spermatozoa in the spermatheca (the highest number found : 2·665 million) than *A. mellifera* queens (average 5·340 million). Nevertheless the queens of *A. c. indica* must copulate with higher number of drones (15 in one flight or 30 in two) than do the *A. mellifera* queens (average 8 matings in one flight).

It is possible to collect sufficient amount of *A. c. indica* semen for artificial insemination. The injection of semen into oviducts of *A. c. indica* queens is not more difficult than in *A. mellifera*. But the number of drones used for insemination must be higher than in *A. mellifera*. Successful artificial insemination with 1 drone is possible in *A. mellifera* but this results in only few of spermatozoa entering the spermatheca of *A. c. indica* queens. Insemination with 1 mm^3 to 4 mm^3 of semen resulted in 0·411 million to 1·21 million of spermatozoa in the spermatheca. Queens inseminated with doses higher than 3 mm^3 of semen produced at the end of the season exclusively worker brood. Two or three inseminations with 3 mm^3 of semen each are recommended. To do this, semen from 40 to 60 drones must be collected, for which 100 to 150 of them must be killed.

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