Journal of Apicultural Research 12(1) : 21–34 (1973)

REPRODUCTION IN *APIS CERANA*
2. REPRODUCTIVE ORGANS AND NATURAL INSEMINATION

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Manuscript received for publication 20th March 1972

Summary

The morphology of the genitalia, and the mating behaviour of *Apis cerana indica* from Peshawar, Pakistan, are described. The morphology of the vagina of the *A. cerana* queen, in particular the open valvefold, makes instrumental insemination easier than in *A. mellifera*. In the drone, both the quantity of semen and the concentration of spermatozoa in it are lower than in *A. mellifera*. In the seminal vesicles about 1·5 million spermatozoa were found per drone, and in the ejaculated fluid about 1·0 million per drone.

One queen, dissected immediately after a second mating flight, had a quantity of semen in the oviducts corresponding to that from 14 drones, so on both flights together this queen may have mated with some 30 drones. A mating sign was observed on newly mated queens, but it lacked the chitinized plates found in *A. mellifera*.

It is concluded that, to ensure full insemination, *A. cerana* queens must mate with more drones than is necessary for *A. mellifera* queens. Evolutionary trends in the genus *Apis* are discussed.

Introduction

The investigations described here are complementary to our observations on mating behaviour in *Apis cerana indica* described in Part 1 of this paper (Ruttner, Woyke & Koeniger, 1972). Studies were made on the male and female genitalia, on the quantity of semen produced in one male, and on the total amount of semen transferred to a queen during a single mating flight. It is hoped that such comparative studies on *A. mellifera* and *A. cerana* will lead to a better understanding of the complicated process and biological background of the storage of sperm in the queen of the genus *Apis*.

Male genitalia and the process of eversion

Eversion of the endophallus

The eversion of the endophallus results from the pressure exerted by the haemolymph when this is compressed by the contraction of the abdominal muscles. First the posterior sclerites of the abdomen, and the penis valves, open. Then the basal part of the endophallus (the so-called vestibulum, VE) everts, up to the hairy dorsal triangular plate HDP (Fig. 1). From the upper basal part of the vestibulum (bursa), the bursal cornua BC and the three pairs of small upper cornua UC start to evert. The bursal cornua evert backwards (posteriorly) and downwards (ventrally), as shown in Fig. 2, and their apices first come close to each other (Fig. 3) and then evert further laterally (Fig. 4) and more ventrally (Fig. 2). These processes occur in the opposite order in *A. mellifera,* where the bursal cornua first evert in a dorsal and lateral direction; then they bend down ventrally and their apices converge.

In the *A. cerana indica* examined, and presumably in *A. cerana* in general, uneverted parts of the cornua still remain inside their apices. All the cornua are covered with

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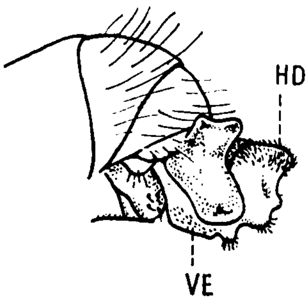

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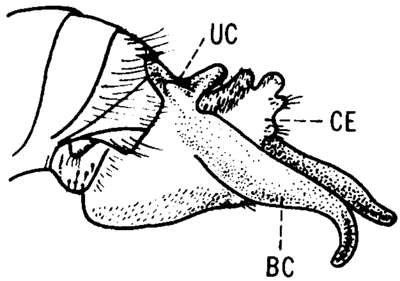

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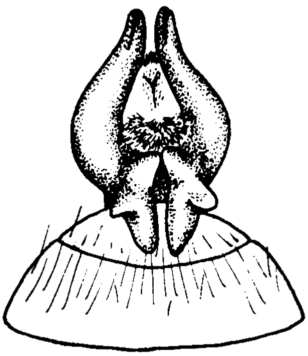

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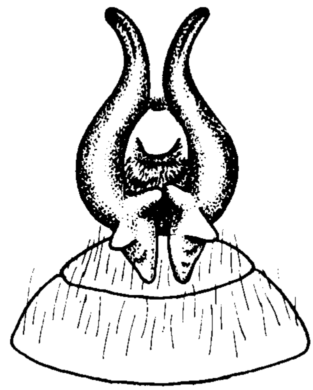

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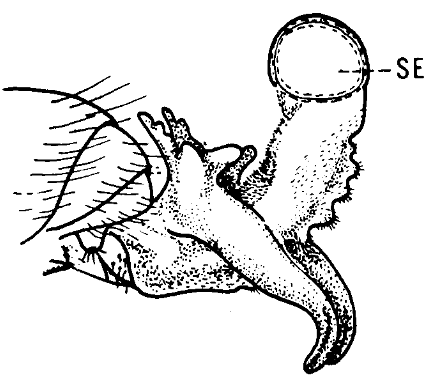

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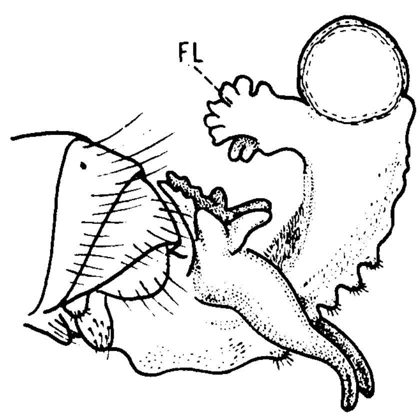

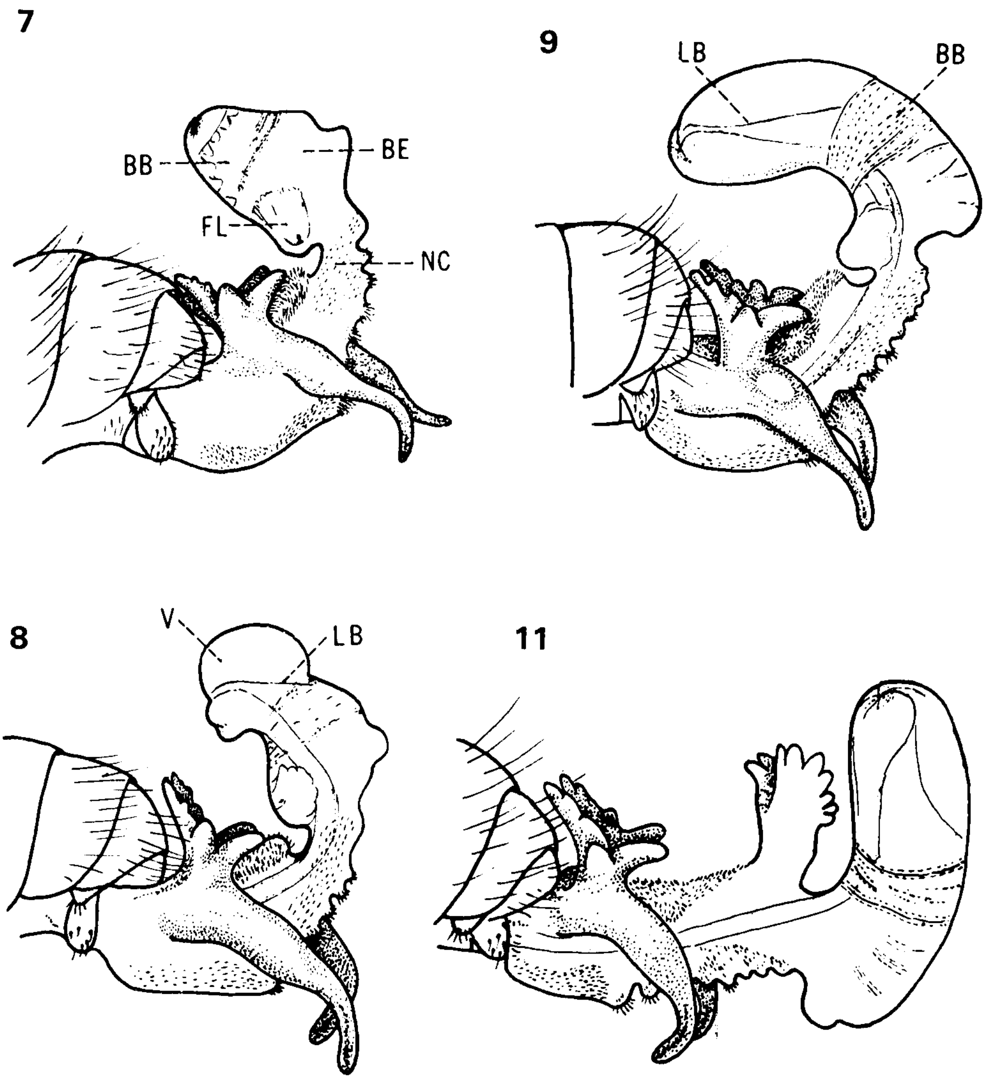

FIG. 1–9, 11. Stages in the eversion of the endophallus of the *Apis cerana* drone. Drawings by J. Woyke; cf. drawings of *Apis mellifera* in *Bee World* (1958).

BB : bow of bulb
BC : bursal cornua
BE : bulb of endophallus
CE : cervix of endophallus
FE : fimbriate lobe
HDP : hairy dorsal triangular plate
LB : lamina basilaris
NC : incompletely everted part of cervix
SE : semen
UC : upper cornua
V : vesicle at end of endophallus
VE : vestibulum of endophallus

an orange layer that is more sticky than in *A. mellifera.* This layer does not burst along the upper wall of the cornua as in *A. mellifera,* but it can be much more easily detached from the cornua. The "movements" of the cornua of the *A. cerana* drone are mostly caused by the different stages of eversion; in *A. mellifera,* on the contrary, the outer layer slides off once it is burst, causing the cornua to bend.

Meanwhile the three pairs of the small upper cornua evert: one pair forwards (anteriorly), one laterally and one backwards (posteriorly). The apices of the last pair meet together (Fig. 3, Fig. 4). The endophallus is now everted roughly as far as half the cervix, the stage shown in Fig. 2. The most difficult part of the eversion, requiring the greatest force, is that of the middle portion of the endophallus (the cervix). After the cervix is everted it can be seen that the dorsal triangular hairy plate is much longer in *A. cerana* than in *A. mellifera.* The bulb of endophallus is now inside the cervix, and when it is filled the sperm begins to flow out (Fig. 5), together with fluid from the endophallus, which is pushed out by the mucus. The sperm is dark yellow, and the fluid is brown. These two components of the semen are not so well mixed as in *A. mellifera*; on the other hand in the course of instrumental insemination it is more difficult to separate the semen from the mucus in *A. cerana* than in *A. mellifera.* An increase in pressure results in the eversion of fimbriate lobe FL (Fig. 6), which looks different from that in *A. mellifera,* being more rosette shaped.

The absence of chitinized plates in *A. cerana* makes the further stage of eversion much easier than in *A. mellifera.* It is therefore more difficult for the experimenter to obtain the correct phase of eversion for instrumental insemination.

In sexually mature drones, sufficiently stimulated, the endophallus everts further before the bulb of endophallus is filled with semen and mucus, and before the fimbriate lobe is everted. The empty bulb passes through the cervix, which is not stretched completely. The part of the cervix behind the triangular hairy plate is therefore narrow (Fig. 7). The everted bulb of the endophallus is now posterior to the cervix, but the fimbriate lobe is still not everted (Fig. 7).

Increase of pressure inside the endophallus causes a peculiar phenomenon not found in *A. mellifera.* In the terminal part of the endophallus, haemolymph enters between the layers of tissue of which the endophallus is composed. Examination of cross-sections showed that the epithelium was disrupted, and the lamina basilaris was detached, with part of the epithelium, from the outer chitinous layer. As a result, a vesicle (V) appears on the end of the bulb of the endophallus (Fig. 8). The detached layer is visible through the transparent wall of the bulb. Further increase of pressure extends the vesicle, making the bulb very large; its end faces in an anterior direction, towards the abdomen (Fig. 9, Fig. 10).

If the bulb was not filled during the partial eversion, the sperm is now (in the course of the experimental procedure) ejaculated on to the final abdominal tergites. The sperm has a high viscosity because it is not mixed with the fluid from the bulb of the endophallus. Droplets of this brown fluid can be found on the bulb in the region near the cervix. The sperm is pushed out of the ejaculatory duct by the mucus. The mucus also has a higher viscosity than in *A. mellifera,* and it solidifies rapidly in the presence of air. As a result, a long thread of mucus comes out of the end of the endophallus (Fig. 10). Examination of cross-sections through the everted endophallus showed that the ejaculatory duct was filled with epithelium. Probably this epithelium was torn off from the mucus glands, as in *A. mellifera.* The epithelium pushes the mucus—and this in turn the semen—from the ejaculatory duct. Inside the everted bulb the internal layer is still visible, detached from the wall of the bulb.

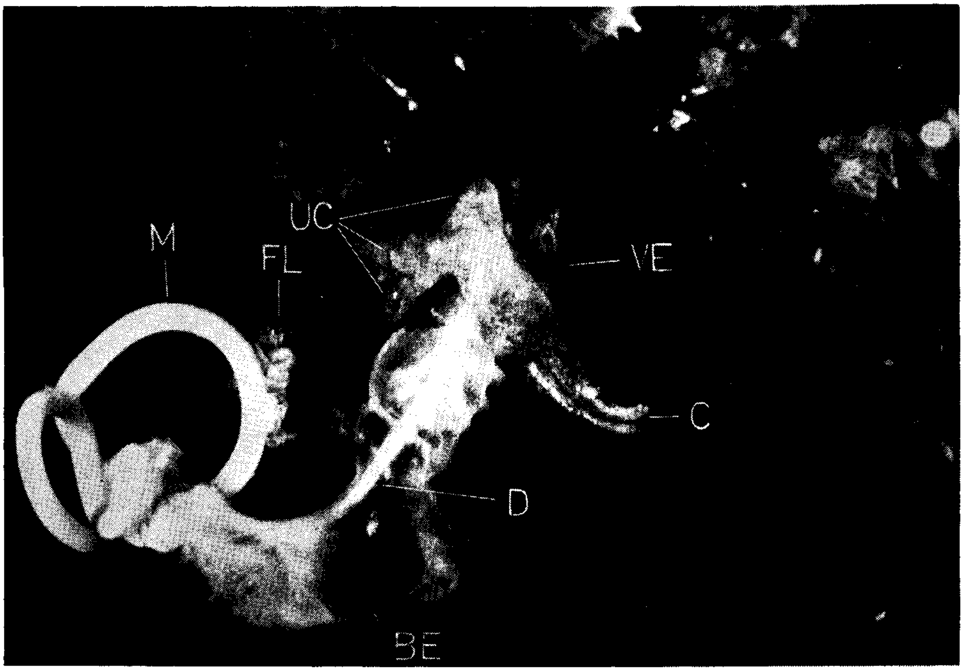

FIG. 10. Fully everted endophallus with threads of coagulated mucus.

BE : bulb of endophallus
C : (lower) cornua
D : ejaculatory duct
FL : fimbriate lobe
M : mucus
UC : upper cornua
VE : vestibulum of endophallus

When the lumen of the everted bulb is not only filled with the haemolymph, but some of it also with air, this layer is ruptured.

The increasing pressure inside the endophallus completes the eversion of that part of the cervix previously uneverted, i.e. the part apical to the hairy triangular plate. This pressure also everts the fimbriate lobe. As a result, the end of the endophallus now turns upwards, in a dorsal direction (Fig. 11). At this stage the endophallus is referred to as completely everted. The haemolymph begins to filter through the thin wall of the bulb, where the membrana basilaris and the epithelium were separated, without rupturing the bulb. Further rise of the internal pressure than ruptures the thin wall of the bulb; the haemolymph flows out, and the end of the endophallus collapses.

At which stage of eversion is the semen ejaculated into the queen's vagina and oviducts? It cannot be when the endophallus is fully everted, because the sperm is not mixed with the fluid of the bulb, and the haemolymph flows out from the end of the endophallus. The only possible stage is when the cervix is everted, the bulb inside it just starting to evert (Fig. 5). Even if the bulb has not been filled at this stage, the obstacles encountered in the queen's sting chamber and bursa copulatrix halt the eversion long enough for ejaculation of semen.

We believe that the greater "stickiness" of the cornua in *A. cerana* helps to unite the pair in copula more firmly, and that the eversion of other parts of the endophallus, such as the fimbriate lobe and the bulb, helps in the separation of the pair.

Volume of semen produced by *Apis cerana* drones

A sexually mature drone that is highly stimulated will evert the endophallus completely, and the sperm is ejaculated violently, separately from the fluid in the bulb, on to the abdomen of the experimentally manipulated drone. In collecting semen for instrumental insemination, it is necessary to prevent this. Mature drones were therefore caught on the flight board and chilled in a refrigerator or in cold water for a while, to reduce the level of stimulation. Only those drones were used which partially everted the endophallus when the thorax was squeezed. Next, by slight pressure on the abdomen, a stage of eversion was attained in which a drop of semen appeared on the end of the endophallus. The semen was completely collected in a calibrated syringe tip for instrumental insemination. Samples were used for further investigation only when it appeared that all the semen had been collected from the endophallus.

Altogether 27·5 mm^3 of semen was collected, from 137 drones. Volumes of the 15 samples measured, each taken from 4–21 drones, were 1–4 mm^3. The average volume of semen produced per drone in these samples ranged from 0·18 to 0·25 mm^3. The overall mean was 0·20 $\pm$ 0·006 mm^3 and the standard deviation 0·023 mm^3.

Concentration of spermatoza in the semen

The concentration of spermatozoa was investigated in semen collected into the tip of the syringe for instrumental insemination. A great many of the spermatozoa remain inside the tip after injection, attached to the walls. The following procedure was adopted to retrieve these spermatozoa. First 1 mm^3 of physiological solution was taken into the tip of the syringe alternately with a bubble of air. This was repeated 5 times, giving 5 columns of solution (each 1 mm^3) separated by 5 bubbles of air. Next the semen was taken from drones with a partially everted endophallus; it was collected from the droplet appearing on the end of the endophallus after slight squeezing of the abdomen, and 1 μl of semen was collected each time. Then the semen was injected into a drop of physiological solution in an embryological dish. The 5 columns of solution in the tip rinsed the spermatozoa from the walls of the syringe tip. More physiological solution was added to the drop, up to 1 ml. The semen was thoroughly mixed with the solution, and then tap water (up to 10 cm^3) was added and mixed. The spermatozoa were counted in 10 large squares of a Fuchs-Rosenthal counting chamber, i.e. in 2 mm^3 of solution.

Altogether five samples of semen (each 1 mm^3) from *A. cerana indica* drones, and 3 samples from *A. mellifera carnica* drones, were investigated. From optical examination, semen from *A. cerana indica* drones appeared to be more dense than that from *A. mellifera* drones. In the 5 samples of semen collected from Indian drones, the concentration ranged from 3·475 million to 5·625 million spermatozoa in 1 mm^3 of semen. The mean was 4·655$\pm$0·397 million per mm^3 of semen. In the samples from *A. mellifera* drones, the concentration ranged from 6·970 million to 7·470 million, and the mean was 7·220 million, spermatozoa per mm^3 semen. This concentration is similar to those given by Woyke (1960) and Mackensen (1964): 7·478 $\pm$ 0·127 million and 7·64 million respectively.

Thus the concentration of spermatozoa in semen of the *A. cerana indica* drones investigated was about 65% of that for *A. mellifera* drones. This difference is probably due to incomplete mixing of the fluid from the bulb of the endophallus with the sperm from the vesiculae seminales.

Number of spermatozoa produced by one drone

The average number was calculated from the average volume of semen ejaculated by one drone (0·20 mm^3), the the concentration of spermatozoa in the semen (4·655 million per mm^3). One drone ejaculated on average about 0·930 million spermatozoa, roughly 1 million.

According to previous investigations, (Tryasko, 1956; Woyke, 1960), the vesiculae seminales of one *A. mellifera* drone contain on average about 11 million spermatozoa. It is, however, not possible to get all these into the syringe of the insemination apparatus by aspiration from the ejaculated fluid. Woyke (1960) obtained 9·3 million in 1·25 mm^3 of semen by this method. Thus the number of 1 million for *A. cerana* must be lower than the true number produced. The average number of spermatozoa in the vesiculae seminales of *A. cerana cerana* was calculated from direct counts to be 1·5 million. The difference between results from the two methods is thus similar

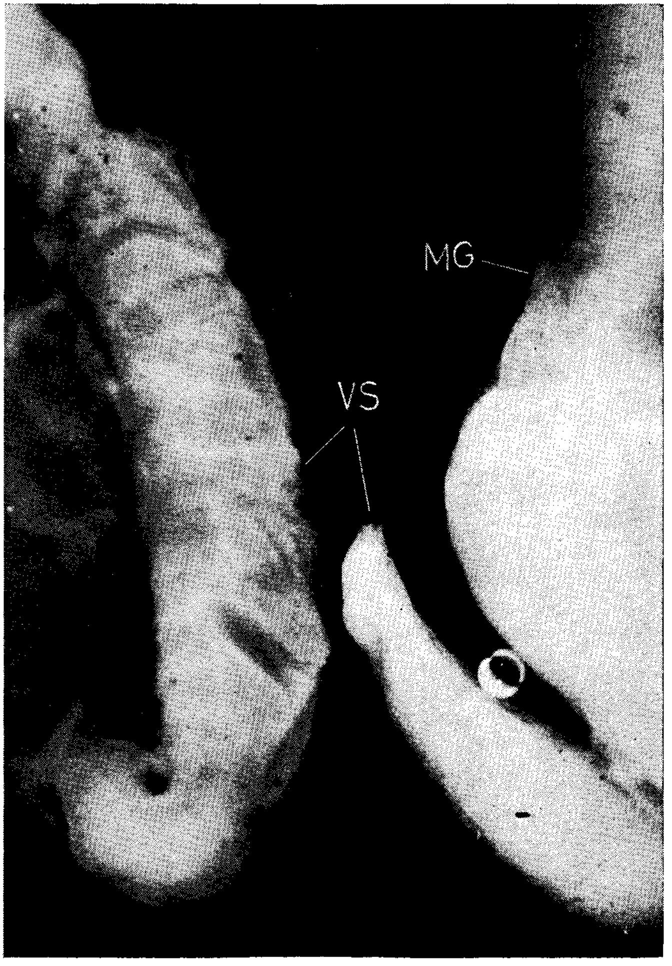

FIG. 12. Seminal vesicle (VS) of *Apis mellifera* (left) and *A. cerana* (right).
MG : mucus gland

in *A. cerana* and *A. mellifera,* and according to both methods, drones of *A. cerana* had only 10–14% as many spermatozoa as *A. mellifera.*

According to the difference in absolute number and in concentration of spermatozoa, the ratio between the volumes of the vesicula seminalis of *A. cerana indica* and *A. mellifera* is 1 : 2·7 (Fig. 12).

Female genitalia

Queens of *A. cerana* are smaller than those of most races of *A. mellifera,* although they are large compared with the workers of their own colonies. The difference between the lengths of the third abdominal sternite of *A. mellifera carnica* and *A. cerana indica* queens was found to be 0·5 mm; between the anterior widths, 1·12 mm (Fig. 13).

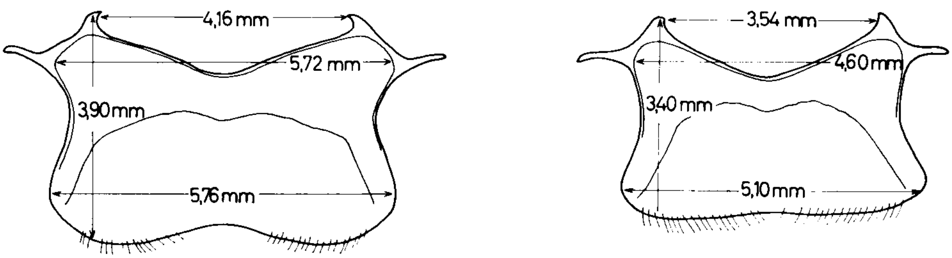

FIG. 13. Third sternal sclerite of queen : *A. mellifera* (left), *A. cerana* (right).

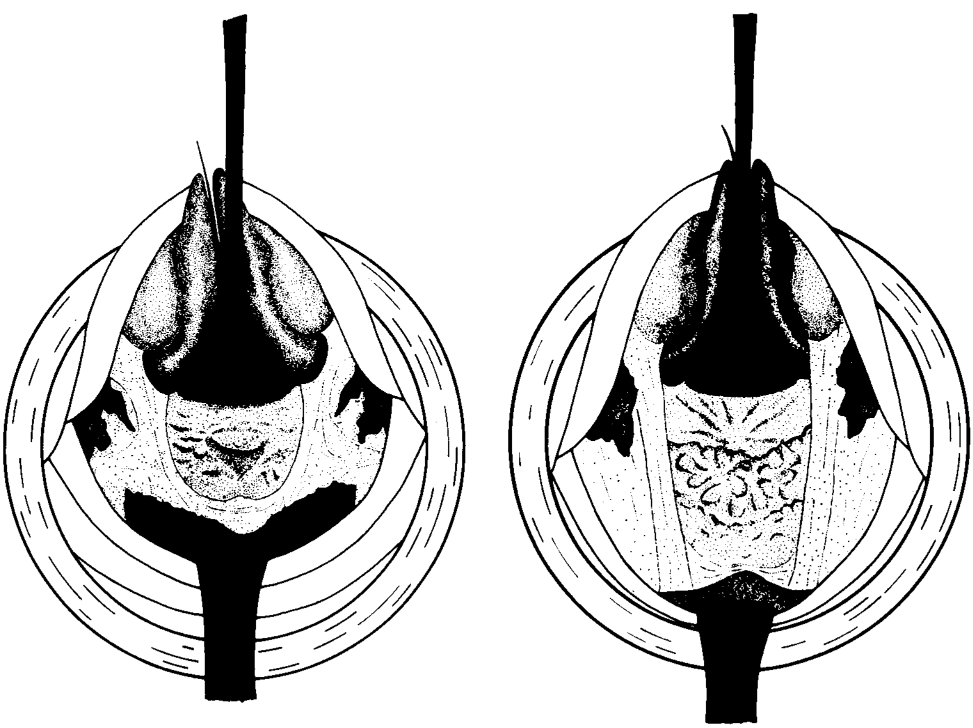

FIG. 14. Sting chamber of queen (opened by hooks for insemination) : *A. cerana* (left), *A. mellifera* (right).

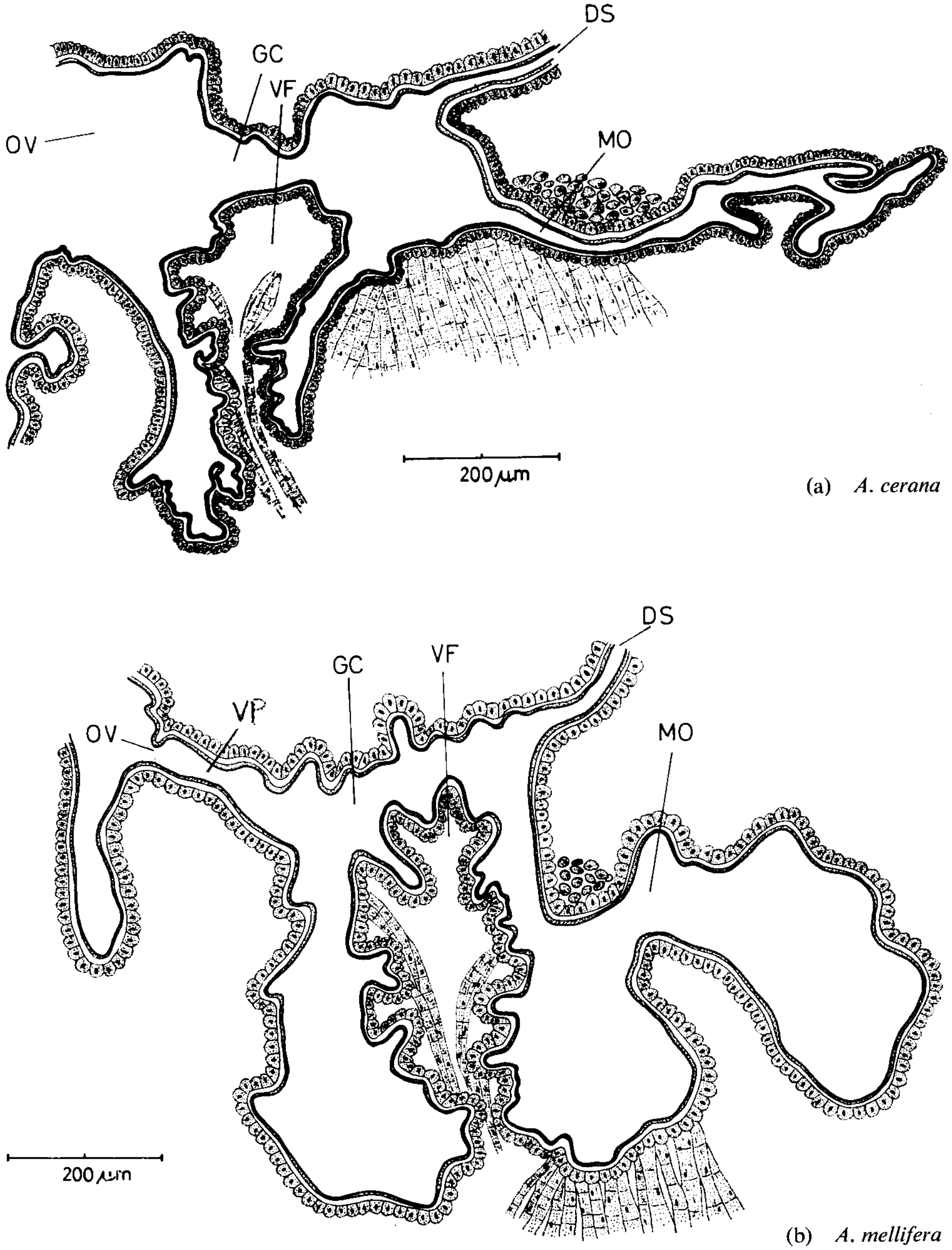

FIG. 15. Vagina and oviduct, median sagittal section.

DS : spermathecal duct
GC : genital chamber
MO : median oviduct
OV : vaginal orifice
VF : valvefold
VP : vaginal passage

When using the Mackensen apparatus for instrumental insemination of *A. cerana,* the difference in size is obvious to the operator, and sometimes it is not easy to insert the hooks. Fig. 14 shows the differences in shape and size of the opened sting chamber for the two species. In the *A. cerana* queens reported on here (as in all races of this species examined), the sclerotized posterior portion of the last intersegmental membrane is rough and black, not thin and brown as in *A. mellifera.* The boundary of the bursa copulatrix is oval, not triangular. The vaginal orifice is closed by a broad pleated valve. The difference in size is evident.

In *A. mellifera,* small queens are more difficult to inseminate instrumentally than larger ones. In *A. cerana,* however, although the instruments (hooks and syringe) look very large in relation to the size of the organs, it is very easy to inject the sperm. Kapil (1962) stated that the female reproductive organs of *A. cerana* are very similar to those of *A. mellifera,* but the new experience gained from instrumental insemination of *A. cerana* queens indicates that differences must exist, at any rate in the genital ducts. Median sagittal sections of queens of both species were therefore examined. As Fig. 15 shows, the vaginal passage, which is narrow in *A. mellifera,* is almost absent in *A. cerana.* In this species the broad orifice of the vagina is closed only by the sting apparatus (removed in Fig. 15*a*) and the free fold in the base, between the bursa copulatrix and genital chamber. No obstruction is therefore encountered to introduction of the syringe into the median oviduct beyond the valvefold.

In *A. mellifera,* the anterior part of the median oviduct has a T-shaped cross-section: a flat horizontal fissure with a "shelf" on each side and a vertical "keel" (Laidlaw, 1944). In *A. cerana* the "keel" is absent; the median oviduct is a horizontal fissure, 14×130 µm in diameter, with a wide funnel on the posterior end and a vertically flattened blind anterior end (transversal diameter 280 µm). The openings to the lateral oviducts are posterior to the end (see Kapil, 1962).

Natural insemination of a *cerana* queen

The "mating sign"

In *A. cerana* the bulb of the endophallus of the drone has no chitinized plates, which in *A. mellifera* are essential parts of the "mating sign" observable on the queen after copulation. Attention was therefore paid to the existence and composition of the mating sign in *A. cerana.*

As reported in Part 1 of this paper (1972), 11 mating signs were observed on queens returning from a flight. All of these queens which could be observed afterwards produced fertilized eggs. On the other hand, two queens which made several flights without showing a mating sign never produced worker brood. So it seems likely that in *A. cerana,* as in *A. mellifera,* the production of a mating sign is a normal process of copulation.

The mating sign of *A. cerana* looked very similar to that of *A. mellifera.* But it was somewhat elongated, protruding from the queen's sting chamber, and the orange surface was more conspicuous (Fig. 16). On closer examination the mating sign consisted of a plug of mucus in the sting chamber (Fig. 17). Orange membranes and an orange sticky mass were attached to the sign; they originated from the orange sticky layer covering the cornua and sometimes also the upper basal part of the endophallus.

One mating sign was found to contain the sticky layers from 22 cornua, i.e. from at least 11 drones. We examined too few to say whether every mating sign includes membranes from the endophallus of many drones.

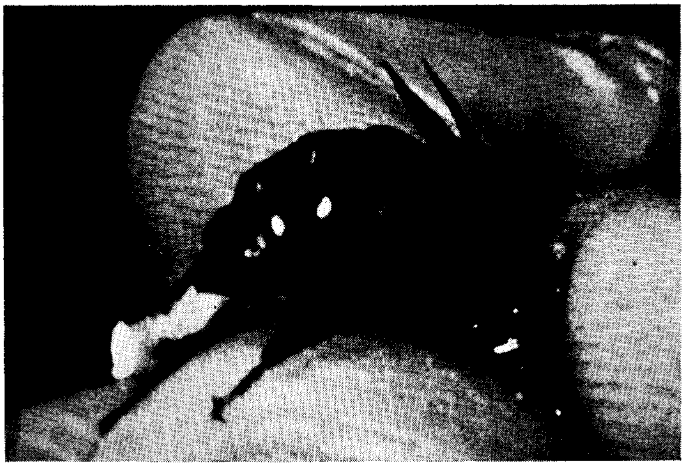

FIG. 16. *Apis cerana* queen with mating sign. *Photograph* : U. Eidam.

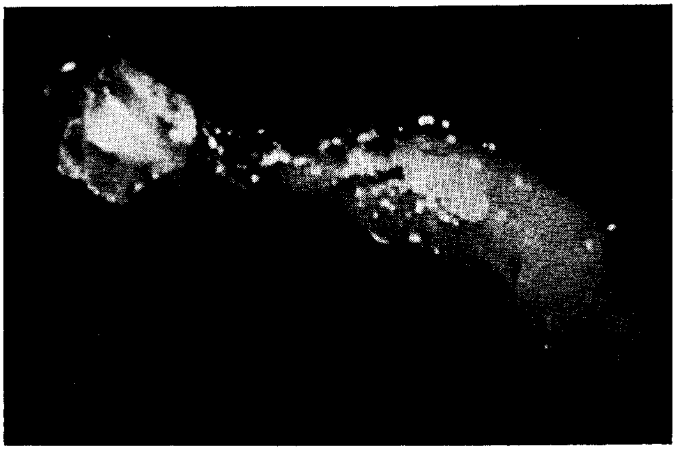

FIG. 17. Mating sign of *Apis cerana* queen. *Photograph* : U. Eidam.
The shining darker mass is the orange layer on the surface.

Volume of semen in the oviducts of a newly mated queen

A queen mated on a previous flight was killed immediately after returning from a second successful mating flight. After opening the abdomen with scissors, the queen was preserved in Weaver's solution (5·0 cm^3 formaldehyde 40 %, 2·5 cm^3 concentrated acetic acid, 20·0 g chloral hydrate, distilled water to make 100 cm^3). The diameter of each oviduct filled with the semen was measured at three places, under a stereoscopic microscope. From the results it was calculated that one oviduct contained 1·83 mm^3 of semen and the other 0·96 mm^3. In one mating flight the queen had thus received 2·79 mm^3 of semen. Since a single drone produces about 0·20 mm^3, the queen probably mated with around 14 drones. The spermatheca was already filled with spermatozoa from the previous mating flight. The number could not be counted in this queen, which had been in a preservative solution. Several other queens also returned twice with a mating sign, and it seems reasonable to assume that some queens of *A. cerana indica* mate with at least as many as 30 drones.

Number of spermatozoa in the spermatheca of a naturally mated queen

A queen mated in Pakistan, and brought to Germany, laid eggs for some months. She was then killed, dissected in physiological solution. The spermatheca was dipped in 1 cm^3 of physiological solution; after removal of the outer tracheal layer, the spermatheca was crushed. The spermatozoa were mixed thoroughly, and 4 cm^3 of tap water added to give a solution suitable for counting. The number of spermatozoa in the spermatheca of this queen was estimated as 2·665 million.

Later two other queens mated in the Graswang valley (Germany) were dissected and the spermatozoa counted. Queen V2, mated on her only flight which lasted 35 minutes, had 270 000 spermatozoa, and queen 135, whose flights were not recorded, had 610 000 spermatozoa. Thus both queens mated during our experiments in Germany had essentially fewer spermatozoa than the one queen mated in Pakistan. This may well have been due to "incomplete mating", a phenomenon well known in *A. mellifera* under similar conditions, i.e. when the weather is unfavourable during the mating period.

Discussion

Only a few observations are so far known on the mating of *A. cerana indica.* Sharma (1960) reported on 9 queens, 2 of which were seen twice with a mating sign. Adlakha (1971) on the contrary stated that "after mating no further flight of *A. cerana* queens has been recorded".

Our observations suggest that repeated matings may occur in *A. cerana indica* about as frequently as in *A. mellifera.* The one important difference is the conclusion from our evidence that more matings occur during one flight than in *A. mellifera.* The flights were longer, and the quantity of semen collected in one flight by a queen corresponded to a higher number of drones.

The number of spermatozoa in the spermatheca of the queen naturally inseminated in Pakistan was about 2·7 million. This is about half the number in an *A. mellifera* queen, but still within the range of variation for the species. The two queens mated in the Graswang valley had very low numbers of spermatozoa, but this is considered likely to be abnormal, and due to bad weather conditions. We may assume that *A. cerana indica* queens have fewer spermatozoa stored in the spermatheca than *A. mellifera* queens, but not less than half as many. Further data are clearly needed on naturally inseminated queens of *A. cerana indica* and, of course, on all the other subspecies of *A. cerana.*

Instrumental insemination with known quantities of semen (data not yet published), showed that the transfer of semen from the oviducts to the spermatheca is not more efficient in *A. cerana indica* than in *A. mellifera*; the contrary is more likely. One may say that in *A. cerana indica* the low number of spermatozoa per drone is counterbalanced only by a higher number of matings per queen.

Two trends may exist in the evolution of social bees, both favoured by selection:

1. Augmentation of the number of matings, to ensure a long period of egg laying and to compensate for the loss of genetic variability originating from the reduction in the proportion of females that are reproductives.
2. Diminution of the number of matings required (by a higher number of sperma-

tozoa per drone, and/or by improving the transfer of semen to the spermatheca), to reduce the risks encountered during mating flights.

These tendencies will tend to counteract each other until an equilibrium is reached.

The degree of relationship of full siblings in a diploid animal is 50%. In honeybees, the degree of relationship of daughters of a queen inseminated with the sperm of one drone only is 75%. In natural mating, if we assume matings with 8 drones, the degree of relationship of the daughters decreases to 53% (Pirchner, Ruttner & Ruttner, 1960). Genetically, further increase in the number of matings per queen has only a small effect (e.g. with 20 drones, 51% consanguinuity). Thus the only remaining factor for maintaining a number of matings higher than 6–8 is the number of spermatozoa stored in the queen's spermatheca. A tendency seems to exist towards an increase in the number of spermatozoa produced by one drone, from solitary bees to social. It would be of interest to know the number of spermatozoa per drone of the less specialized *Apis* species, *florea* and *dorsata*.

Acknowledgements

Dr. M. Rashid Khan, Agricultural Research Station, Tarnab, Peshawar, Pakistan, most kindly helped one of us (N. Koeniger) in many ways, both during his stay in Pakistan and in preparing the transport of colonies of *A. cerana* to Germany. Other institutions or persons have also been most helpful in sending colonies:

Institute of Apiculture, Chinese Academy of Agricultural Science, Peking, People's Republic of China. Prof. R. P. Kapil, Punjab Agricultural University, Hissar, India. Central Bee Research and Training Institute, Poona, India. Dr. O. Klockenhoff and Dr. B. Nogge, Department of Zoology, University of Kabul, Afghanistan.

Dr. F. K. Böttcher, Erlangen, Germany, kindly gave us the permission to use the mating station of the Bayerische Landesanstalt für Bienenzucht near Graswang. U. Eidam and B. Gessner provided valuable help during the experiments on mating flights, the latter also by preparing the microscope sections.

The work described in both Parts of this paper was done with the aid of a grant of the Deutsche Forschungsgemeinschaft. One of us (J. Woyke) was supported by a grant from the Deutscher Akademischer Austauschdienst.

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