Dr. R.F.A. MORITZ

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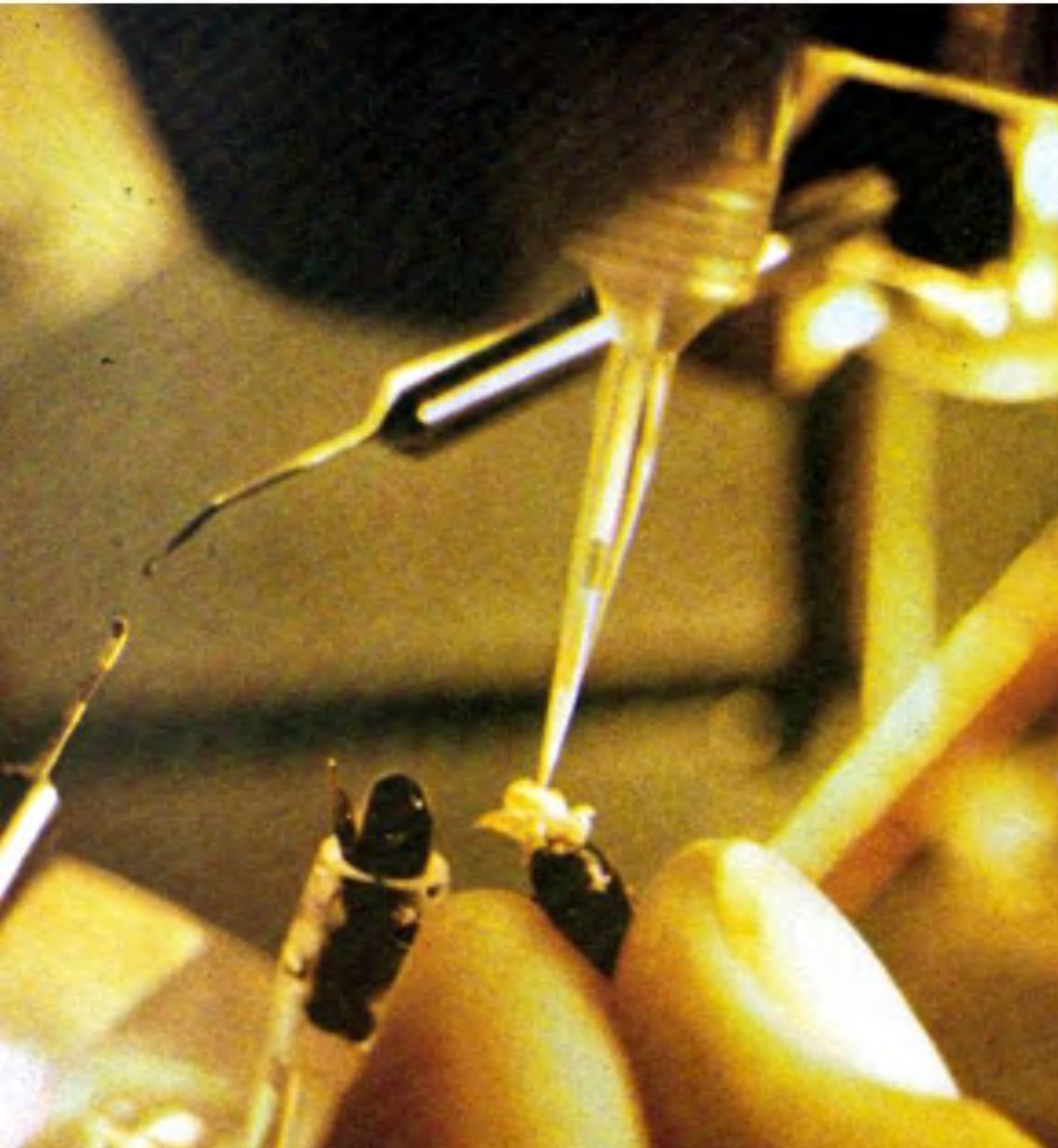
The Instrumental Insemination of the Queen Bee



APIMONDIA
THE INTERNATIONAL BEEKEEPING TECHNOLOGY
AND ECONOMY INSTITUTE

THE INSTRUMENTAL INSEMINATION OF THE QUEEN BEE

The book was edited under
the guidance of Dr. R.F.A. MORITZ

1989

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PREFACE

Instrumental insemination of the queen bee has become an integrative part of beekeeping in the last few decades. It has application in both beekeeping practice and the research activity. Further advance of the technique is being promoted by many bee breeders and the scientists. Today, many apparatus for instrumental insemination have become available, meeting both the requirements of the experienced operator and of the beginner, who only inseminates a few queen bees annually. In addition to the improvement of the apparatus for instrumental insemination, new methods of obtaining and storing semen have been developed in the last few years. New approaches are already operational in storage of semen-a major problem of planned beekeeping. New strategies of selection have been developed, seeking for ever higher efficiency and based on modern methods of insemination, which have opened new, most promising vistas in apiculture. In order to maintain continuous record of these fast developments and to make them widely known another fully revised edition of "The Instrumental Insemination of the Queen Bee" was required. This new edition, designed as a handbook for both the beginner and skilled operator, is thought as a reference source and guide on the benchtop in the insemination laboratory. It will help the operator to trouble shoot mistakes in his insemination method and will, at the same time, be of help in planning and conducting the selection projects.

I do hope this book will be a guide to instrumental insemination and beebreeding, and that it will stimulate further advance in this domain.

Robin F. A., MORITZ,
PD, Dr. Erlangen,
November 1987

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ORIGIN AND DEVELOPMENT OF INSTRUMENTAL INSEMINATION OF QUEEN BEES

Harry H. LAIDLAW, Jr.

Anton JANSHA, appointed in 1770 as Royal Teacher of Bee Culture to Empress Maria Theresia of Austria, was probably the first to observe and record that virgin queen bees fly from their hives when young and return bearing a part of the copulatory organ of the drone. He observed further that they will fly again only if they do not return with the "mating sign" (ALFONSUS, 1931). Jansha made other astute observations but apparently displayed no curiosity about the mating fligh itself.

First attempt to artificially inseminate a queen bee

A Swiss beekeeper, François HUBER, also in the late 1700's, made the same observation as JANSHA and surmized that queens mate outside their hive rather than within the hive (HUBER 1814). To test this, Huber confined young virgin queens along with drones to their hives by means of a glass tube in their hive entrance that permitted workers to pass through but not drones or queens. Such restricted queens did not lay within several weeks following confinement. This excited his curiosity about how and where queens mate. His friend, the biologist Charles BONNET, in whom HUBER confided, proposed that HUBER place a hive with a virgin queen and drones in a tall cage so the mating of the queen could be observed. HUBER did this but no matings took place.

BONNET then suggested that HUBER try to artificially inseminate virgin queens by introducing semen into the vagina with a hair pencil. HUBER reported unsatisfactory results; and he was no more successful in observing queens' matings with clipping part of the queens' wings to prevent them from flying so rapidly or so great a distance, or by covering part of the queens' eyes with opaque varnish to render their sight less acute. These experiments leave little doubt that HUBER was the first to attempt to artificially inseminate a queen bee, and to try to observe natural mating.

Other early efforts to inseminate queens artificially and control mating

After HUBER, interest in artificial insemination of queens was practically nonexistant until WANKLER (1927 — 1957), a German beekeeper and clock maker, demonstrated in 1883 the introduction of semen into a queen by means of an "artificial penis" he had invented and which he constructed of silver. This device was a type of syringe and was, with little doubt, the first to be used in artificial insemination of queen bees. But it was not established that WANKLER's insemination efforts were successful.

WANKLER may have had a dual interest in artificial insemination: to determine if it could be done, and to control mating for practical reasons. Since then, interest in artificial insemination has resided almost exclusively in searching for a method to control mating for genetic and breeding purposes.

Shortly after Wankler's insemination attempts, Nelson McLAIN (1886 a, b, 1887) apiculturist of the United States Department of Agriculture, began a series of controlled mating experiments, including artificial insemination. At first, semen from an ejaculated drone was dropped upon the "open vulva" of a virgin queen as she was held back downward in the hand. Later, the queen was held in a wooden "queen clamp" and semen was collected and injected into the queen with a modified hypodermic syringe. None of these attempts could be considered truly successful.

The meager success of his artificial insemination experiments prompted McLAIN (1888) to investigate mating queens in enclosures. Six queens in the scores of trials may have mated. McLAIN's disappointing results and the appearance and development of commercial queen rearing in the United States caused the then feeble attention to artificial insemination of queen bees to disappear.

The honey bee continued to interest insect morphologists, however, and early in the 20th century two contributions were made to our knowledge of honey bee reproductive morphology that are important to artificial insemination. The first of these was the description by E. BRESSLAU in 1905 of the flaplike invagination in the vagina of the queens which he called the "Ventilwulst" or valvefold. The second

was the thorough description and illustration of the morphology of the honey bee by R. E. SNODGRASS in 1910.

The first of the 20th century efforts to artificially inseminate queen bees were made by Francis JAGER and C. W. HOWARD in 1914. They used a modified hypodermic syringe, and they failed, as did the experimenters that followed them until 1927 when Lloyd R. WATSON (1927 a, b) demonstrated the first repeatably successful method of artificial insemination of queen bees.

Beginning of success and the use of modern laboratory instruments

L. R. WATSON's demonstration proved that artificial insemination of queen bees is possible and it elevated the procedure to a skilled laboratory technique. It was, however, only the beginning of development; the attainment of highly successful routine inseminations was accomplished by others after his demonstration. Nevertheless, to WATSON belongs the honor of proving to a skeptical apicultural world that in spite of a long history of failures artificial insemination of queen bees is a fact and can be accomplished in the laboratory.

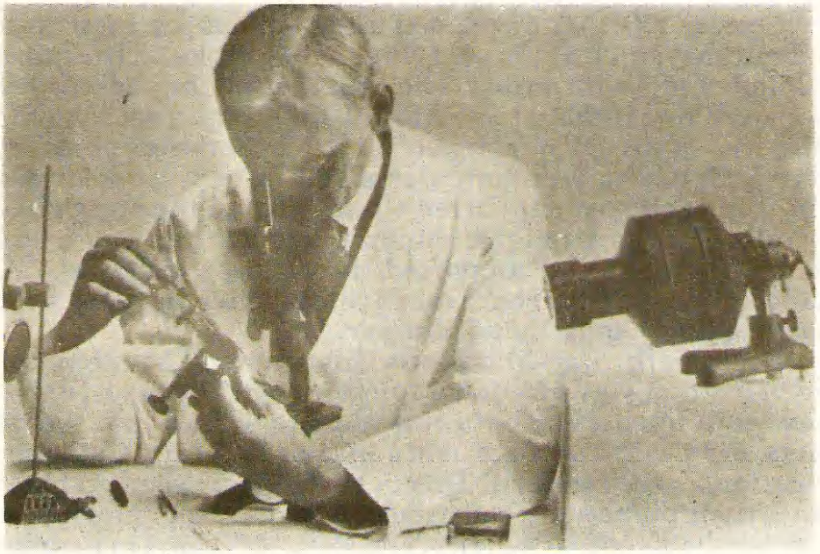


Fig. 1 — Watson instrumentally inseminating a queen bee.
From: Watson, 1927. Controlled Mating of Queenbees.

WATSON first tried methods of artificial insemination that had been claimed to be successful, and failing, he turned to the use of a capillary syringe of his own design and construction for collecting and injecting the semen, and of a binocular stereoscopic microscope to which he attached a Barber pipette manipulator to provide fine control of syringe movement (Fig. 1). Light from a microscope lamp was deflected onto the queen by an adjustable mirror. A cradle for the queen and fine forceps completed the equipment he used. WATSON's instruments were enormous improvements over prior equipment, and he chose the term "instrumental insemination" as being more descriptive of his method and more appropriate for honey bees than "artificial insemination".

WATSON (1928) made other contributions that have remained elements of instrumental insemination. Among these were repeated injections of semen and the recognition that queens should be kept warm following semen injection.

Genesis of the basic design of most modern instruments

W. J. NOLAN (1929, 1932 a, b, 1937 a, b) learned instrumental insemination from Watson and was the first to use WATSON's instruments and procedures. Over a period of several years NOLAN developed an insemination instrument and microsyringe of his own (Fig. 2). He replaced the queen-holding cradle with a glass tube, and devised the back-up tube now used to get the queen into the tube. The queen-holding tube was eventually fastened on a bar between posts that were attached to a rectangular metal false stage near each end, and each post supported a hook to pull the sting chamber plates away from the midline, but neither was a sting hook. One post also supported the syringe which NOLAN made inexpensively from a mechanical pencil he fitted with a glass capillary and a metal plunger.

Because the flap-like ventral invagination of the vagina, the valvefold, was unknown to either WATSON or NOLAN they were unsuccessful in introducing semen into the oviducts, even with a syringe, and consequently only slight inseminations were attained. This is particularly regrettable because their pioneering contributions to instrumental insemination established its validity, and NOLAN's final instrument was the first instrument designed especially for instrumental

insemination of queen bees. It was the original and seminal model for most of the current insemination instruments.

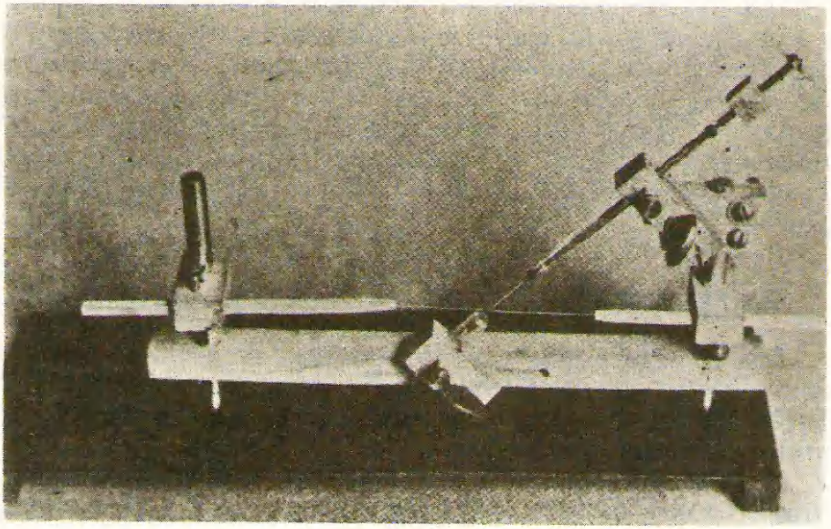


Fig. 2 — Nolan's final queen bee insemination instrument.
From: Nolan, 1932. Breeding the honeybee under controlled conditions. U.S.D.A. Technical Bulletin, Nr. 326

The valvefold and stinghook

Coincident with NOLAN's experiments, H. H. LAIDLAW (1931) with the aid of a binocular stereomiscroscope and lamp was endeavoring to inseminate queens by direct eversion, with ejaculation, of the coital structure of drones into the reproductive canal of virgin queens, and also by modification of direct eversion. He fastened the queen in NOLAN's queen-holding tube, anesthetized the queen with carbon dioxide and later tried other chemicals, and to expose the vaginal orifice pushed the base of the sting dorsally by means of a semicircular wire with a loop bent downward at the middle of the wire to fit against the base of the sting. This was the prototype of the sting hook he devised later. The same wire held the sting chamber open.

Persistent partial successes led LAIDLAW in 1932 to search for the cause of failure to get normal inseminations and he made dissections of queens just returned from their mating flight. The oviducts of all were filled with semen, as

had been recorded earlier by BISHOP (1920). Dissections of queens that had been subjected to insemination attempts (LAIDLAW, 1933 a, 1933 b) revealed no semen in the oviducts, and the valvefold was erect and completely obstructed the median oviduct. In 1934 LAIDLAW pushed the valvefold ventrally with a probe and successfully deposited semen in the oviducts of the queens treated. It was evident that the key to successful artificial insemination is placing the semen beyond the valvefold into the oviducts. All else is "fine tunning".

MACKENSEN's model for most insemination instruments

The insemination instrument that is most familiar to practitioners of instrumental insemination is MACKENSEN's and it is his instrument that is the classical model for similar ones that have appeared.

Otto MACKENSEN learned instrumental insemination from NOLAN in 1936. NOLAN's instrument was basically well designed, Fig. 2, but difficult to use because its construction was quite crude. MACKENSEN built his own instrument in 1936 as a refinement of NOLAN's, and it has retained the same design, including the "slip" supports for the sting chamber opening hook handles and the syringe, except for the 1939 modification of the dorsal opening hook into a sting hook (MACKENSEN, 1939; MACKENSEN and ROBERTS, 1948) and the metal replacement of wooden parts (MACKENSEN and TUCKER 1970). A valvefold probe was also included with the instrument in 1939. In 1944 MACKENSEN (1945) began using carbon dioxide as an anesthetic that was conveyed to the queen-holding tube by rubber tubing, and in 1945 he discovered that carbon dioxide caused instrumentally inseminated queens to begin oviposition nearly as soon as naturally mated ones (MACKENSEN, 1947).

LAIDLAW micromanipulator insemination instrument

Concurrently with MACKENSEN's development of his instrument LAIDLAW (1936, 1937, 1939, 1944) made the prototype of his current instrument in 1936 and 1937. The queen was held between "leaves" of a clamp on a movable base and

the sting chamber was opened by a ventral hook similar to Nolan's and a sting hook that fitted between the bases of the sting lancets. The leaves of the queen holder and the movements of the opening hooks were controlled by screw mechanisms. The syringe was held in a "slip" holder similar to MACKENSEN's that was supported on an adjustable rod fastened to the stage of the microscope.

The final design was made in 1948; and a modification of the anesthetization chamber in 1949 is the only change (LAIDLAW, 1949, 1953). This instrument differs from MACKENSEN's in that the movements of the opening hooks are controlled by racks and pinions, and the queen is adjusted for insertion of the syringe by moving the queen manipulator with the queen over the microscope stage, rather than by moving the syringe. The syringe movement is also controlled by the rack and pinion mechanism of a syringe micromanipulator that is borne by a rod attached to a plate on which the microscope rests and the syringe is independent of the queen manipulator.

Syringes

MACKENSEN used NOLAN's syringe until 1948 when he designed and made a syringe without a tight-fitting plunger and with a tapered tip. The metal syringe barrel had a threaded rod that could push a rubber diaphragm into a cone-shaped cavity in the basal end of a plastic removable insemination tip. The tip was filled with fluid that served as a plunger.

The first removable and plastic tip originated with W. C. ROBERTS (1947) who also invented a plastic syringe mechanism. A plunger rod extended into the tip and caused wear. MACKENSEN's syringe avoided wear in the tip because the plunger rod did not extend into it, and fluid served as a plunger.

LAIDLAW designed and LORENZEN made in 1957 an adapter for MACKENSEN's syringe so that glass tips could be used, and this was enlarged and slightly modified by RUTTNER (1975). MACKENSEN's syringe and the adapter make a versatile and efficient combination.

John HARBO (1979, 1985) made a simple and efficient syringe from a glass capillary tube, a drawn capillary point, fine

tubing, and a Gilmont micrometer syringe. The micrometer can accurately measure the amount of semen taken into the capillary and injected into the queen.

A glass capillary with attached point can be connected with fine tubing directly to a Gilmont micrometer syringe to make a satisfactory syringe for use with instruments provided with a syringe micromanipulator.

RUTTNER, SCHNEIDER, FRESNAYE instrument

The evolution of queen bee instrumental insemination instruments reached a probable economic limit with the addition of the instrument of RUTTNER, SCHNEIDER and FRESNAYE (1974) to those of MACKENSEN and LAIDLAW. The three are about equal in efficiency and ease of operation. The RUTTNER, SCHNEIDER, FRESNAYE instrument is modeled after MACKENSEN's and has a manipulator for the syringe similar to LAIDLAW's but with an added tilting movement. The syringe manipulator was first added to a Mackensen-type instrument by V. V. TRYASKO (1958). A special apparatus designed by RUTTNER (1964) to receive the queen-holding tube and to direct the CO^2 is an improvement of the Mackensen-type instrument.

Semen must be placed beyond the valvefold into oviducts. This can be done with any of the queen manipulators or the syringes. The valvefold can be pushed aside with the syringe tip (VESELY 1965) or with a probe (LAIDLAW, 1934). LAIDLAW (1977) by properly pulling the sting base dorsally with the sting hook inserted the syringe directly past the valvefold into the vagina.

Sanitation

In 1951, 1952 and 1953 MACKENSEN and LAIDLAW, suffered severe losses of instrumentally inseminated queens. The cause was diagnosed as bacterial contamination of the semen as it was collected in the syringe. RUTTNER experienced the same problem. When strict sanitation was adhered to and an antibiotic was added to the plunger fluid of the syringe the problem subsided (LAIDLAW, 1956; RUTTNER, (1976). Strict sanitation is essential for successful instrumental insemination.

Multidrone matings

Until 1944 when W. C. ROBERTS showed that it is common for queens to make more than one mating flight, and V. V. TRYASKO in 1951 proved by measurement of semen in the oviducts of queens returning from their mating flight that the queens had mated with more than one drone, it was believed that queens mate with only one drone. The discoveries of ROBERTS and TRYASKO explained partially at least why it is necessary to inseminate queens with semen from several drones to obtain normal or near normal inseminations.

Sex determination

The "spottiness" that characterized the brood of so many inseminated queens before 1952 was considered a reflection of deficiencies in the insemination technique. MACKENSEN's 1951 report on viability and sex determination in the honey bee established that spottiness is not a defect in insemination technique but is a consequence of the peculiarity of sex determination in bees. The multiple mating and sex determination contributions are of such fundamental importance to instrumental insemination as well as to bee genetics and bee breeding that they should be considered part of instrumental insemination methodology.

MATING BEHAVIOUR AND ANATOMY OF THE REPRODUCTIVE ORGANS

G. KOENIGER
F. RUTTNER

In many insects there is a certain interval of time between the mating of males and females and the fertilization and laying of eggs. Often, the optimum conditions to seek for a mate are altogether different from the optimum conditions required for egglaying and rearing of the offspring. Females have a special organ, the spermatheca, where semen is stored until it will be necessary for fertilization of eggs.

In honey bees too, mating and egg laying take place under completely different conditions. Egg laying takes place only in the hive, where the queen and eggs are protected and constant conditions are available for brood rearing, irrespective of the weather and foraging conditions at one time or another. But, in order to mate, the queen flies to the drone congregation areas. On warm and sunny afternoons (approximately between 12.00 and 17.00 hrs, at temperatures above 20°C) several thousand drones from different apiaries gather in such places waiting for the queens which come to mate (JEAN-PROST, 1957; RUTTNER and RUTTNER 1963; ZMARLICKI and MORSE, 1963). They usually fly at 10-25 m above the ground. The sizes of such congregation places vary between 30 m diameter and over 200 m diameter. In general, the flight range of drones is on average 3—6 km, and over 10 km at the most. Queens will usually fly 1—3 km (PEER, 1957; RUTTNER and RUTTNER, 1972).

Because of this behavioural pattern, the possibility of the queen mating with her brothers is substantially reduced. In addition, the queen can mate with many drones from different apiaries in quite a short period of time, which provides for a profitable combination of hereditary characteristics. And, for the same reason, the beekeeper has no control over the queen and cannot determine with which drone she mate.

Not only selection of queens is a prerequisite for selection programmes, but also the selection of drones. It can be provided for as follows:

1. Queens are left to fly and mate freely in isolated mating stations. When such stations are not on islands but on the

mainland, the area within a 10 km radius around them should be free of any bees (flight ranges are added).

2. By using the instrumental insemination. Instrumental insemination weds the advantage of using drones of different lines, obtained on purpose. In mating stations only one type of drone is available. The disadvantage of the instrumental insemination is that it requires proper equipment, rigurously aseptic conditions and high skill in performing it.

The instrumental insemination technique requires thorough knowledge of the anatomy of the sexual reproductive and of their natural mating behaviour. All attempts of instrumentally inseminating queens have failed before the role played by the valvefold was discovered (LAIDLAW, 1944); the ascertainment of the fact that the queen mates with 10—17 drones was also very important (ADAMS et al., 1977; ROBERTS, 1944; RUTTNER, 1955; TABER, 1954; TRYASKO, 1956; WOYKE, 1955).

We shall first describe the anatomy of the reproductive organs, then the mating behaviour, fertilization and laying of eggs and in conclusion we shall point out the differences between the natural and the instrumental inseminations.

The reproductive organs of the queen

1. The sting chamber and Bursa copulatrix (Fig. 3).

The sting chamber and bursa copulatrix (Bc) lie between the last sternite and tergite. The sting chamber (S) lies in the centre, and above it, in a pocket, is the rectal opening (R); below it — the vaginal opening. When the queens is relaxed, the vaginal opening is covered by the projecting base of the sting and by the sting plate (Fig. 3,7).

A proeminent skin fold short brown chitinous spines on it, leads from the outside of the sting apparatus, over the floor of the sting chamber, to the ventral sclerite. It separates the copulatrix from the sting chamber.

Three structures open into the bursa copulatrix: in the centre the vagina, and two bursal pouches — one on each side (St). All three are covered by skin folds.

The bursal pouches on each side of bursa copulatrix are prominent structure from which muscle fibres extend to the sternite, which probably help in opening the vagina during mating (Fig. 5a).

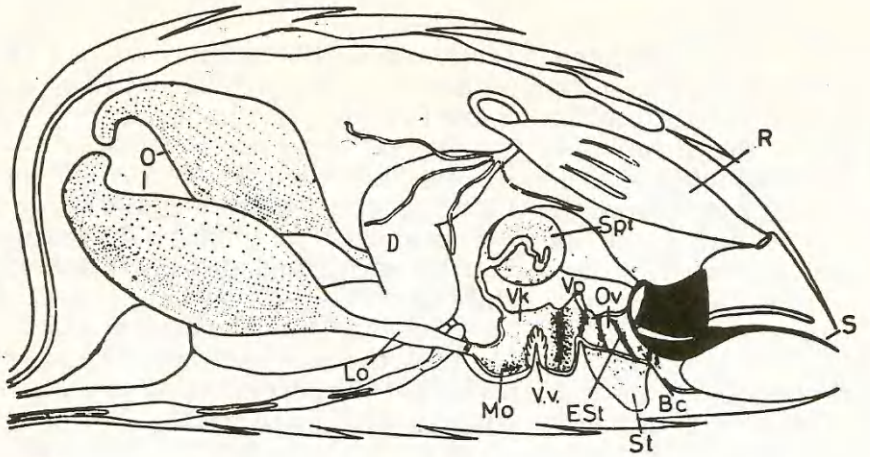


Fig. 3 — Longitudinal section through the abdomen of an unfertilized queen
Bc — Bursa copulatrix (Vaginal vestibulum); ESt — entrance to the lateral copulatory pouch of Bursa copulatrix; D — intestine; Lo — lateral oviducts (ovarian tubule); Mo — median oviduct (ovarian tubule); K — rectal opening; O — ovary; Ov — vaginal orifice; S — sting; St — lateral bursal pouches; Spt — spermatheca; Vk — vaginal chamber; Vp — vaginal passage; Vv — valvula vaginalis

2. The vagina

The stretchable wall of the vagina has many folds and protrusions so that its shape changes depending on the expansion or contraction of the abdomen. The vaginal orifice is a horizontal slit which is closed when the queen is relaxed, and when seen from the outside it looks like a hump.

A short narrow segment (the vaginal passage, VP) leads into a sack-like enlarged section (the vaginal chamber, VK), into which a muscle flap (valvula vaginalis, Vv) extends ventrally, from the sternum. Its end extends to the dorsal part of the vagina, blocking the passage to and from the oviducts. During mating and egglaying the valvefold is folded back by the queen towards the vaginal orifice in order to allow the passage of semen and eggs.

3. The ovary and oviducts

The pair of ovaries (O) consist of approximately 360 ovarioles where eggs will mature after mating. The ovarioles lead into two lateral oviducts (LO) which both end into the median oviduct (MO). The lateral oviducts are highly expandable so that they can hold large amounts of semen during mating (Fig. 6). Unlike them, the median oviduct is a narrow passage with a T-shaped cross section, which is imbedded in strong muscles which also tightly fixes it to the last sternite.

4. The spermatheca

The spermatheca (Spt) lies above the median oviduct. Its shape is spheric, with a 1.2 mm diameter. It consists of a thin and translucid chitinous membrane covered by a one-cell layer. The covering layer consists of a thin network of tracheae which makes it look whitish. Above the network of tracheae lie two glandular ducts (the spermathecal gland) which branch out of the spermathecal duct just before it reaches the spermatheca. In unfertilized queens the spermatheca contains a clear fluid, while in fertilized queens it is full of spermatozoa (Fig. 9). When filled up, the spermatheca holds 5—6 million spermatozoa. It is the tracheae and the spermathecal gland which provide for the preservation of the fertilizing capability of spermatozoa for up to 5 years.

The spermatheca is linked to the oviduct by the spermathecal duct (ductus spermaticus) shaped like an arch. At the bending point it is covered by thick muscle bundles — the Bresslau seminal pump — which help the semen to reach the spermatheca and play a role in the fertilization of eggs as well.

The reproductive organs of the drone

1. The copulatory organ (endophallus):

The endophallus is a long, soft membranous duct which lies inside the abdomen, with 3 distinguishing zones:

a. The vestibulum (V) is close to the genital orifice, with a thick hairy area on the ventral side — the rhomboidal field (RF). On the dorsal side two apendices, with an orange colour coating are tightly folded up, which will subsequently be everted — the horns (cornua) (CO) (Fig. 4 and 8 respectively).

b. In the middle, a more slender section — cervix (C), there also is a hairy area (TF) and one folded appendix impaired, — the fimbriate lobe.

c. The bulb (B) is the innermost section, with a membrane ring (WOYKE and RUTTNER), 1958). Its ventral side is very slender, while on the lateral and dorsal sides lie very thick plates of a specific shape — the chitinuous plates (CD).

2. The testes

The spermatozoa develop inside the oval, cream-coloured testes (T) that lie in the front part of the abdomen, on both sides of the gut. They consist of approximately 200 testioles

— (testicular tubules). The seminal duct (vas deferens) enlarges into the bottle-shaped seminal vesicle whose walls consist of strong muscles.

Spermatozoa start migrating from testes to the seminal vesicle when about 2—3 days old (KURENNOY, 1953; MINDT, 1961). They attach their head to the gland cells of the wall, undergoing a second physiological process during this second stage of maturation. Meantime the gland cells empty their contents into the vesicle among the spermatozoa. When the drone is 12 days old the spermatozoa are mature and may be used for inseminating the queens.

3. The mucous glands

At the rear of the seminal vesicle, the mucous glands (MD) open into the seminal duct. The left and right mucous glands are so tightly bound to one another as they form a U-shaped body. The outer layers of the wall are formed by strong muscles, while the inner layer of the wall consists of

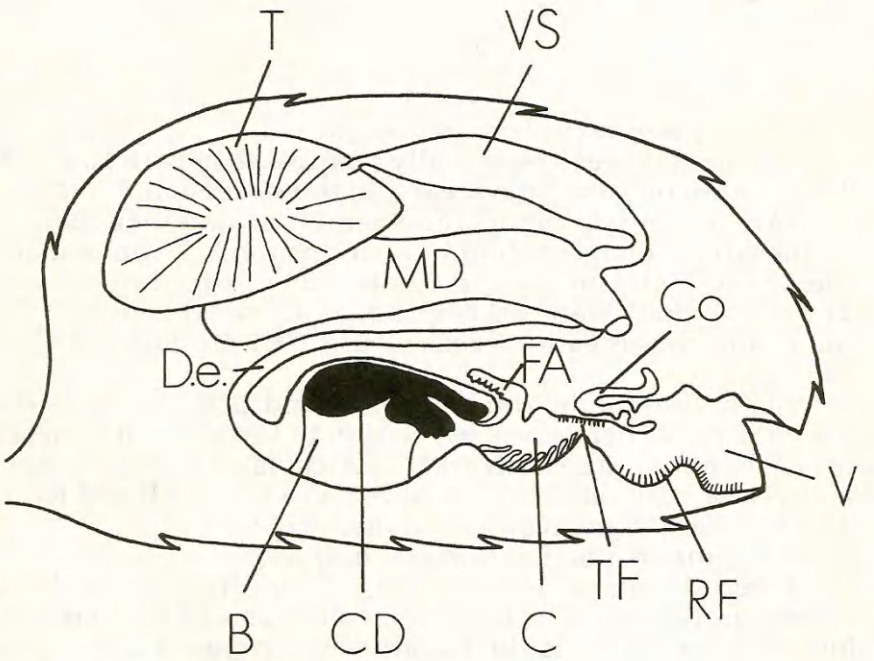


Fig. 4 — Longitudinal section through the abdomen of a drone
B — bulb; C — cervix; CD — dorsal chitinous plates; CO — the horns (cornua); FA — feathery appendage; MD — mucous gland; RF — hairy rhomb field; TF — hairy triangular area; V — vestibulum; VS — vezicula seminalis

secretory epithelial cells. They secrete a white mucous substance which will be one of the component elements of the mating sign. Secretion of mucous starts immediately after emergence of the drone. After the 5th day the mucous glands dissolve but the mucous is still too fluid and it takes a couple of days to ripe.

4. Semen

The semen consists of two components, from different sources:

The first are the spermatozoa from testes. They are 250 m (1/4 mm) long filaments that make snake-like movements when active. The heads incorporating the nucleus are very small.

The second element is the semen fluid from the seminal vesicles.

The colour of the ripe semen is yellowish. Its structure is also specific because spermatozoa are in bundles. The semen is easily distinguishable from snow-white homogenous mucous.

Mating

The flight of sexual reproductives

The queen bees are sexually mature when 5—6 days old. They fly out on sunny days, early in the afternoon. First they perform one or two flights for orientation, and then they fly to the drone congregation area for mating. Drones make their first flight when 6—8 days old, but only for orientation. The are sexually mature when 12 days old and then they fly to the drone congregation areas (DRESCHER, 1969, MINDT, 1962).

In the mountain zones, drones and probably also the queens orient themselves in relation to the horizon in order to reach the congregation area. In Austria, the congregation areas have been the same for 20 years (RUTTNER and RUTTNER, 1966; personal observations, 1987).

Recognition and mounting of the queen

When the queen reaches the congregation place she is immediately spotted by the drones, first as a flying, dark coloured object. This is the reason why drones also fly after butterflies and thrown stones. But in the end she is identified to be a queen by her smell (the queen substance) (GARY, 1962; PAIN and RUTTNER, 1963). She will be followed by

many drones. One of them will grasp her while flying, mount on her and hug her tight with its 6 legs. By means of the sensory cells in the genital area he will find the queen's sting chamber (RUTTNER, 1962), and introduce its organ of reproduction into the sting chamber — mating being thus completed.

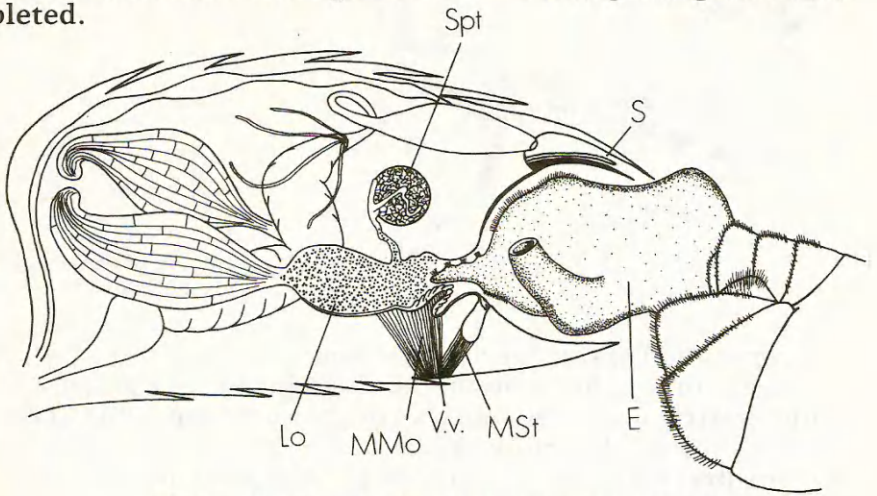


Fig. 5a — Copulation of the queen and drone at eversion;
E — copulatory organ; Lo — lateral oviduct filled with sperm; MMo — muscle of lateral pouch of Bursa copulatrix; S — sting; Spt — spermatheca

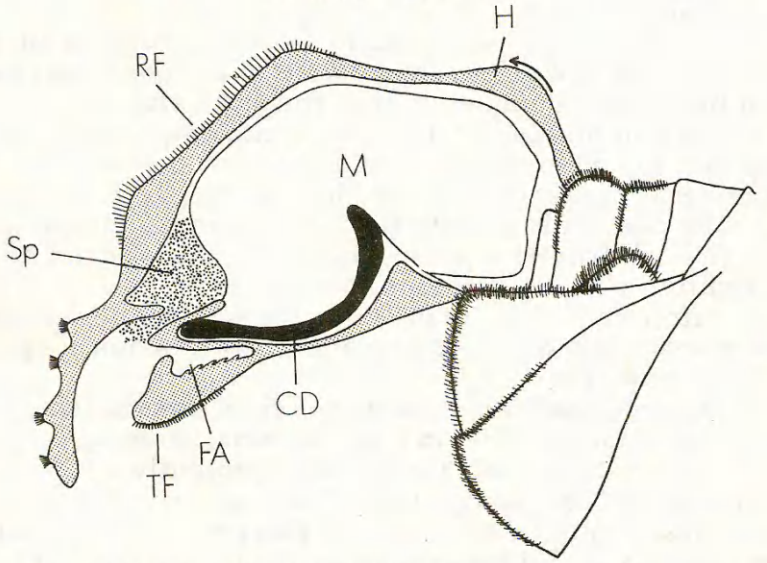


Fig.5b — Longitudinal section through the endophallus during eversion
CD — chitinous plates; FA — feathery appendage; H — haemolimph; arrow indicating the haemolimph's course; M — mucous; RF — hairy rhoms field

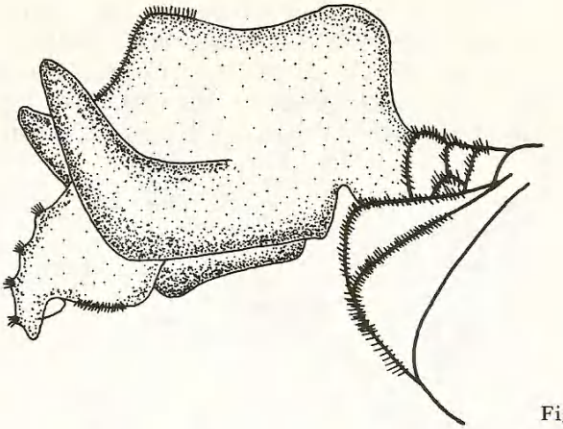


Fig. 5c — Unsectioned endophallus

Copulation of the queen and drone

Eversion of the endophallus is induced by the simultaneous contraction of all muscles of the abdomen, which compresses both the haemolymph and the air in the air sacs. Under this pressure, the of chitinous plates around the genital orifice raise up, and the endophallus is everted and pressed out. (For better understanding the process of eversion see footnote 1.)

First the hairy vestibulum comes into sight which was pushed out by eversion. In fractions of a second the vestibulum penetrates completely into the sting chamber, and the drone is motionless because the haemolymph had been pushed into the endophallus (Fig. 6). At this moment the sting chamber is utmostly distended by the half everted endophallus. The contact is so tight that the benumbed drone cannot detach itself from the queen, although its legs do not hug her so tightly any more, and it is now turned on its back (Fig. 6). By contraction of the muscles of the seminal vesicle and of the mucous glands the semen and white mucous are pushed into the bulb (Figs. 6 and 7).

The endophallus is so large and rigid that the sting chamber, which normally lies in the abdominal opening, is now pushed upward (Figs. 5a, 7a and b). Consequently, the chitinous membrane of bursa copulatrix and of the sting chamber stretches so much that the ribbed flap covering the orifice of the vagina is folded back thus clearing the entrance (Fig. 7b). The queen is probably also involved in this process. The slen-

der cervix penetrates into the vagina at this moment (Fig. 5a), the drone being benumbed and motionless.

Note 1. The eversion of endophallus is understood by pushing inside the fingers of a thin plastic glove. When inflating the glove, the compressed air presses the fingers on all sides and little by little pushes them out. In order to obtain the necessary pressure the drone compresses haemolymph and air into the endophallus.

By the sustained pressure of the queen, the eversion of the endophallus is completed and semen is ejaculated. First the semen reaches the median oviduct after the queen has folded back the valvefold (Fig. 5) (KOENIGER, 1984, 1986).

Concomitantly, the bulb with its chitinous plates and the white secretion of the mucous glands is pushed through the slender cervix.. The chitinous plates and mucous separate themselves from the bulb and when eversion is completed they are left in the queen as the mating sign (Fig. 7b). Now the endophallus is so long (Fig. 8a and c) that there is no room for it in the sting chamber any more. First the vestibulum is pushed out, with horns completely eversed. Their orange colour covering was left inside the queen. With the penetration of the chitinous plates and mucous in the sting chamber the drone detaches itself from the queen and falls on the ground. The orange covering was left on the mating sign.

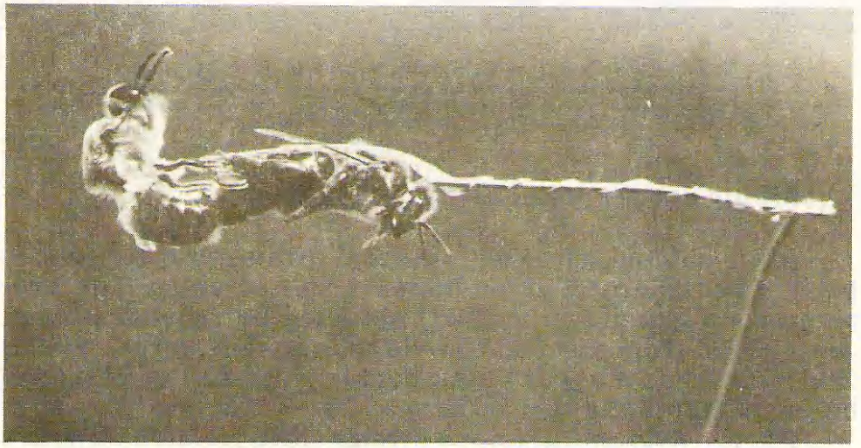


Fig. 6 — Movement of vulnerable organ during mating
Above: Vulnerable organs in normal state (the plates are vertically placed and cover the vaginal orifice);
Below: Vulnerable organ during eversion (the plates are oblically pushed upward. The vaginal orifice is free and enlarged through dilatation)

During a mating flight the queen mates with up to 8 drones. The next drone removes the mating sign left by the preceding drone with the hairy area of the vestibulum (Fig. 7c). The semen penetrates into the lateral oviducts filling them up (Fig. 6a).

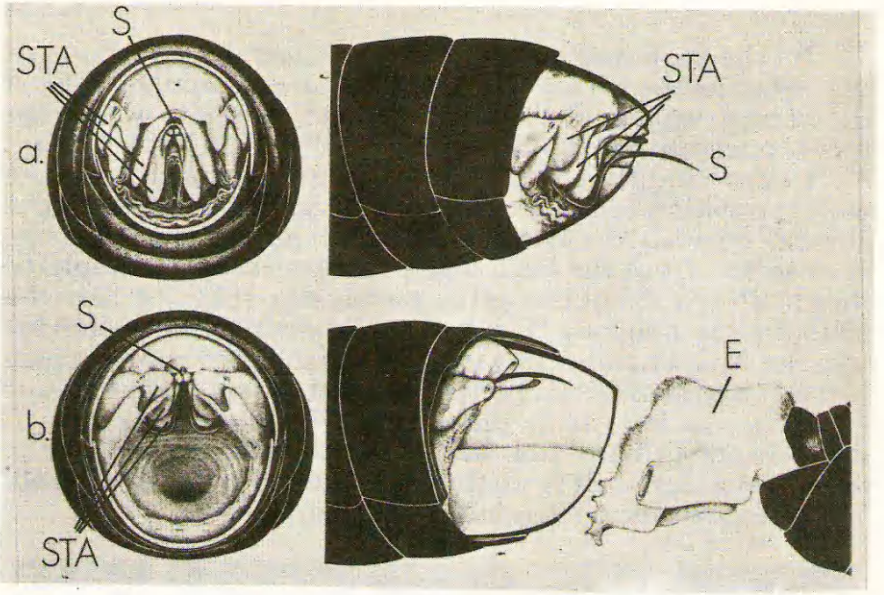


Fig. 7 — Movement of vulnerable apparatus during mating
above: the vulnerable apparatus at rest (its plates are vertically placed covering the vaginal orifice)
below: the vulnerable apparatus while pushing forward sperm (its plates are pushed transversally) upwards. The vaginal orifice is free and enlarged by stretching.

Filling of the spermatheca with semen

After having mated with several drones, the queen flies back to her colony. The oviducts are full of semen. The large amount of semen (up to 20μl) obtained by several matings was pushed from the median oviduct into the lateral oviducts (Fig. 5a). Almost always both oviducts are full with semen, but often not to the same extent.

But the oviducts are not a proper store room for spermatozoa, and therefore a transfer is made during the following 24 hours, as follows:

Shortly after the return of the queen, she presses the abdomen by adequate movements. The semen is pressed from

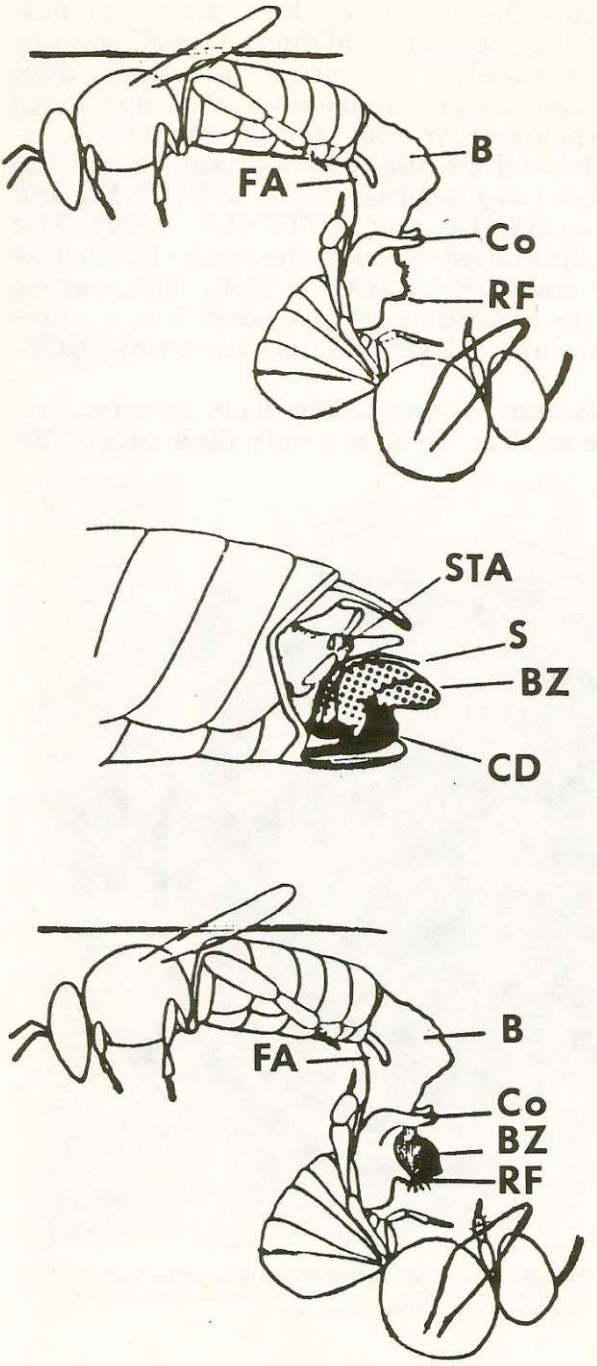


Fig. 8 — a) Last eversion stage of the endophallus of first drone. The mating sign is introduced into the queen; b) Position of the mating sign into the queen; c) Last stage of eversion of the endophallus of the second drone: the mating sign of the first drone is glued to the one of the second drone, while it gives off its own mating sign;
B — bulb; BZ — mating sign; CD — dorsal chitinous plates; Co — horns (cornua); FA — feathery appendage; RF — hairy rhomb field; S — sting; STA — vulnerable apparatus

the lateral oviducts into the median oviduct, the semen passing by the orifice of the spermathecal duct. A small amount of the semen (approximately 1/10) enters the narrow duct (0.01—0.25 mm in diameter and approximately 1 mm long) and reaches the spermatheca: the passage of semen is the result of both the individual motility of spermatozoa and the pressure from the Bresslau seminal pump (RUTTNER and KOENIGER, 1971; GESSNER and RUTTNER, 1977). The rest of the semen is eliminated through the sting chamber as dry, white-brownish sticks (HAMMANN, 1956). The process is not controlled by the central nervous system. It still continues even after sectioning the ventral nervous trunk (KOENIGER, 1985).

Some of the beekeepers are mistaken in considering that the semen sticks are mating signs; not only their origin dif-

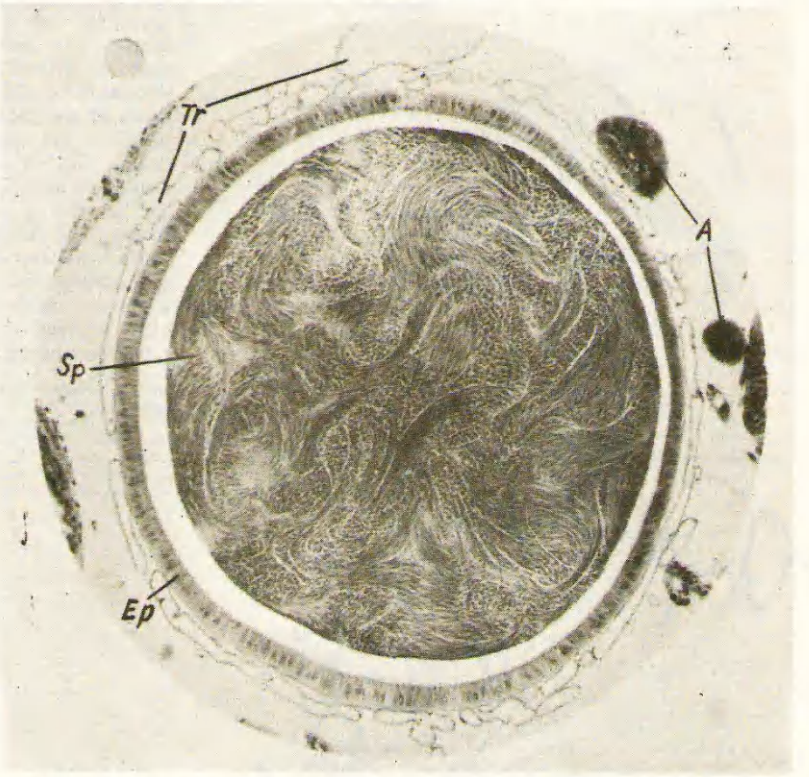


Fig. 9 — Transversal section through the spermatheca of a mated queen
A — gland; Ep — spermatheca wall made up of high cells; Sp — spermatozoa gathered in interdependent clusters; Tr — extern cover of tracheae

fers, but also their aspect. The mating sign is l mm long, with one side bordered by brown chitinous plates. The white mucous, the main element of the mating sign, is coated by a thin, orange and sticky layer. Shortly after the queen has returned from the mating flight, the worker bees remove the mating sign.

The filling of the spermatheca depends on how the worker bees take care of the queen. That is why the mating hives must be stocked with enough bees (WOYKE, 1983).

Storage of semen

After 24 hours there is no semen in the oviducts, the 5—6 million spermatozoa being stored in proper conditions into the spermatheca (Fig. 9) This "store room" endowed with tracheae and a spermathecal gland provides for the preservation of the fertilizing capability of spermatozoa for up to 5 years. The processes involved are not yet precisely known. It is known that an alcaline medium (pH=8.6) is maintained inside the spermatheca, by sodium and kalium ions. It is likely that the one-cell layer actively controls the supply of ions (GESSNER and GESSNER, 1976; VERMA, 1973). After surgical removal of the spermathecal gland, the spermatozoa are still alive after 90 days, but 30 days later the queen will only lay unfertilized eggs. If the network of tracheae is removed, after 30 days all spermatozoa in the spermatheca are dead (G. KOENIGER, 1970).

In the spermatheca the number of spermatozoa originating from different drones is not the same. From some drones more semen has penetrated into the spermatheca, and less from others. In the practice of instrumental insemination evidence was obtained that the semen which entered the last into the oviduct and hence the first to reach the spermatheca is slightly more favoured. In contrast to previous reports, it has been lately found that the semen is uniformely mixed in the spermatheca (MORITZ, 1986; PAGE et al., 1984). Consequently, the number of the descendants of different drones is not the same, but the ratio of these different numbers is always the same. For example: more than 1/7 of worker bees will be yellow although only one of the ten drones with which the queen had mated was yellow. This finding is important in queen rearing. With artificial insemination, the drones whose semen was injected the last contribute more semen in the spermatheca (MORITZ, 1986).

Maturation and laying of eggs

When the queen has mated with the necessary number of drones (10—17) during one or two mating flights, the neurosecretory changes and the maturation of egg begins (BIEDERMANN, 1964; HERRMANN, 1969). No precise evidence has been obtained of the stimuli during mating which are decisive in starting egg laying. Neither the active flight of the queen, nor the degree of filling of the oviduct and of the spermatheca have any influence. It is likely that the extreme dilatation of the queen by the endophallus of the drone plays the decisive role (G. KOENIGER, 1981). As early as 2 days after the last successful mating flight the queen already starts egglaying.

In the ovaries of queens there are approximately 360 ovarioles, and 30 egg follicles in each of the latter. A queen well attended by the worker bees can lay up to 1500—2000 eggs daily, in peak season. This daily amount of egg production exceeds the queen's body weight. She is able to lay so many eggs because she includes the proteins from the food with which she is fed by worker bees directly into eggs, without breaking them up into the component elements (amino-acids) (LACOMB, 1977; RUTZ and LÜSCHER, 1974).

When a worker bee egg must be laid, then several spermatozoa will be released from the spermatheca into the median oviduct. The queen always releases only limited amount of semen from the spermatheca. The number of spermatozoa depends on the degree of filling of the spermatheca (HARBO, 1979a). 1—10 spermatozoa penetrate into an egg, but only one unites with the egg nucleus (NACHTSHEIM, 1914; WOYKE et al., 1966).

When an egg is to be laid into a drone cell, the queen is aware of this fact because of the different sizes of the cell, and does not release semen from the spermatheca. She determines the sizes of the cell by inspection with her front legs (KOENIGER N., 1969).

Differences between instrumental insemination and natural mating

With instrumental insemination, many actions taking place during natural mating are eliminated. For example, the queens are not left to fly freely to avoid undesired semen. But

it is important to take into account as much as possible of what is already-known about the natural mating. For example, instrumental insemination will be efficient only if the age of queens and drones is the age at which they mate under natural conditions.

Differences with respect to queens

During instrumental insemination the queens are anesthetized, so that the operator must perform all actions instead of the queen. The sting chamber is kept open by hooks, and the skin folds at the vaginal orifice must be drawn aside. The folding back of the valvefold from the vagina, is of utmost importance because otherwise the semen will not reach the oviduct (Fig. 6a).

The many skin folds in the vagina may have different shapes depending on the degree of opening of the queen and of the pressure on her. The operator must try to be as close as possible to the natural conditions (Fig. 5b). At present, the insemination instruments are being modified so as to enable a dilatation of the queen similar to that induced by the drone endophallus.

Also, during instrumental insemination, one of the natural mating stimuli inducing egg laying is available. A double anesthetization with CO_2 has the same effect (HERRMANN, 1969).

Differences with respect to drones

In drones, too, none of the stimuli which induce mating under natural conditions are available. Contraction of the abdominal muscles, prerequisite of the eversion of the endophallus, is induced in laboratory in two ways by pressing the thorax between fingers and concomitantly lightly touching the abdomen, or by anesthetization with cloroform. In general, the endophallus is everted up to the cervix, hence up to the moment when, during natural mating, the benumbed drone has turned on its back. At this moment the semen and mucous must already be in the bulb.

Now the operator must again perform the activities of the queen: pressure for ejection of the semen and of the mating sign. This operation is never performed as during natural mating because the endophallus is not under the pressure of the queen's sting chamber and vagina. The resistance opposing the eversion during the natural mating is absent. In ge-

neral, by pressing the abdomen, when the bulb is everted the semen and mucous are also ejected, while the chitinous plates — the outer coating of the mating sign — remain inside the bulb.

Attempts are also being made for optimizing the collection of semen.

In conclusion, it is obvious that instrumental insemination is a highly advanced technique having been successfully and routinely utilized on a large scale, for several decades. However, constant perfection by new findings and by bringing it the closest possible to the natural mating are important targets.

REARING AND MAINTAINING QUEENS AND DRONES

V. MAUL

A. Rearing and maintaining queens

A large range of thoroughly experimented procedures have been available for a long time in rearing queens of known origin. H. RUTTNER (1980) made a detailed survey so that I shall not dwell on details. In principle, it must be pointed out that for the instrumental insemination to attain its target, special care must be taken in providing for optimum rearing conditions. The large and vigurous queens are easier to inseminate and also justify the special insemination work by their longevity. Therefore, particular attention must be paid to the methods of rearing in normal colonies. Grafting may also be used as it is an efficient technique, the essentials being a 9 mm diameter for the artificial queen — cell cups and an age of up to one day for the grafted larvae (WEISS, 1980).

Confinement and marking of queens

Although according to some experts (for example ADAM, 1969) the highly performing queen should emerge in the mating nucleus prepared for her, which means that queen-cell cups with queens ready to emerge should be introduced into the nuclei prepared for this purpose, this is not possible with instrumental insemination because of technical reasons. Catching the unmarked young queen takes a long time and very often confusion is possible. That is the reason why another method is widely used, namely confinement in emergence cages on the 10th or 11th day which are left in the nurse colony until the emergence of queens, or are introduced into adequate incubators. H. RUTTNER (1980) made a general survey of the adequate procedures in this respect too.

Marking of queens, which is indispensable for identification, may be done very easily before introducing the queens. The mark must provide as much information as possible. Therefore, the use of numbered opalite labels is recommended, in the colour of the current year. Approximately 1000 individual markings are posible if not only the number, but also the clockwise position are registered (see Fig. 10), in the re-

cord of the reared queens. Even when the mark is lost, the wing clipped during insemination provides another possibility to locate and verify it as the inseminated queen.

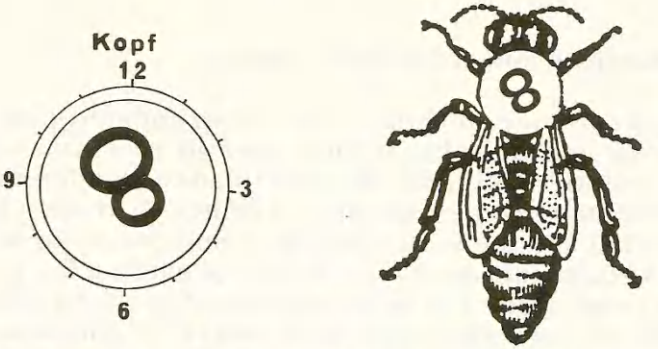


Fig. 10 — Position of the marking plate with additional information
Figure's base is estimated like the clock's hand. We take 8/11 for our example

Maintenance of queens after emergence

Just as in rearing and maintaining of young queens, favourable conditions should be provided so that the optimum insemination results are obtained. First, one must remember that for successful migration of the semen injected into oviducts to the spermatheca, a temperature equal to that in the brood chamber is necessary (VESELY, 1969, 1970; WOYKE and JASINSKI, 1973). On the other hand, the successive operations must be carried out in their normal order. The queens to be inseminated will be transferred to a place close to the insemination laboratory. It is desirable to provide them with everything they need so that permanent watching should not be necessary.

For this purpose, storage of the queens in mating nuclei with a few combs is advantageous. Good results are also obtained when storing them in cages together with several attendant bees in an incubator, at a temperature 34°C (WOYKE and JASINSKI, 1973), but this method is applicable only for small batches of queens to be inseminated for experimental purposes, as they require much work for maintenance. In the United States unmated and inseminated queens are mostly stored in "queen banks" (in cages, in special queenless colonies), but following recent experiments the efficiency of this method of storage is questionable. WOYKE and JASINSKI (1986) reported many cases of queen's arolia (foot

pads) having been harmed by bites of worker bees, which resulted in atrophy and the removal of segments of legs. In this way even young queens, reared under optimum conditions, become queens of poor quality.

The Kirchhain mating nucleus as a mating nuce with several combs deserves special mention as it was particularly developed for instrumental insemination (MAUL, 1971). Being made of stryofoam it always ensures, a favourable climate inside even with a small amount of bees. The hive entrance is on the bottom and an excluder may be attached to it. The new types are provided with optional entrances so that the hive entrance may be left either open, or closed, or with the queen excluder in place. The secondary entrance in the darkness under the bottom board discourages the queens to fly and so they can be stored there long before insemination. In addition, robbing and overheating when placed in the sun is prevented with this nucleus. In the other types of nuclei the entrance and aeration are usually unprotected being on the front wall (as for example the Austrian mating nucleus or the "Taunus-Apidea" nucleus, both made of plastic material. But if they are placed in the shade, and not used for long term the queens they are also most adequate for the purposes of instrumental insemination. Absolutely inappropriate are the single-comb mating nuclei still widely used in Central Europe. Very much time is wasted catching the queens. As no bee way exists (having one comb only) the temperature inside the nucleus decreases when it is cold. This entails insufficient filling of the spermatheca and consequently blocking of the oviducts with the remaining semen (VESELY, 1969, 1970).

As a rule, these small mating nuclei are stocked with artificial (shaken) swarms. For the initial stocking of a Kirchhain mating nucleus for example, 4 small combs with approximately 120 g of bees are necessary. The queen will be introduced into a cage with candy. If not so, the queen may be introduced into the nucleus 1—2 hours after it has been stocked. It is very important that the young queens move freely inside the nucleus during the first few days.

After being kept in the dark for 3 days, the mating nuclei are arranged in rows, close to one another, hence in a different manner than in the natural mating (see Fig. 11). However, alternating the flight direction is recommended, as well as painting the front walls with different colours to avoid heavy drifting of bees. Hive stand of the height of usual ta-

bles are very efficient as they facilitate subsequent operations.

The mating nuclei widely used in the United States are larger. They are formed at the beginning of the season with standard combs which can be divided in two halves. Such nuclei are used several times for a batch of queens. This is also

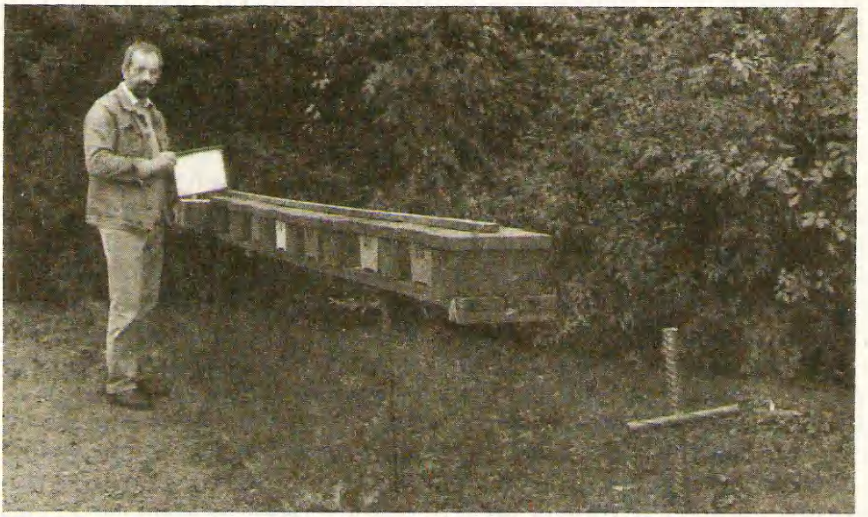


Fig. 11 — Kirckhain mating nuclei on rows, at a most convenient height for work. It has construction steel bars for support, transversally linked by other fused bars.

possible with the baby-mating nuclei used in Central Europe, with the emergence of queens early in the season. Good results are obtained when the young queen is introduced one week after removal of the laying queen and if the cage with queen in it is kept closed for 24 hours. In the Kirchhain mating nucleus, one can even store laying queens for several months. After removing the queen excluder, two more little combs may be introduced into the feeder section. For stocking new mating nuclei, bees and combs with brood may be taken from strong nuclei.

B. **Rearing and maintaining drones**

In selection programmes instrumental insemination provides for the decisive advantage of immediate and multiple combinations of queens and drones of different origins. But

successful accomplishment of such combinations requires careful planning and co-ordination of queen and drone rearing, as well as reliable techniques, in order to make sexually mature drones, of the desired type and age available.

The timing of rearing drones and queens in critical, because of their different development to sexual maturity. The queen is sexually mature when 5—6 days old, which means on the 21st—22nd day of the general development, whereas drones are sexually mature when 12—14days old, i.e. on the 36—40th day of the general development respectively (it takes a longer time after sealing of the brood). In practice queen development is considered to start on the day of grafting, which reduces this time by 4 days. As a general rule, drone rearing must start at least 3 weeks before the day of grafting. If it is impossible to determine the date of egglaying, the age of the sealed drone brood can be estimated by the colour of eyes (see Table 1), and then the time to start the batch of queen's can be determined.

Table 1

Progressive colouring of eyes of drone brood depending on development, in days

No. of days after egglaying	No. of days before emergence		Colour of pupa eyes
15	approxim.	10	white
16	"	9	yellowish
17	"	8	light pink
18	"	7	light pink
19	"	6	dark violet
20	"	5	the simple eyes are visible
21	"	4	black
22	"	2—3	colouring of the body begins

What is true for queens, is also holds for drones: high-quality individuals can be obtained only *under optimum rearing conditions.* This means on the one hand the abundance of food stores in the nurse colony, and on the other hand the "inner impulse" to rear drones. During the build-up phase (spring/early summer) both conditions are available, and the impulse to rear drones may be only supressed when many drones exist in the colony (WEISS 1962). The impluse gradually declines as the colony development slows down, the driving out of drones being the manifest evidence. The time when this occurs depends on the weather and flow condi-

tions, but differences between colonies may also exist, depending on race and propensity.

If drone brood must be obtained during a "reluctant" period, changes in the internal conditions of the colony must be made. Stimulative feeding is just as important as is the increase of the number of nurse-bees by introducing combs with brood. If in this critical period the queen can be induced to lay drone eggs by confining it in frame cage, then a normal drone nurse colony must be available, to take care of the brood. It has to be a strong recently dequeenced colony, with a great amount of brood and food. This colony may also be used for rearing queens. But care must be taken to remove all queen cells before emergence. Only a young unmated queen confined in a cage is left in the colony, and if needed young bees are added by introducing extra combs with brood, but not with no drone brood on them.

Identification

Providing for precise identification of the newly emerged drones is as important as in queens, but it is much more difficult. Coloured marking is possible (see below) but seldom used in practice because it requires much work. Usually, the first step is *confinement of the drone brood* (so that newly emerged drones should not get mixed with other drones). The time of confinement is established by random checks of the eye colour of the sealed drone brood. The drone brood may be stored in confinement in the honey chamber of the drone-rearing colony, in a reserve (stocking) colony, in drone free nuclei in frame cages in colonies, or in incubators.

Storage in the honey chamber

Confinement in the honey chamber is mostly used in top-opening hives (the multiple-storey- hives for example). All drones and bees must be brushed down into the brood chamber. The honey chamber can be kept out of reach of undesired drones by placing on an excluder screen; introduction of combs with bees must be avoided. The comb with brood from which drones will emerge is placed in the centre of the honey chamber as well as two combs with the youngest possible brood certainly after all bees have been brushed off. These bees will subsequently walk up back into the honey chamber

very quickly. In strong colonies, instead of brushing the bees from combs, a bee free super can be placed on the excluder, the next operations being the same.

If the honey- chamber of a strong colony is used, the combs with worker brood must be taken from the parent colony. If not, the drone brood from the foreign colony must be destroyed. In any case, the safest way is to "behead" the sealed drone brood cells every 8—10 days.

The emerged drones may safely stay for a longer time in the isolated honey- chamber. The honey chamber must be opened only early in the morning (up to approximately 10.00), because otherwise drones will fly out. In the build-up period, it is better to introduce the newly- emerged drones in separate nuclei already after a few days. This can be easily achieved by placing the honey chamber (super) with all the bees in it on a new hive stand (with the excluder at the bottom of the super), and by transferring it to a place outside the previous flight range. If no emergency- brood is available, unsealed brood is added again, or an unmated queen is introduced in a cage. This method cannot be used with drone-rearing

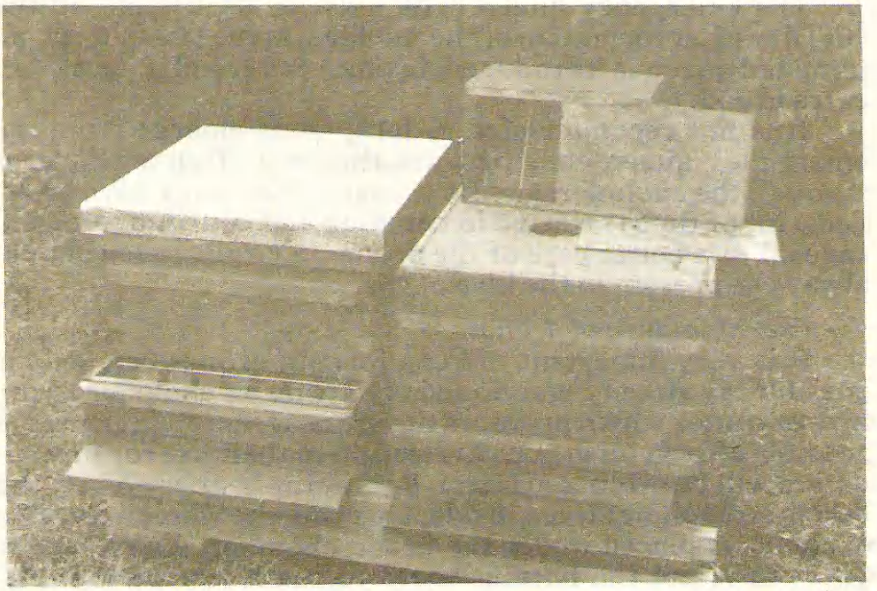


Fig. 12 — Drone management in multistoreyed hives.
On left, hive with queen excluder over a very high bottom;
On right, an intermediary lid provided with an orifice that can be closed
Behind, a verandah with an excluder to keep the drones that want to fly.

colonies which are tested for production efficiency. In such cases storage in reserve colonies is preferable. If necessary, a collective brood colony can be formed with small amounts of drones and bees, in a nucleus with combs with food stores and with brood.

Storage in nuclei

When confining drones in nuclei the procedure is on principle similar. The nucleus will be stocked with honey combs and 2—3 combs with brood (the foreign drone brood must be destroyed!). The bees necessary will be taken from different colonies (mostly young bees if possible, to be active as nurse bees) and sifted to remove drones. For 5—6 standard combs, approximately 1kg of bees is needed. The emerged drones are kept in the nucleus until needed. With an average or poor honey flow, continuous stimulative feeding is required. Not more than one comb full of drone brood should be provided/kg of bees. Whether one wishes to obtain drones from several sister colonies in a single nucleus, then half frames can be be fastened together to form normal frames with mixed brood.

The emergency queen cells must be destroyed, on the 10th day after formation of the nucleus, at the latest. Next, an unmated queen is introduced, inside a cage, and if necessary extra brood.

It is not recommended to let queens emerge from the emergency queen cells because they are often small, pass through the excluder, and fly to mate. After they have emerged a lot of time is wasted in catching and confining them in cages. For a better care of the drones, a virgin queen should always be present in the hive.

Free of undesired drones

It is very important to keep foreign drones off. This is possible, as already mentioned, by fixing an excluder to the hive entrance which prevents the drones from flying out (the possible effects on drones in relation to their use for insemination will be discussed later). For multiple storey hives, the best is an excluder fixed inside, covering the whole area. The excluder is placed under the brood chamber, which means between it and the bottom board of the hive (see Fig. 12). High bottom boards are preferred. The confined drones stay in complete darkness, which allows storing them for a longer time. This method is preferable to the entrance appliance

whereby the excluder extends outward and upward at a 45° angle, which was widely used previously (see Fig. 13). In spite of the projecting roof, the drones staying there in the sunshine were very excited during their active period and lost vitality much sooner than those confined in the dark. The sizes of the passages of the excluder and its inclination are necessary in order to let worker bees pass through. This appliance had the advantage that several drones ready to fly could be caught, as it covered the hive entrance entirely.

Out of the smaller nuclei for maintaining drones (up to 5 combs) the "Benjamin" nucleus having the shape of a super is very useful (Fig. 13). At the opening on the bottom of the hive, (for ventilation during transport, instead of the ventilation screen) an excluder may be attached which prevents the drones to fly out. The regular hive entrance is closed in this case. In this nucleus drones are also confined in the dark.

If drones are confined in the lower part, the death rate must be checked each week (in the morning, the super is easi-

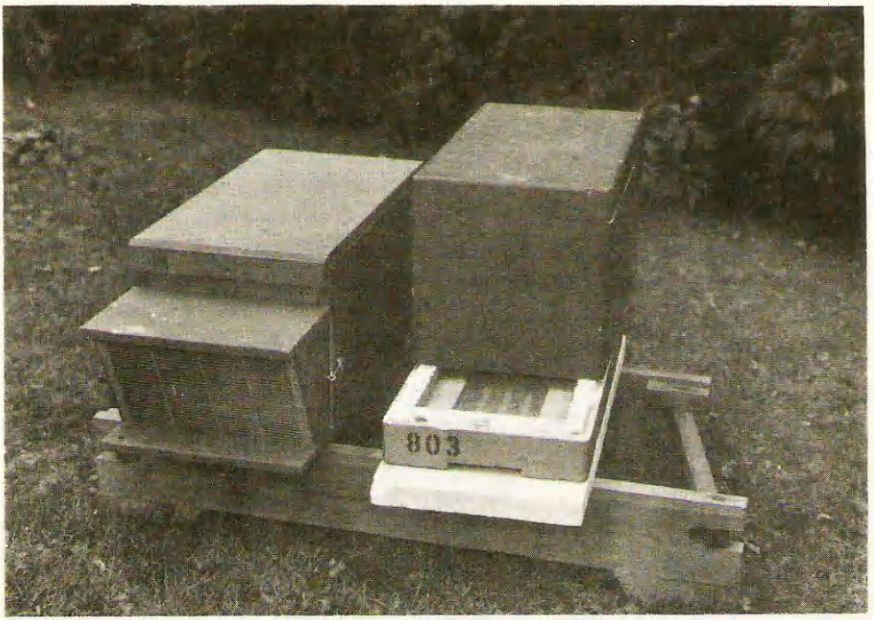


Fig. 13 — Drone management in nuclei.
On left, an attached verandah (advisable only for a short period of time);
On right, "Benjamin" hives with excluder in the ventilation orifice, placed on the bottom of the hive.

ly turned over!) because it is known that the life span of drones in confinement is short. In case the death of drones occurs very early and the death rate is high, the utilization of the remaining drones is questionable.

Taking out of drones

Drones may be taken out very easily by using the entrance appliance with excluder at a 45° angle, or an empty super, with an excluder on top. In this case, the nucleus in the shape of a super must be provided with an inner cover with an excluder at the hive entrance (Fig. 12). A hole is bored in the front wall of the empty super which is closed with a cork. The drones caught on top of the entrance appliance with excluder are transferred in wire cages (Fig. 14) onto the window glass of a closed cabinet and are used inmediately, or are left in the cages for a short time, in a nurse colony (LAIDLAW, 1954). After the transfer into the cabinet, drones will heavily defecate during the excited flight against the window glass. Therefore, drones must be taken one by one from the entrance appliance with excluder to prevent heavy reciprocal splashing with faeces. A glass cage, heated and provided with light is most useful for the purpose. Up to 10.00 hrs. a.m. adult dro-

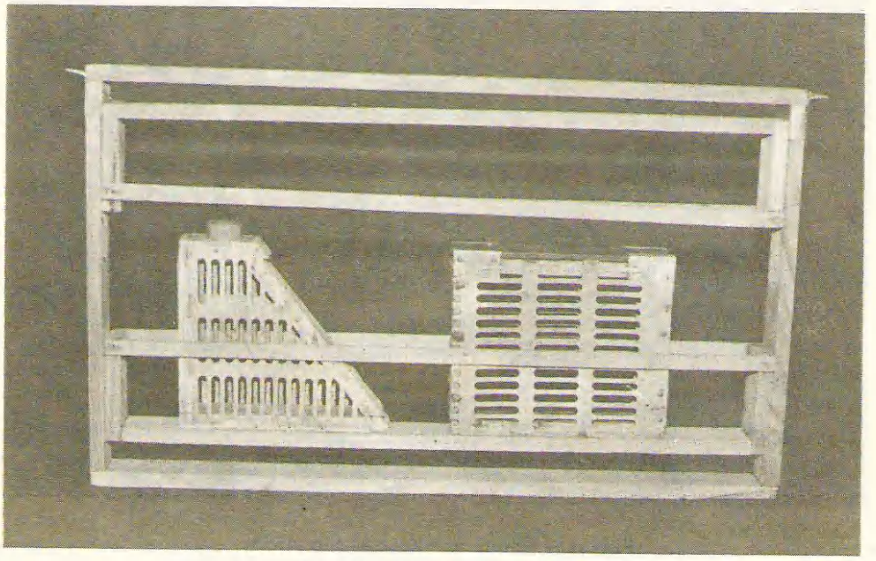


Fig. 14 — Drone cages
On left, triangular shape with closing cork (very handy)
On right, rectangular shape with sliding lid (very quickly propolized)

nes can be taken directly from the nuclei, without great loss. They are usually found on the outer combs, outside the brood area.

Except occasional difficulty in collecting semen because of incomplete defecation, no other obvious disadvantages of permanent confinement were recorded. It seems that many drones would release faeces in the hive. Where a yard far from other apiaries is available, (outside the flight range of the drone nuclei) the drones could be carried there for their cleansing flight. A second bottom board on which the nucleus will be placed in the new yard, without excluder, is useful for this purpose, the initial bottom board with excluder and with closed hive entrance (not to let out the foreign drones trapped) is placed close to it. In the evening, it is fixed back to the nucleus. Also useful is an ampty super with an excluder on top of the open inner cover. During their flight in this "flight cage" drones can release faeces (do not forget to replace the papers!) and in the evening they are brought back to their colony.

Special storage methods

Compared to the methods described above, confinement in a frame cage of excluder material is only an intermediary solution. Inside, only little room for movement is available for all drones obtained from an entire brood comb. Therefore, at short intervals of time, the emerging drones are transferred to another place, after being marked with different colours depending on their origin and age. LAIDLAW (1954) recommends storage of small groups of drones in excluder cages which hang between the brood combs in the drone nurse colony. The same method should be used when the drone brood comb, together with the nurse bees, is introduced for emergence of drones in the incubator (JAYCOX, 1961; MORIMOTO, 1963). But confinement in the frame excluder cages or incubators requires much work, being of interest for experimental purposes only, because only very few drones are necessary, and only for short periods of time.

Spraying the sifted drones with different colours, as recommended by SCHLEY (1987), would be useful only if drones of a few origins were stored concomitantly. The colour scale is limited and, in addition, the different ages of drones should also be individually marked. Also, because of the free flight the percentage of the drones lost is always very high.

INSEMINATION APPARATUS

J. R. HARBO
V. MAUL

Equipment used for the instrumental insemination of queen bees can be divided into three categories: (1) beekeeping equipment (cages for queens and drones), (2) some standard laboratory equipment (tank of carbon dioxide, regulator, gas flowmeter, dissecting microscope with about 10X magnification, and a microscope light, and (3) specialized equipment (insemination stand, hooks and syringe). This chapter will deal only with the specialized equipment. This equipment varies throughout the world, so two authors, one from the USA and one from the FRG, will describe the equipment used in their respective countries.

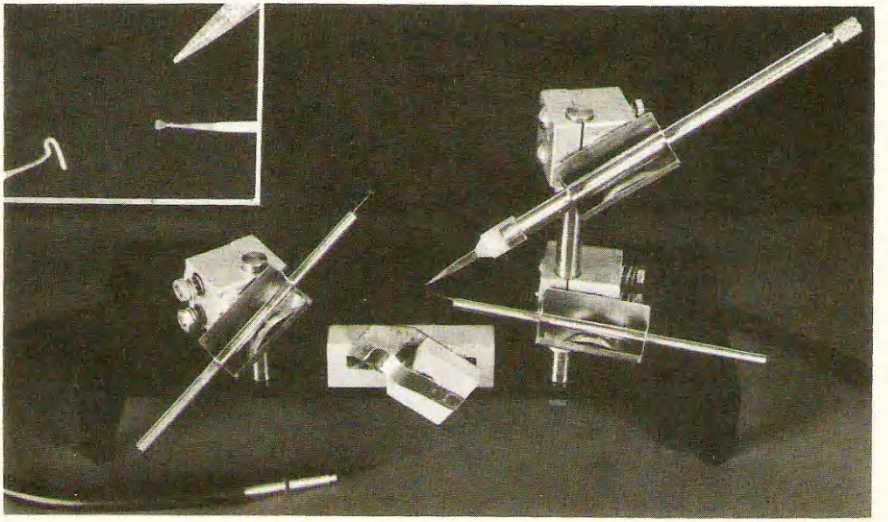


Fig. 15 — Mackensen insemination stand and syringe.
The ventral hook is on the left and the sting hook is on the post with the syringe. The posts can be reversed for left-handed use of the syringe. Upper left is an enlargement of the sting hook (top view), ventral hook (side view), and tip. The plug for the queen holder is attached to the end of the insemination hose. The mountings for the hooks and syringe are identical except for the size of rod that they will hold. These mountings allow for three types of movement: (1) a horizontal turning around the vertical post, (2) vertical turning around the horizontal axis where the mounting attaches to the box that holds the syringe or hook, and (3) in-out movement. Measurements and detailed mechanical drawings of all parts of the syringe and stand are given by Mackensen and Tucker (1970).

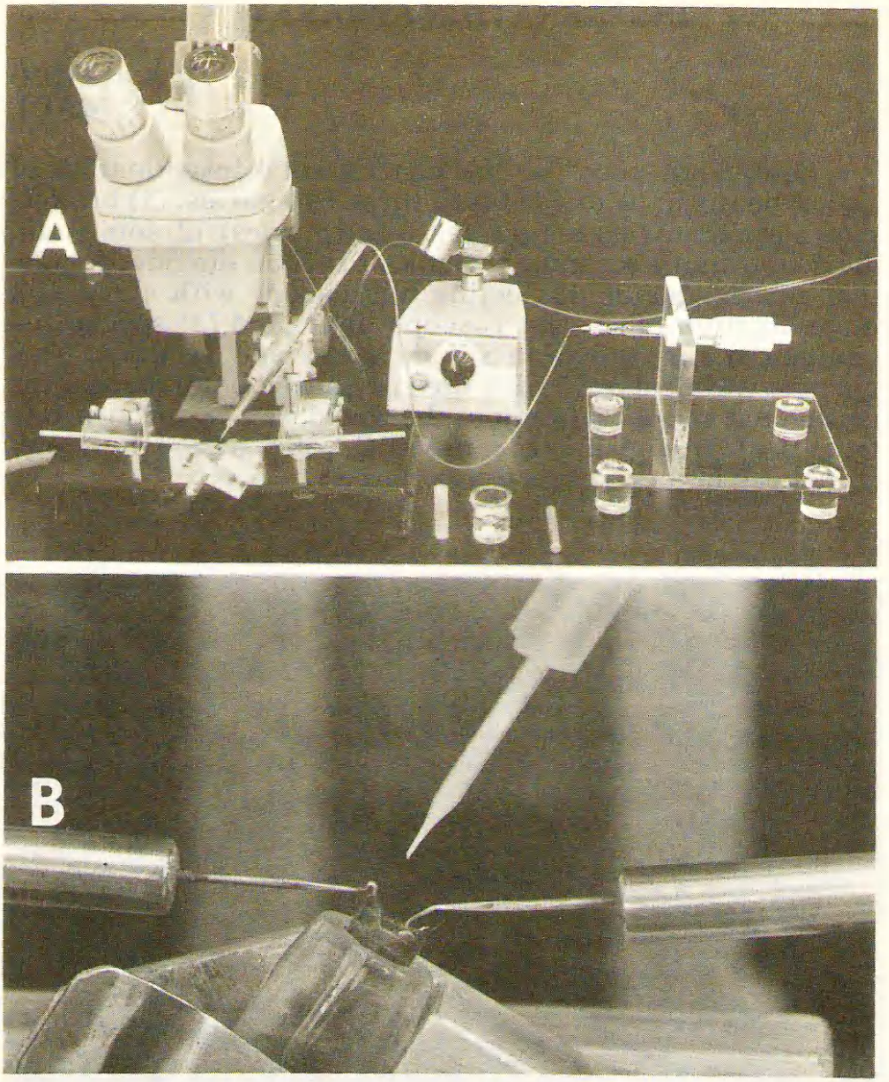


Fig. 16 — Insemination equipment in use:
(A) A Mackensen insemination stand holds the syringe shown in Fig. 3. The micrometer for the syringe (200 µl capacity) is mounted in a stand made of acrylic plastic. (B) Side view of a queen ready for insemination.

A. Equipment used in the USA

1. The stand

The MACKENSEN stand (Fig. 1) was first described by MACKENSEN and ROBERTS (1948). It was modified slightly by MACKENSEN and then redescribed in detail by MACKENSEN and TUCKER (1970). This stand is used by nearly everyone doing instrumental insemination of bees in the USA. The device aligns the holders of the ventral hook, sting hook, syringe and queen into one vertical plane. Within this plane, the operator can set the heights of the syringe and the hooks as well as the angle and horizontal placement of the queen.

The mountings for the syringe and hooks are similar and allow for horizontal and vertical rotation as well as in-out movement of the syringe and hooks (Fig. 15). The absence of gears enables the operator to feel the contact between the instruments and the queen. The mounts hold the syringe and hooks in place when the hands are removed.

Although separate pieces, the plug and queen holder (shown in Figures 1 and 2, respectively) are part of the insemination stand. After a queen backs into the acrylic holder, the plug positions the queen so that about 2 1/2 abdominal segments protrude (Fig. 16 B). Carbon dioxide gas (about 35 ml/min) passes through the plug to immobilize the queen. To keep each queen in the same position relative to the hook and syringe, one must align the end of the queen holder with the top of the block as shown in Fig. 16 B.

2. The syringe

Two types of syringes are described here, but there are many types used in the USA (MACKENSEN, 1954; KAFTANOGLU and PENG, 1980; HARBO, 1985; LAIDLAW, 1985). All can be used with the Mackensen stand.

The Mackensen syringe (Fig. 15) has been the standard syringe since 1954. It is made on a lathe and is composed of stainless steel and acrylic plastic (MACKENSEN and TUKKER 1970). The Mackensen syringe is used throughout much of the world and is the most commonly used syringe in the USA.

A syringe designed by HARBO (1985) is shown in Figs. 16 and 17. It is an hydraulic system that consists of a tip made from glass capillary tubing connected by plastic tubing to a

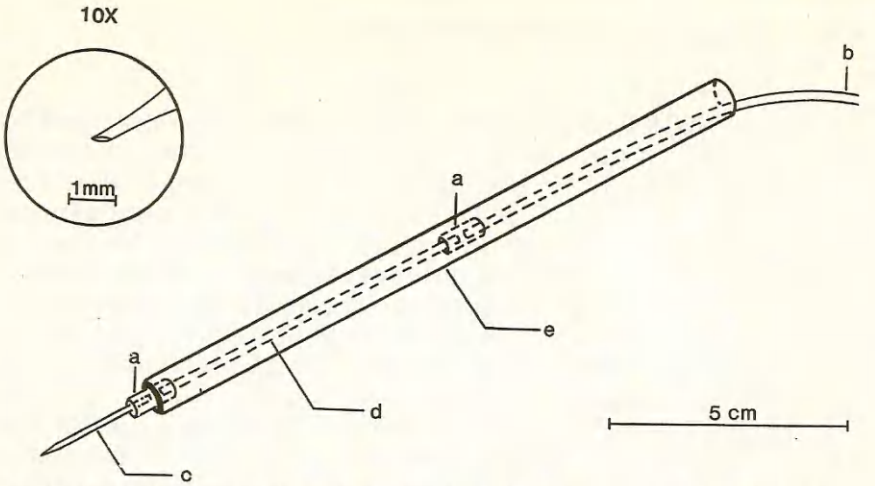


Fig. 17 — The barrel and tip portions of an insemination syringe designed by Harbo (1985)

The tip (c) is a glass capillary tube that was drawn to a point with heat and polished to an angular point (see enlargement). The inside diameter (ID) at the orifice of the point is 0.16—0.20 mm; the outside diameter (OD) at the orifice is 0.26—0.32 mm. The storage tube (d) is a glass or plastic tube (usually about 1 mm ID) that receives semen when large amounts are collected and that can be removed and stored, if desired. The syringe barrel (e) is glass tubing with an ID 5—6 mm and an OD 7—8 mm. The opening at the tip end of the barrel is reduced to a diameter of ca. 4.2 mm. This reduced orifice forms a tight fit around the latex connector that holds the tip. Thus the tip is held firmly for inseminations and yet is flexible enough to avoid breaking if bumped. The connectors (a) are sections of latex tubing ID 1.2, OD 4.5 mm). Tube b (polyvinyl tubing; ID 0.5 OD 1.5 mm) forms the hydraulic connection to the syringe micrometer shown in Fig. 2A. To assemble the parts, the storage tube, followed by tube b, is pushed into the barrel until the storage tube protrudes out the end where the tip attaches. The tip and the latex connector are attached to the storage tube, and then these parts are pushed back into the barrel until the connector fits as shown. The syringe and plastic tubes are filled with saline that has been boiled to remove dissolved air, thus keeping the hydraulic system responsive. However, the saline in the storage tube and tip and that used during insemination should not be boiled.

syringe micrometer. The system has a 200 μl capacity that can be expanded after this limit has been reached by disconnecting the tubing where it attaches to the syringe and discharging fluid from the micrometer. The syringe was originally designed for use in semen storage but is also useful when large quantities of semen are collected and mixed, or when an operator simply wants to collect all the semen before beginning the inseminations.

3. Insemination tips

The tip for the Mackensen syringe is made of acrylic plastic and has an inside diameter of 0.13 mm and an outside diameter of 0.23 mm at the point (MACKENSEN, 1954; MAKKENSEN and TUCKER, 1970). Unlike glass tips, the plastic tip enlarges rapidly from the point.

Glass tips for the syringe in Fig. 17 are described in the figure caption. The tip is filed to an oblique point (see 10X in Fig. 17) and put briefly into a flame to polish the cut surfaces

(HARBO, 1973). The angled point eases the insertion of the tip into the median oviduct and thus allows the operator to use a tip that has a relatively large outside diameter at the point.

B. INSEMINATION APPARATUSES USED IN EUROPE

The standard apparatus

When the insemination technique was introduced in Europe, the basic design of the insemination apparatus developed by MACKENSEN and ROBERTS was adopted. But the interest for easier handling resulted in many subsequent in-

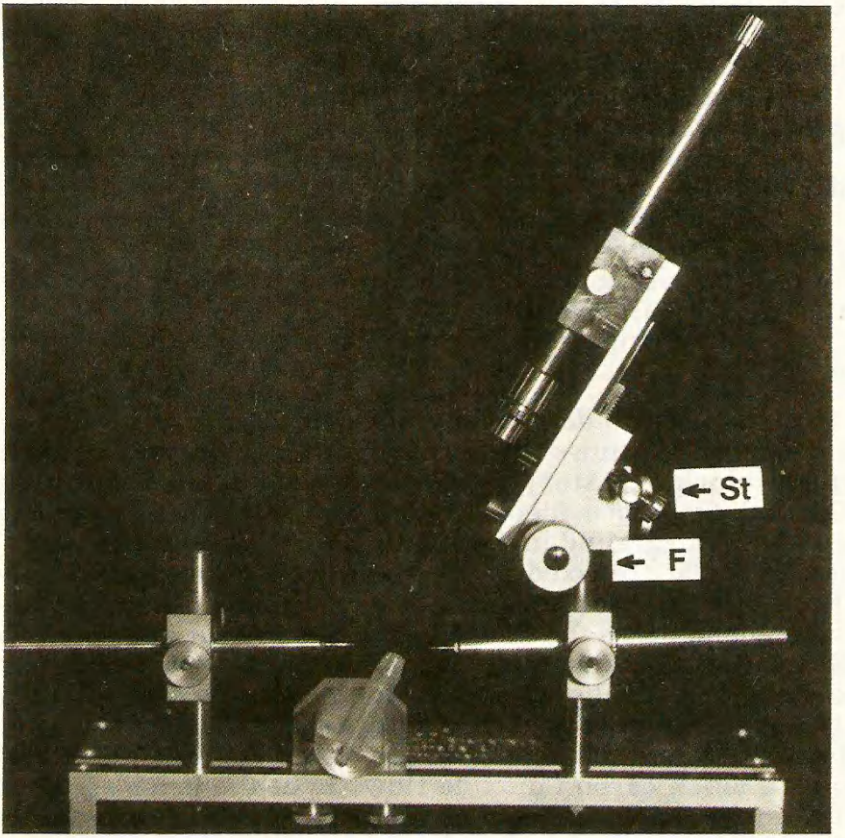


Fig. 18 — Ruttner, Schneider, Fresnaye standard device. Stand's columns and queen support are fixed to the longitudinal orifices from the foundamental plate. Their position can be changed. The handles for the ventral hook (left) and for sting's hook (right) are passed through certain balls and could be revolved anyhow. The fine F adjustment is moved through the syringe's support frontward and backward, and the St adjustment screw modifies the inclination. The syringe is provided with a larger adapter for glass points.

dividual developments. Almost all constituent parts have been substantially modified to provide for technical improvements (i.e. the queen holder devised by RUTTNER, including fine adjustments for alignment of the various parts). Special heed was paid to the introduction of the glass tips to replace the plastic tips used by Mackensen. The reason was their lower cost, but primarily providing for better hygienic conditions.

The "standard" apparatus (RUTTNER, SCHNEIDER, FRESNAYE, 1974) — brings together several of the above improvements. Since then, the standard apparatus has asserted itself for its performances many times. It will be described with its latest improvements.

The insemination apparatus (Fig. 18) consists of the following basic elements:

1. A stand with horizontal adjustment of the vertical supporters (posts) and of the queen block;
2. The queen block with the gas tube and the queen holder (Fig. 19);
3. The sting hook and the ventral hook, each of them fixed in a ball joint in the vertical support;
4. The syringe block with the rack-and-pinion drive and the adjustment screw;
5. The syringe (Mackensen type) with adapter for plastic or glass tips.

The queen holder developed by Ruttner

Figure 19 shows the position of the queen-holder in the queen block. The stopper is tightly fitted into the fixing plate. Gas supply is provided through the openings in the main component part and in the fixing plate. The queen block and the gas tube are fixed in the stand with tightening screws. The queen is first introduced into an auxiliary tube, with the same diameter as the queen holder, opened at one end only. She will walk inside the tube, and when she reaches the closed end of the tube she will go back, into the queen holder. The stopper will be pushed in before she has a chance to move forward again. When the queen holder is attached to the queen block carbon dioxide flows in at the prior adjusted. The inclination of the queen block can be afterwards adjusted, if necessary, after first loosening the nut M. For fine adjustment, the queen block can be moved or turned to the left or right, which is an important improvement as compared to the older types.

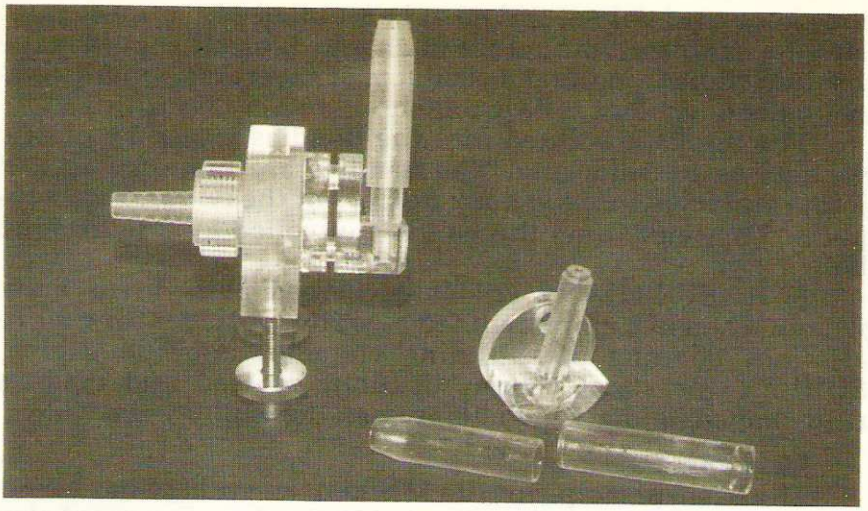


Fig. 19 — Queen's block (according to Ruttner).
Assembled on left, from lateral position. On right without stopper, having in front queen's support and entering tube.

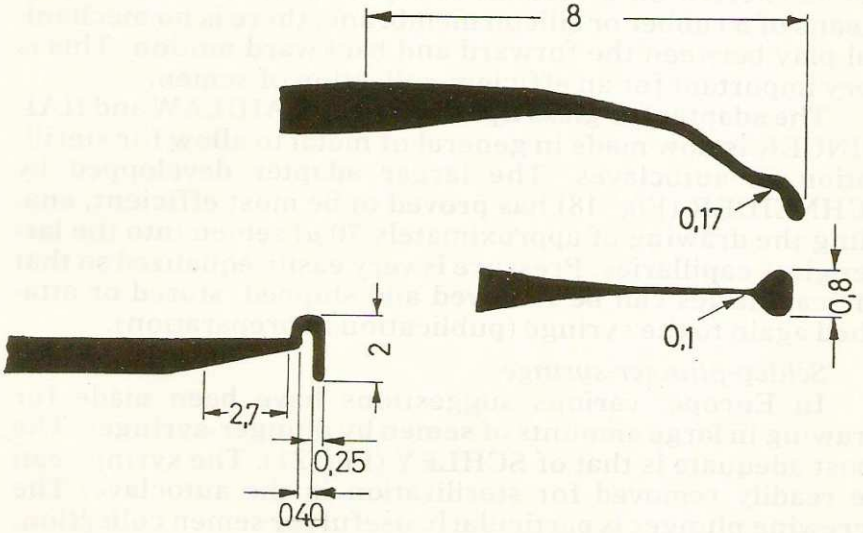


Fig. 20 — Ventral hook (left) and sting's hook (right)

Hooks and their mounting

The standard sizes of the hooks are given in Fig. 20. Particularly the sting hook must be made so accurately as to precisely fit into the sting chamber. Because its very thin neck it

may be easily broken, and must be handled with utmost care. He who cannot manufacture the hooks himself should make a sound choice among the products of several suppliers. Spare hooks must always be available.

The hooks are fitted into the handles either by plastic sockets or by soldering. The handles are mounted in ball joints and fixed into the hook holders of the two vertical supports. The tension on the ball is controlled by a tightening bolt.

SCHAFFERHANS (1987) developed a modified hook which, when in a more inclined position, provides for a more efficient opening of the sting chamber and the vagina. SCHLEY (personal communication) devised and made a ring-shaped holder which helps open the vagina even wider.

Mackensen syringe

The basic principle of the Mackensen syringe has asserted itself as the most adequate and it was therefore used also for the standard apparatus. Because the pressure of the pusher is exerted on a column of liquid with no air at all, by means of a rubber or silicon membrane, there is no mechanical play between the forward and backward motion. This is very important for an efficient collection of semen.

The adapter for glass tips devised by LAIDLAW and HAIDINGER is now made in general of metal to allow for sterilization in autoclaves. The larger adapter developped by SCHNEIDER (Fig. 18) has proved to be most efficient, enabling the drawing of approximately 70 μl semen into the larger glass capillaries. Pressure is very easily equalized so that the capillaries can be removed and shipped, stored or attached again to the syringe (publication in preparation).

Schley-plunger-syringe

In Europe, various suggestions have been made for drawing in large amounts of semen by plunger-syringes. The most adequate is that of SCHLEY (Fig. 21). The syringe can be readily removed for sterilization in the autoclave. The screwing plunger is particularly useful for semen collection. Additional facilities include a fast tensioning device and a dosimeter (for details see Schley's description, 1987).

The syringe block

The syringe block is attached to the right vertical supporter (stand post), above the hook holder. The syringe holder is a groove in which the syringe is easily and quickly introdu-

ced. It is fastened to a rack-and-pinion drive serial product of microscope mechanical stages screw enables fine adjustment of the inclination angle. When the tip is initially introduced incompletely and more inclined and is next slightly moved ventrally by means of the adjustment screw, it can be further inserted past the valvefold without using a vaginal probe (VESELY, 1967). To ease this operation, SCHLEY (1987) added another adjustment screw for parallel lateral control of the syringe movements. Another adjustment screw enables diagonal positioning, which previously could be obtained only by revolving the whole syringe block round the vertical support.

The tension of the rack-and-pinion drive of the syringe holder is adjusted so that the holder may be lightly moved and yet remain steadily in place. If it happens to slip down slowly, when the temperature is too high, the use of a harder vaseline is recommended for greasing the rack-and-pinion drive.

The distance between the insemination syringe and the queen block is relatively short in the standard apparatus so that the use of larger capillaries as syringe tips is not possible. But the distance can be slightly increased by attaching the syringe block to a third vertical support post (longer than the other two) (SCHNEIDER, publication in preparation). For this position the Mackensen syringe with a short barrel in current insemination laboratories is recommended to be used.

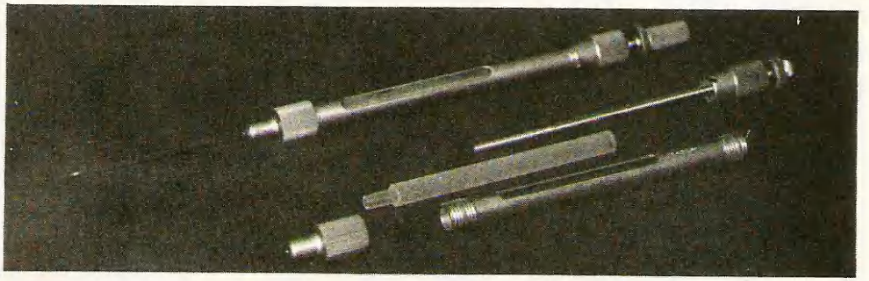


Fig. 21 — Piston syringe (according to Schley).
Assembled backward. On constituent parts frontward.

Glass tips

Glass tips are mostly used and are available on the market at fair prices. Their advantages are smooth surfaces which

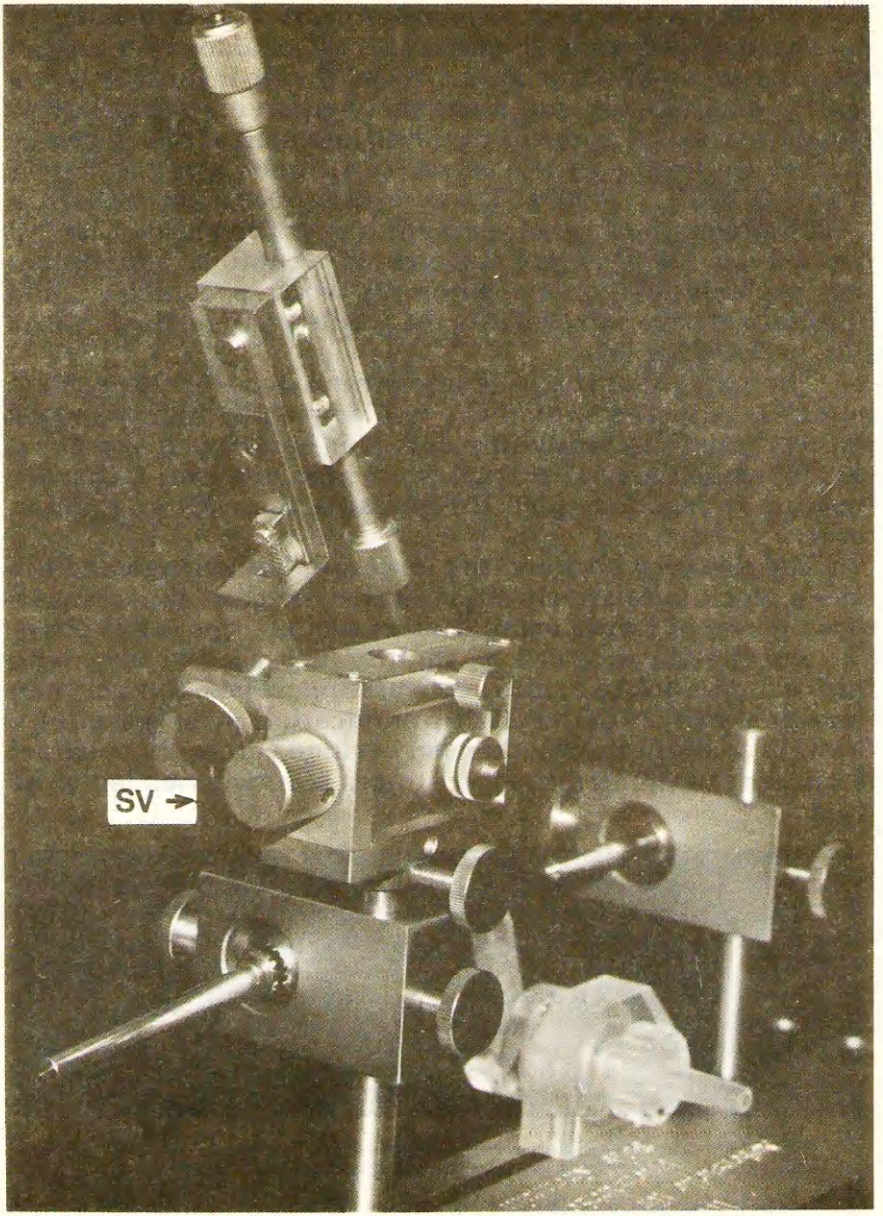


Fig. 22 — Syringe's block (according to Schley) (seen from the back)
Additional screw Sv makes the horizontal movement of the entire syringe's block easier, so that the point might be introduced without using the probe. Below is shown the ball for hooks' handles.

enable easier introduction, and efficient cleaning and sterilization.

SCHLEY (1981, a, b) devised a special apparatus for automatic stretching of the usual micropipettes into a desired tip shape which is also equipped with a device for re-sharpening the tips. The inner diameter at the end of the tip is 0.18 mm, and the outer diameter is 0.3 mm. The micropipettes of the initial, normal size may be used for storage or shipping of semen, being provided for this purpose with a cap made of a silicon or tygon tubing.

Other individually devised insemination apparatuses

In eastern Europe the apparatus devised by VESELY is widely used. A rack and pinion drive is directly attached to the syringe barrel and provides for back and forward movement.

A.and C.WINKLER (1981, 1986) integrated the insemination apparatus, the microscope and CO_2 supply into a single, portable unit. Plunger syringes are used, similar to the Schley syringes.

In Denmark, HOLM (1986) has developed an apparatus, initially devised by SWIENTY, in which the movements of hooks and syringe are controlled by micromanipulators. Finally we mention a Dutch model as representative for all original individual achievements. It is devised almost entirely with elements of the Fischer technique toy kit and works perfectly well.

Microscope, illumination and CO_2 supply

When making a choice from the very many stereoscopic microscopes available, special heed has to be paid to

— the height to which it can be adjusted during insemination,
— the magnification range,
— the field of view and
— the sharpness of spot focus.

At present, fluorescent lamps with glass fibres are preferred instead of the low-voltage lamps with heat-proof filters. As the light is projected from the end of the flexible "swan neck" it can be brought very close to the queen without requiring much space for that.

The carbon dioxide necessary for anesthetization is generally available in cylinders, the flow being adjusted by a valve. Small cylinders are easier to handle than the large ones and can be re-filled with gas from the large cylinder.

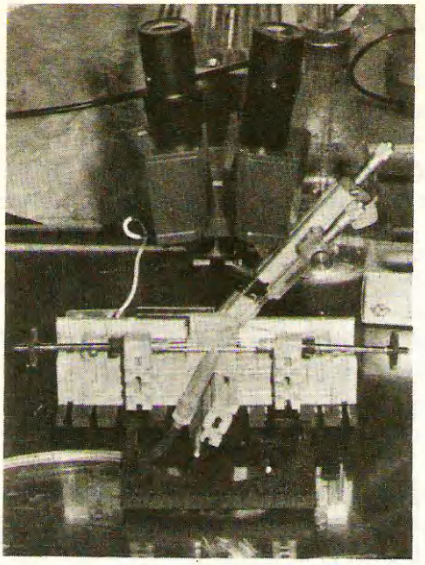


Fig. 23 — Insemination apparatus (anyone can make it), according to Fischer's technique

Addresses of suppliers of insemination apparatuses and auxiliary equipment

W. Seip, Hauptstr. 34—36, D-6 308 Butzbach-Ebersgöns; the standard apparatus with various modifications according to Schley, with plunger syringe, all component parts, and courses of instrumental insemination.

W. Uhl. Loherstr. 7, D-6 334 Aßlar; standard apparatus with Mackensen syringe (large adapter with threads in centimeters), and all auxiliary equipment and parts.

K. Burmeister, Bonebüttelar Weg 12, D-2350 Neumünster: glass tips.

Hessische Landesanstalt für Tierzucht, Abteilung für Bienenzucht, Erlenstr. 9, D-3575 Kirchhain: larger adapter for Mackensen syringes (with threads in inches); instrumental insemination courses.

Vaca Valley Apiaries G745 Buchtown Lane, Vacaville CA 95688 USA — Mackensen/Dowe apparatus, hydraulic syringe, glass tips, Schley instrument

Sweetwater Apiaries Box 449, Tylertown MI 39667 USA — Mackensen/Dowe apparatus, hydraulic syringe, glass tips

Honey Bee Genetics Box 1672 Vacaville CA 955696 USA — Modified Mackensen apparatus, glass & plastic tips with metric threads and threads in inch.

SPERM STORAGE

J. R. HARBO

A. Natural Storage

Storage of sperm in a specialized pouch (the spermatheca) is a normal process for queen honey bees, as it is for most female insects. The spermatheca of a queen bee receives sperm from a natural insemination (when a queen is 1 — 2 weeks old), or during instrumental insemination. Once insemination occurs and egg laying begins, no more sperm will enter the spermatheca; breeders have not been able to successfully reinseminate queens after they have been mated and laying, and queens will not mate naturally again. A queen releases sperm from her spermatheca as she lays eggs, so all sperm are stored in the spermatheca for at least 2 days (the time from insemination until the laying of the first egg) and some are stored for the entire lifetime of the queen, often as long as 3 years.

Thus the storage of honey-bee sperm is standard practice and occurs in every mated queen. Since sperm can be taken from the spermatheca of one or more queens and used to inseminate a virgin queen (CALE and GOWEN, 1964), a bee breeder has the opportunity to use this natural storage system. Sperm that have been in a spermatheca for over 2 years are still able to migrate to the spermatheca of a second queen and produce worker progeny. Therefore, one could inseminate a group of queens with sperm that are to be stored and then recover these sperm (possibly 1 or 2 years later) from the spermathecae of the surviving queens.

Little is known about the metabolic activity of sperm while in the spermatheca, but they are probably stored at a very low metabolic state (TABER and BLUM, 1960; LENSKY and SCHINDLER, 1967; POOLE, 1970). Operating on live, mated queens, KOENIGER (1970) removed 50 — 70% of the tracheal net that surrounds the spermatheca and discovered that sperm became immobile in about 3 weeks. POOLE (1970 and 1972) concluded that the function of the dense tracheal network surrounding the spermatheca is to supply oxygen to the columnar cells that form the spermathecal wall. By operating on live queens, POOLE (1972) learned that the columnar cells died in areas where the tracheal network had been

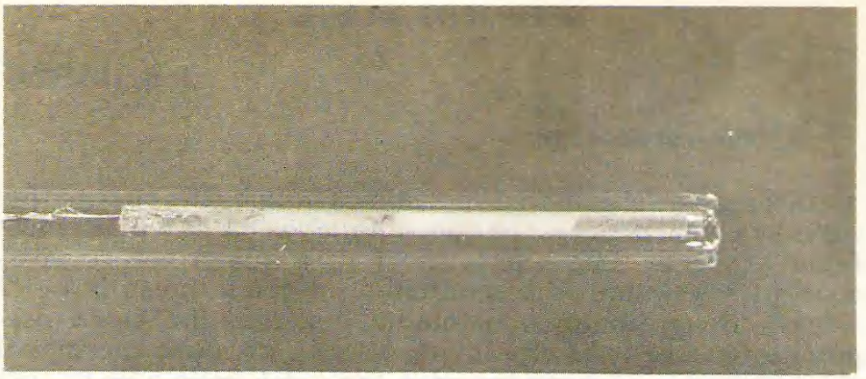


Fig. 24 — A glass capillary tube of semen that is ready for storage in liquid nitrogen.

This tube is inside of a larger plastic tube (glass was used for the photograph) that has an inside diameter of 2 mm and a length of 29 mm. The large tube is sealed at the bottom and keeps the tube of semen from becoming lost in the tank of liquid nitrogen.

Nearly all methods of storing semen use capillary tubes as containers for semen and seal the ends by various means. The semen in the tube above was sealed between plugs of petrolatum. No visible air was allowed between the semen and the plugs. A plug is formed by moving a column of semen to one end of the capillary tube and then pushing that end through a mound of petrolatum. The column is then pushed to the other end of the capillary (the plug of petrolatum follows, intact), and the other end is pushed through the mound of petrolatum. When it is time to use the semen, the plug that leads the column is collected as it emerges from the tube; the plug following the column acts as a piston to reduce the amount of semen that coats the inside of the capillary.

The tube above has a thermocouple entering from the right and ending near the center of the semen. When storing many tubes at once, only one tube needs a thermocouple. The rates of cooling and warming can be monitored by connecting the thermocouple to a millivolt, strip-chart recorder.

removed, and he concluded that the spermatheca serves as an isolating structure for sperm. The survival of sperm *in vitro* for 8 months in a sealed glass capillary tube (POOLE and TABER 1970), support this hypothesis by showing that sperm can survive long periods with little oxygen and no additional nutrients.

B. *In Vitro* Storage

1. Storage methods

Nearly all methods of storing honey-bee sperm use glass capillary tubes as containers for the semen and seal the ends by various means. TABER (1961) observed that it was better if the tube contained less air than semen rather than more air than semen. Therefore, HARBO (1973) began storing semen in tubes that contained no visible air (Fig. 24).

There is no standard diluent for honey-bee semen, nor is there a standard rate of dilution for semen that is to be stored. POOLE and TABER (1970) did not dilute semen when they successfully stored it for 35 weeks at 13 — 15°C. They simply

dusted a dry capillary tube with an antibiotic (streptomycin sulfate) before semen was collected into the tube. HARBO (1979 a) diluted semen 1:1 when storing in liquid nitrogen ($-196^{\circ}C$). Others have diluted semen at a much higher rate in order to test diluents (CAMARGO, 1975; VERMA, 1978; WILLIAMS and HARBO, 1982; WILLIAMS, 1983; MORITZ, 1984). Nearly any diluent is adequate when semen is diluted only slightly (such as 10:1, semen: diluent), but the composition of the diluent becomes very important at higher dilution rates (1:10 or 1:100). POOLE and EDWARDS (1970) found that if sperm motility is used as an evaluation criterion, sugar must be present in the final diluent.

2. Storage at nonfreezing temperatures

Studies have show that sperm should be kept between 13° and $25^{\circ}C$ when stored at nonfreezing temperatures. Cooler temperatures, 2°(TABER and BLUM, 1960), 5° and $10^{\circ}C$ (HARBO and WILLIAMS, 1987) cause high mortality of sperm. For long-term storage (13 — 35 weeks), POOLE and TABER (1970) showed that 13° — 15° was far better than room temperature (about 24°), but for short-term storage (2 days), HARBO and WILLIAMS (1987) calculated the optimum temperature to be $21^{\circ}C$.

There is evidence that honey-bee sperm can survive for at least one week with little or no loss in viability. Vesely and Titera (unpublished) stored undiluted semen in capillary tubes at room temperature. While in a capillary tube, a column of semen in their study was sealed at each end with an air space, a short column of semen, another air space, and a mechanical plug. Seven days of storage did not reduce the number of sperm entering the spermatheca; however, 12 and 16 days of storage showed a 37 and 75% decline, respectively. In a similar study, Harbo (unpublished) sealed diluted semen with petrolatum (Fig. 24) and stored it at $15^{\circ}C$ for 1, 3, 6, 10, 15 and 30 days. Semen was diluted 8:1 (semen: saline), and the saline consisted of 0.85% NaCl and 0.25% dihydrostreptomycin sulfate. Samples stored 15 days or less did not differ from controls (diluted but not stored) in the number of sperm entering the spermatheca; samples stored 30 days had 27% fewer ($P < 0.01$).

3. Storage at subfreezing temperatures

The first successful reports of storing honey-bee sperm at subfreezing temperatures were those of SAWADA and

CHANG (1964) for storage in dry ice (−79°C) and MELNICHENDO and VAVILOV (1975) for storage in liquid nitrogen (−196°C). Since that time, others (HARBO, 1977; KAFTANOGLU and PENG, 1984) have produced progeny from sperm that have been stored for 1 (KAFTANOGLU and PENNG, 1984) and 2 years (HARBO, 1983) in liquid nitrogen.

When storing sperm in liquid nitrogen, the diluent often contains a chemical that protects the sperm from freeze damage. Melnichenko and Vavilov (1975) and Verma (1983) used hemolymph of the honey bee as a cryoprotective material; Harbo (1977) and Kaftanoglu and Peng (1984) used dimethyl sulfoxide (DMSO). The most common dilution with DMSO consisted of 10% DMSO, 40% saline and 50% semen.

When used with honey bee sperm, both hemolymph and DMSO have undesirable qualities. Hemolymph makes inseminations difficult because it causes the semen to coagulate (VERMA, 1983). DMSO caused sterility in about 3% of the queens that were produced from spermatozoa that had been in a 10% solution of DMSO (HARBO, 1986).

The rate at which sperm are cooled from room temperature to liquid nitrogen (−196°C) and the rate of warming back to room temperature are important. All agree that rapid warming allows the best survial for honey bee spermatozoa, but researchers have found success with various cooling rates. MELNICHENKO and VAVILOV (1975) found that a rapid rate of cooling and warming was far better than either gradual cooling and warming or rapid cooling and gradual warming. A fourth possibility, gradual cooling and rapid warming, was recommended by HARBO (1979) and KAFTANOGLU and PENG (1984). The gradual cooling rate used by Harbo was between 4 and 40° per minute (usually about 25° per minute) throughout the 220°range. Cooling rates used by Kaftanoglu and Peng ranged from 0.5 to 30° per minute, and they plunged the sample into liquid nitrogen when it reached −40°C.

4. Damage to sperm

Sperm sustain various levels of damage as a result of being stored in liquid nitrogen. A sperm may be dead or perfectly viable after storage, but many express partial damage. Five examples of partial damage are arranged below in a progression from the most to the least severe. A typical sample that has been stored in liquid nitrogen will contain sperm

from each of the categories below plus dead and perfectly viable cells.

a. Sperm show at least some motility when examined microscopically but do not enter the spermatheca after insemination. A sample contains sperm with this level of damage and worse (dead and immobile) only when there is some motility and no sperm enter the spermatheca. In another example, if some sperm enter the spermatheca but the number is fewer than expected, then the sample contains sperm with this level of damage, possibly some dead cells, and certainly some cells that are damaged less or undamaged.

b. A sperm enters the spermatheca but is not able to fertilize an egg. One can expect 100% worker brood from a young newly-inseminated queen that has 100,000 or more sperm in her spermatheca (HARBO 1985). However, queens inseminated with sperm stored in liquid nitrogen often contained 600,000 in their spermatheca and these produced only 55 or 75% worker brood (HARBO, 1979 a; KAFTANOGLU and PENG, 1984).

c. A sperm enters the spermatheca and apparently enters an egg, but causes the egg not to hatch. Nearly all queens produce a small percentage of eggs (about 5%) that do not hatch (HARBO, 1981). However, queens inseminated with sperm that have been stored in liquid nitrogen sometimes produce more non-hatching eggs than control queens (HARBO, 1979b). After entering the egg, the sperm pronucleus probably combines with the egg pronucleus to produce a nonviable zygote.

d. A sperm enters the spermatheca and enters the egg, but it does not combine with the egg pronucleus, and both the sperm pronucleus and the egg pronucleus develop into male tissue. This results in the production of normal mosaic males which were detectable because of the use of genetic eye and body markers in the stored sperm (HARBO, 1980). Thus a sperm pronucleus contributed to the development of a mosaic male because it did not unite with an egg pronucleus. But when queens were inseminated with semen from these mosaics, the identical genetic replicates of this sperm pronucleus did unite with egg pronuclei to produce normal worker bees, and no mosaic males. Therefore, if freezing in liquid nitrogen caused some sperm to be unable to unite with an egg pronucleus, this inability was not expressed by their genetic replicates in the next generation.

e. An apparently normal female is produced from stored sperm, but the queen is sterile and produces only non hatching eggs. Harbo (1986) found that this sterility, found in about 3% of the queen progeny, was caused by the cryoprotecting chemical, dimethyl sulfoxide (DMSO), and not by the freezing process.

5. Present Use of Sperm Storage

Routine long-term storage of bee sperm (storage for 6 months or more) is not used anywhere. Although problems remain for storage of bee sperm in liquid nitrogen, nitrogen storage shows more promise than other methods, and considerable progress has been made in the past 15 years. For example, honey bee sperm can survive the harsh transition from room temperature to $-196^{\circ}C$, methods for handling and storing semen have been developed, and various levels of damage to sperm have been identified and can be used as guidelines for evaluating future results.

In contrast, short-term storage (storage for 1 week or less) at nonfreezing temperatures is widely used. This includes semen that is shipped, semen that is collected into syringes or storage tubes and used the following day, and semen that is diluted and mixed (perhaps centrifuged as KAFTANOGLU and PENG [1980] and MORITZ [1983] suggest) and used later the same day.

THE INSEMINATION PROCEDURE

R.F.A. MORITZ

With thorough knowledge of the anatomy of the reproductive organs, the artificial insemination technique is not difficult. But with cheaper insemination apparatuses the operator must be skilled not to hurt the queen during insemination. The latest advances in the field have made the work easier for beginners, as the micromanipulators with which they are equipped move the syringe and the sting hook almost without the slightest shaking. Experienced insemination operators often prefer simpler models because when working intensively they are more resistant and more quickly handled.

Recommendations to beginners

Under the guidance of an experienced operator it is not difficult to learn how to perform instrumental insemination. In several countries, private or state institutions regularly organize training courses. But often such courses are not available, so that the beginner must travel by himself along the difficult road which leads — after trials, error and accomplishments — to the successfully instrumentally inseminated queen. Guidelines for instrumental insemination are given here to minimize mistakes and to provide for actually successful inseminations from the very beginning.

Preliminary practising

First, the simpler technique of drawing semen into the syringe must be practised. This provides experience for subsequent handling of the insemination apparatus and of the different mechanisms controlling the movement of the micromanipulators. Coordination of the movements under the microscope is quite elaborate and requires practising. We recommend the beginner to draw semen into the insemination syringe by using a 10—12 x magnification. When he can draw semen satisfactorily (8μl semen in 15 minutes) he can start handling the queens.

First of all the fixing of the hooks must be practised with queens. After several successful trials, attempts to introduce the insemination syringe can be made. For the first trials the syringe must be filled with saline solution (Table 2) not with

semen. With an adequate positioning of the syringe the saline solution should be readily and easily injected into the vaginal orifice. If strong resistance is opposed, the angle of inclination must be changed and then another trial to introduce the syringe must be made.

The large queens are the most adequate for training. First, one should try to inseminate only the queens in which the valve fold is easily located. Great differences may exist in this respect depending on the queens' origin. A 20x magnification makes the work easier.

At the beginning, positioning of the queen and proper adjustment of the position of the syringe might take a very long time. There is a great danger in drying of the exposed tissues. In such a case, the syringe cannot be inserted without problems and the queen is very likely to be hurt. That is why, the beginner must work in conditions of high air humidity. Also, the semen may dry and clog in the insemination syringe. This may be avoided by regularly moistening the tip with sterile buffer solution (Table 2).

Table 2

Buffer and dilution solutions to be used during instrumental insemination (in % weight). The substances will be dissolved in sterile distilled water. The pH value is adjusted by adding a few drops of NaOH. Be careful! Solutions should always be prepared only shortly before use; storage may alter thei pH value.

Physiological saline solution according to Hyes	Varhom or Kiev diluant	Tris buffer solution
0.9% NaCl	—	1.1% NaCl
0,2% KCl	0,04% KCl	—
0,2% CaCl	—	—
0,1% $NaHCO_3$	0,21% $NaHCO_3$	—
—	0,3% glucose D	0,1% glucose D
—	2,43% trinatrium-citrate 2-hydrate	—
—	0,3% sulphamilamide	—
—	—	0,01% arginine
—	—	0,01% lysine
—	—	0,61% tris
pH 8,5	ph 8,7	pH 8,8

Obtaining and drawing semen into the syringe

First the insemination syringe must be prepared: it must be filled with sterile buffer solution in keeping with the type of syringe and directions for use given by the producer (see Table 2). The plunger is pushed down until enough space is available for drawing in the necessary amount of semen. Then the syringe is mounted into the holder of the insemination apparatus. The microscope is adjusted to a 10x magnification and is focused on the tip of the syringe. Now drones are prepared.

Obtaining the semen for instrumental insemination is often the most difficult problem. Even in routine practice of instrumental insemination the necessary number of drones is often not available, or the drones are not sexually mature when needed (see the chapter "Rearing and maintaining drones"). Therefore, utmost care should be taken of the drones before insemination, and above all they should not be kept in cold places. The acclimation boxes are now widely used, inside which drones can fly freely and release faeces. Even when using this method, the amount of semen obtained from different drones will not be the same as it considerably depends on the conditions in the drone rearing colony. For the first attempts one should select the drones ready to perform the mating flight, caught in the first afternoon hours from the hive entrance. Obtaining semen from drones kept for a long time in cages is always difficult. Eversion and ejaculation may be induced manually or by using chloroform.

Inducing eversion manually

The drone is grasped by the thorax with the thumb and fore finger of the left hand, with the drone's ventral surface upwards. While the head and the anterior part of the thorax are squeezed with the left hand, the dorsal part of the abdomen is teased or repeatedly squeezed lightly with the thumb and forefinger of the right hand. In a sexually mature drone this usually causes contraction of the abdominal muscles and a partial or complete eversion of the endophallus. If no semen is visible after eversion the abdomen is squeezed from the dorsal to the ventral part in order to continue the eversion by force until the semen is ejected. Usually, no semen is obtained without abdominal contraction. When the abdomen contracts without partial eversion, semen can be obtained by enhanced pressure on the abdomen.

Inducing eversion by using chloroform

Contraction of the abdomen may also be induced by using chloroform. A narcosis bottle (approximately 100 ml) with large neck is used. It is important that the bottle is closed air tight with a glass cap. The walls and bottom are lined with filter paper. This is very convenient because by taking out the filter paper, the drone faeces are readily and properly removed. A cotton ball on which a few drops of chloroform are poured is placed between the filter paper and the bottom of the bottle. Drones are introduced into the bottle, one at a time. First they are very agitated. After few seconds the abdomens of adult drones become rigid and eversion takes place. If eversion is only partial the process can be completed by squeezing the abdomen until semen is ejected.

When using chloroform utmost care should be taken. The use of a breathing protection mask and gloves recommended is because chloroform is a highly toxic substance for man. It is readily absorbed through skin and can produce lesions of the liver, if constantly used. In addition, it is inflammable and one should by no means smoke (which in general is desirable, not only when using chloroform)! While a number of experts assess that use of chloroform substantially facilitates starting of eversion, others say that too much time is wasted. Taking into consideration its undesirable effects, chloroform should only be used by way of exception, as for example with highly valuable drones, but by no means in routine insemination operations.

Mucus and semen

After successful eversion, semen and mucus appear on the endophallus. The amount of semen and its distribution may vary greatly. Usually, the cream-coloured semen lies on a layer of white mucus. Immature drones produce only mucus but no sperm. Before the beginner collects the semen, he must be sure which is semen and which is mucus; It is helpful to distinguish both on a few drones before collecting semen for the first time. If mucus is drawn into the syringe it will hinder insemination; this is one of the most frequent mistakes made by beginners.

After eversion, semen quickly spreads in a thin layer over the mucus, and can only be collected with difficulties. It is therefore important to avoid any delay in drawing the semen into the syringe.

After eversion, the drone is brought between the thumb and forefinger of the left hand, with the endophallus near the tip of the syringe. The plunger is slightly withdrawn to provide an air space so that semen cannot mix with the buffer solution and be diluted by it. The surface of the semen is then made to touch the point of the syringe at about a 45-degrees

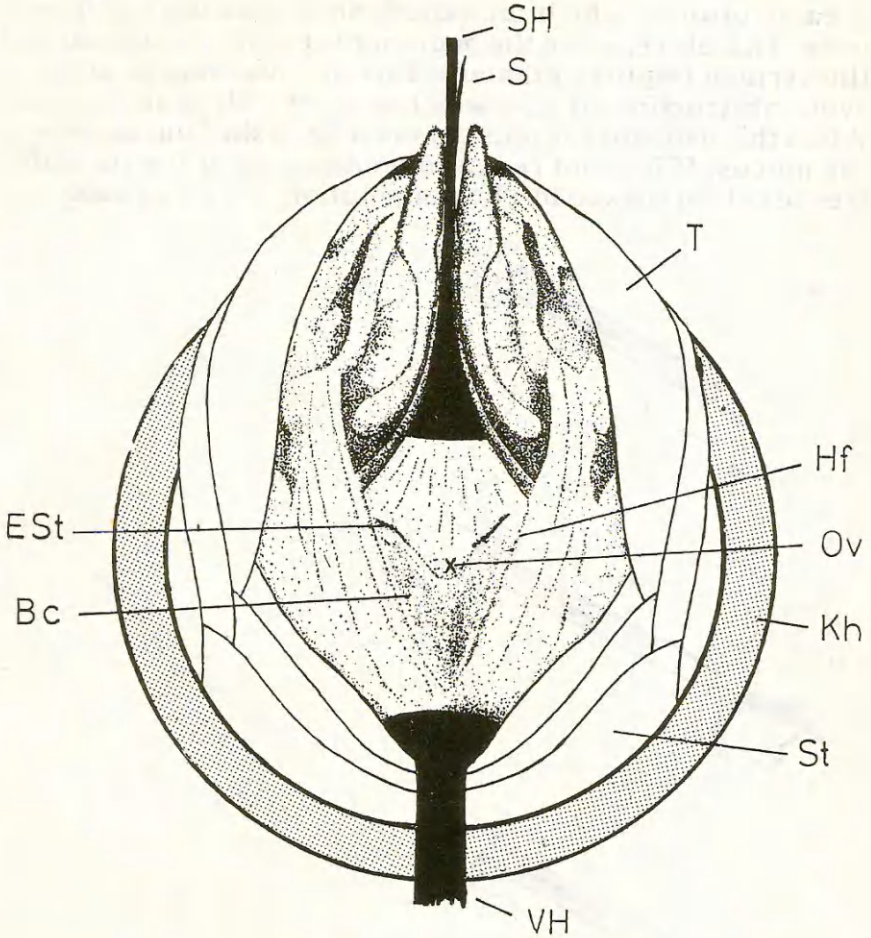


Fig. 25 — For insemination, the queen's sting chamber is opened using the VH ventral hook and the SH sting's hook. The Hf skin folds form an isoscele triangle. The vaginal opening a is found on the site marked with x and underneath is the vaginal valva, which cannot be seen from the outside.

Bc — Bursa copulatrix; ESt — entrance to the lateral pouches; Hf — skin folds; Ch — queen's support; a — vaginal orifice; S — sting; SH — sting's hook; St — sternite; T — tergite; VH — ventral hook

angle (do not take up semen Fig. 26). When semen adheres to the tip, the drone is pulled away from the syringe slightly without breaking contact so that the semen will continue to adhere to the tip. When withdrawing the plunger the semen will flow easily and fast toward the capillary tip. This procedure avoids taking up the mucus into the syringe. Mucus is too viscous to pass into the tip. Taking up mucus requires greater efforts, which an experienced operator notices at once. Therefore, when the beginner sees that the loacking of the syringe requires greater effort he must stop in order to avoid obstruction of tips which are very difficult to clear. When this happens the plunger must be pushed out to remove the mucus. If it is not removed, moistening of the tip with a sterile cotton soaked in a buffer solution will often help.

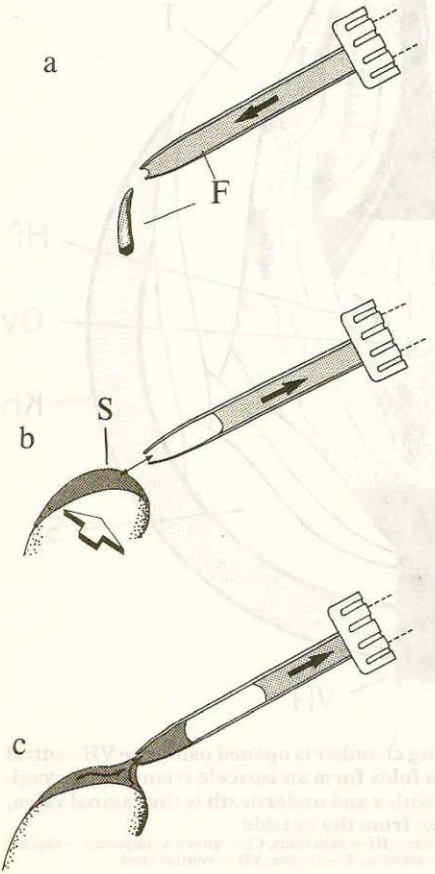


Fig. 26 — Drawing of sperm
a) first the buffer solution is drawn out from the syringe (F) and afterwards an air bubble is sucked up; b) the S sperm surface found on the drone's endophallus is very carefully drawn nearer the point of the insemination syringe; c) the sperm is absorbed, yet without introducing the point into it. The air bubble should be found in between the buffer solution and the sperm

After the entire amount of semen is collected from a drone, the plunger is withdrawn to let a little air in. No semen should be left in the syringe tip until handling the next drone, to prevent obstruction of the tip. Depending on the capacity of the syringe and on the amount needed, semen can be successively collected from several drones. In general, 1μl of semen is obtained from one adult drone. It was found that for an efficient insemination of a queen 8μl semen are necessary. In routine operations, much larger amounts are necessary for mating many queens with the same paternal line or for preparing semen samples to be used for the mixing technique (Figs. 25 and 26).

The first insemination

After the operator gets to know the construction of the insemination apparatus and is conversant with all clues in handling it and when he is able to collect semen fast, he may try the first inseminations.

If the syringe is not already positioned in the holder from collecting the semen, the first step is to mount it in the apparatus according to the directions given by the producer. The insemination tip should be moved sideways out of handling area under the microscope to avoid tip damage while installing the queen holder.

Next the queen narcosis has to be prepared. The carbon dioxide supply tube is connected to the queen holder and the gas flow is adjusted to approximately 80 ml/min. The gas flow should be controlled with a wash bottle by observing the gas bubbles passing through the water.

After the gas flow is adjusted, the queen is mounted in the holder. She is forced to enter a plastic tube of the same diameter as that of the queen holder. At the other end of the tube there is a small hole. When the queen reaches this end she will try to escape walking in opposite direction. The queen holder is placed above the large opening of the plastic tube and the queen will walk on her own into the proper position in the queen holder. Before she has a chance to move forward again, the queen holder is mounted in the insemination apparatus and if the gas flow is correctly adjusted, narcosis sets in shortly afterwards. The queen's abdomen will make a few deep respiration movements which will decline after a few seconds. Finally the queen remains still even when touched.

Opening of the sting chamber

The queen is correctly positioned in the queen holder when only the last three abdominal segments are visible. The hind legs must be placed inside the queen holder. The holder has to be turned so that the dorsal side of the queen faces the insemination syringe to the right. The two hooks for opening the sting chamber are brought close to the queen. The wider (sting) hook is to the right and the slender one, for the ventral sclerite, to the left of the microscope field. A 20x magnification is necessary for a good view of the structure of the sting chamber of the queen. First the ventral hook and then the sting hook are inserted into the sting chamber. During the subsequent operation it is essential that the end of the queen's abdomen outside of the holder is level with that of the queen's body, without any torsion. Therefore the hooks must be handled very carefully not to hurt the queen. Opening the sting chamber is indeed one of the most critical stages of instrumental insemination. Opening it too widely or incompletely will make insertion of the syringe in the vaginal orifice difficult. In Fig. 25 a correct opening of the queen's sting chamber is shown. As a basic rule, the triangle between the bases of the sting lancets must be equilateral.

Due to technical constraints the opening of the sting chamber is never optimal. During natural mating, all sides of the sting chamber are stretched out and the vaginal orifice, in the center is much more widely open. Otherwise the drone would not even be able to mate with the queen during the mating flight. Bidirectional opening is unnatural, and an essential impediment, as it is the result of the utilization of all types of insemination apparatuses available at present. Because of the stretching of the tissue, the vaginal orifice is narrowed rather than enlarged. New types of hooks have been developed, to provide for less tension and easier access to the vaginal orifice.

After opening the sting chamber, the hooks should not be touched any more, and the tip of the insemination syringe is adjusted to be a little above the vaginal orifice. With insemination apparatuses with only vertical adjustment of the syringe, the valvefold must be pushed to the left with a vaginal probe and then the tip is inserted, after which the probe is removed. With other types of apparatuses, with horizontal adjustment of the syringe, this operation is performed with the syringe tip itself: the tip is inserted about 1/2 mm into the visi-

ble vagina, is slightly moved to the left and the syringe is inclined a little more. Then the tip is inserted another 1.5 mm into the vagina. If the surrounding tissue begins to move before the syringe had reached this depth, it has probably been inserted into one of the bursal pouches that lie to either side of the vagina. The syringe must be taken out and inserted again after slightly adjusting the inclination angle. The same thing must be done when the column of semen in the tip does not begin to move immediately. In such cases, either the tip has been improperly positioned, or it was obstructed. But when the semen begins to move properly without any leakage around the tip the injection can proceed rapidly. Usually, 8 μl semen is injected into one queen.

If semen cannot be injected after several trials, one must start all over again, from the beginning, namely with the positioning of the queen. If one fails again, the queen must be introduced back into the nurse colony and one must continue with another queen. Most often, on the next day the insemination will be successful. Irrespective of the size of the queens, some of them are more difficult to inseminate than others. (Fig. 27A-D).

Preventing infection

Spreading of diseases is a great threat with instrumental insemination. The agents involved are not germs of human diseases dangerous to bees, but specific pathologic agents on the bees' bodies or from their faeces. Mucus and semen handled during insemination are an ideal culture medium for these germs as they are rich in carbohydrates and proteins. Moreover, after insemination, the queen is introduced into an "incubator" — the brood chamber of the bee colony, where the temperature is precisely 35°C; no wonder, that death of queens after insemination is often caused by bacterial and virus infections as a consequence of poor hygiene.

Bacterial septicemia develops very fast. The pathogenes are usually *Pseudomonas* bacteria (for example *(Pseudomonas antiseptica,* BAILEY, 1981). The disease is easy to recognize from the first symptoms: the slow and unsteady movements, and the swollen abdomen are typical. The queen will not start oviposition or will stop it shortly after these symptoms appear. Finally she will fall from the comb and stay alive

for some time on the bottom board before dying. Dissection of dead queens reveals black spots on various internal organs — venom gland, ovaries, the Malpighian tubules, etc. (symptoms which FYG described in 1936 as "bacterial melanosis"), which are equivocal symptoms of the disease. With acute di-

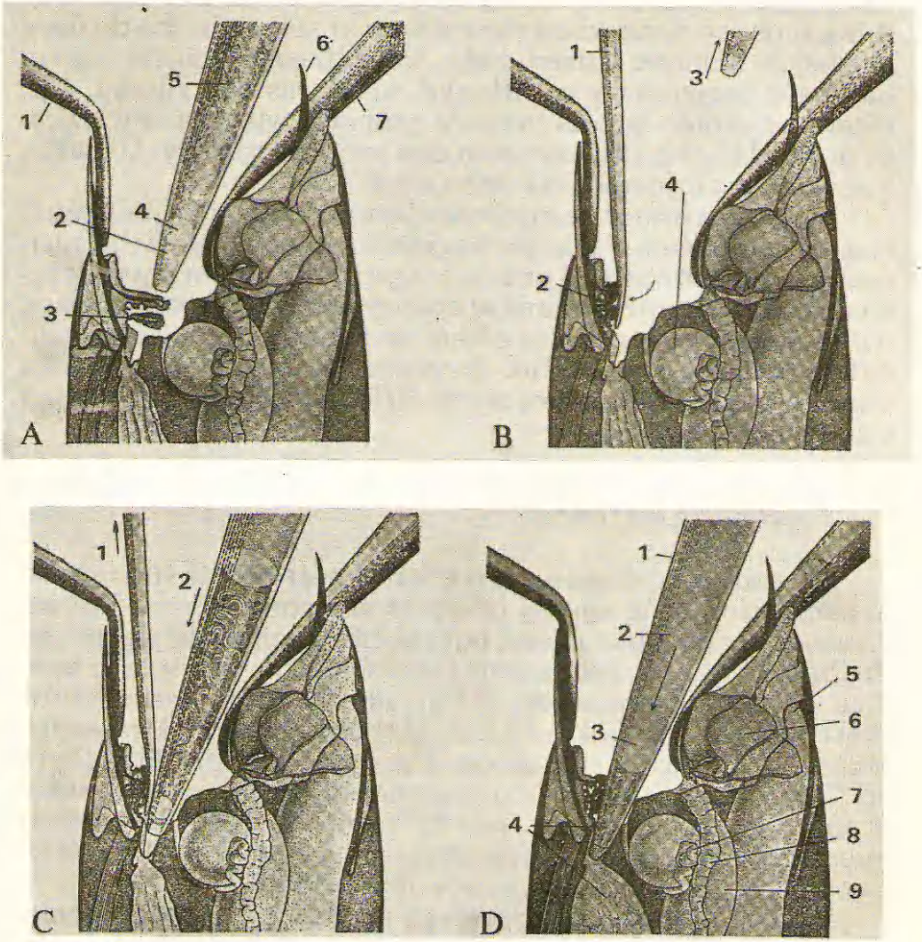


Fig. 27 — The four stages of instrumental insemination

A — adjustment of insemination syringe; 1) ventral sting; 2) buffer solution; 3) vaginal valva; 4) air bubble; 5) sperm; 6) sting; 7) the sting's hook;

B — introduction of the probe (if necessary): 1) the probe; 2) vaginal valva; 3) point of the insemination syringe; 4) the spermatheca;

C — introduction of the insemination syringe point (1) the probe (if previously used) is now withdrawn (2);

D — injecting the sperm 1) the point of the insemination syringe; 2) buffer solution; 3) air bubbles; 4) the sperm; 5) the rectum; 6) vulnerable apparatus; 7) spermatheca's gland; 8) annex gland; 9) venom pouch

sease the queen will die in 1—2 days (MACKENSEN, 1969). In the final stage the body disintegrates at the joining points of the head, thorax and abdomen.

The symptoms of chronical paralysis of bees are similar, but its progress is slower. Several days after infection queens are still on combs, sluggish and bloated with body fluid. Some will even lay a few eggs before they become sick. Finally they fall on the bottom board where they stay alive for a few days. The disease develops but much more rapid in the acute paralysis of bees, but the symptoms are similar.

Preventing such infections is not simple. Particularly with virus infections the possibilities are limited. Only the symptoms of bee diseases are known and the pathogenes are described only morphologically. Not much knowledge is available about the causal relationships between pathogene and the disease, and about the vast domain of physiopathology of the honey bee as well. Consequently, infections may be avoided only by rigorous hygienic conditions.

Nosema disease of the queen can be prevented. It is a slowly developing disease which considerably shortens the queen's life because of its associate symptom, diarrhea. Infection occurs not during insemination but in the nurse colony or the mating nucleus. *Nosema apis* spores spreads through the faeces of diseased insects which they release inside the hive. Therefore, under natural conditions, mostly worker bees will be infected as they clean the hive. Infections of queens occurs rarely, because usually they do not take food from combs (BAILEY, 1972). Infection of the queen only occurs when she is fed by infected worker bees. This can easily be prevented by stocking the mating nuclei with young bees from healthy colonies. The mating nuclei must always be filled with bees from the best colonies. This is often painful, but this is the only way that failures and mass infection of highly performing inseminated queens will be prevented.

The steps taken outside the insemination laboratory for preventing bee diseases are as equally important as the hygiene requirements in the insemination laboratory. Mating nuclei must be disinfected before being used, by sterilization in flame or immersion into hot lye wash. The mating nuclei must be stocked with bees from strong and healthy- colonies. The queen cannot be more vital than the nurse colony; sparing of the bee stock is in no case advantageous.

Consequently, rigorous observance is required of specific rules in the insemination activity and with respect to the laboratory equipment. Absolute sterile conditions in an insemination laboratory are impossible, but aseptic work conditions considerably reduce number of pathogenes and hence also the possibility of an infection. Cleanliness itself plays an essential role: hands must be washed whenever necessary with a disinfectant solution which is not harmful to the skin, and the desk top and all equipment and devices used must be regulary cleaned with alcohol 80% or with other efficient disinfectants. No bees must fly around in the laboratory (as a matter of fact bees should never be introduced). They are usually the major cause of infection. The desk top material must be easy to clean and therefore wood is not recommended. The best are strong plastic materials or stainless steel. Faience is resistant enough but difficult to be cleaned between tiles and, in addition causes frequent breakage of glass ware.

An absolutely necessary equipment in an insemination laboratory is the autoclave (or at least a pressure cooker). Before inseminating a new batch of queens, all equipment with direct contact with the queen and semen must be kept in the autoclave for 20 minutes, at approximately 120°C. Even the distilled water and the saline solutions should be sterilized in the autoclave, or at least boiled again.

Items of plastic material cannot be introduced into the autoclave, and some of them do not tolerate alcohol and other organic diluting substances. Such items will be immersed into a special aqueous disinfectant solution. After taking the items from the autoclave, they must be kept in alcohol 80% before being used. But neither the alcohol, nor the disinfectant solutions must reach the queen or semen.

Preparation of the buffer solution

The saline solution with which the syringe is filled before taking up semen substantially contributes to the success of insemination. The pH value and the concentration of salts in the solution (osmolarity) are of primary importance. WILLIAMS (1983) reported that the saline solution with low concentrations have a negative effect on the viability of semen. If semen is not diluted for use, and if the solution is only used as buffer solution, the most adequate is the physiologi-

cal saline solution of Hyes (see Table 2). The pH value of the solution is adjusted to 8,5 with soda lye. It is therefore desiderable that an insemination laboratory be also equipped with a pH-meter. But the pH can also be determined quite accurately by using an indicator paper. The solution must be prepared in a sterile glass beaker and be subsequently boiled or sterilized in the autoclave. Inside the autoclave, the beaker must be covered by a sheet of aluminium foil, in order to avoid excessive evaporation of the solutions. The amount of solution lost by boiling must be replaced by sterile distilled water.

In many insemination laboratories a diluent solution containing citrate is also used: "Varohm" or Kiev diluent (Table 2). It is also used in insemination of swine and is available on the market. Unfortunately, this diluent has the disadvantage that it cannot be sterilized in the autoclave or boiled without setting a brownish colour. This can be avoided by heating it to only 90°C. For avoiding infections, some inseminators also add antibiotics after sterilization (penicillin G and streptomycin). But the most important rule for the prevention of infections by use of buffer solutions is to always have freshly prepared solutions. They should not be usedafter three days storage in the refrigerator.

Before filling the insemination syringe with the buffer solution, it must be thoroughly rinsed with sterilized water to remove possible traces of alcohol. It is also recommended to have a small dish with sterile buffer solution at hand to soak the cotton tip for cleaning. The sterilized equipment, the queen and semen should by no means be touched with the hand. Especially when mounting the syringe tip sterile forceps or sterile (medical) gloves must be used. After each inseminated batch, the capillaries and tips must be thoroughly cleared of semen and mucus. This is not only a hygienic requirement. They are indeed an ideal medium for pathogenic agents, but it is also very difficult to remove dried mucus from the thin capillaries (for example with a stranded wire).

Anesthetization of queen with CO_2

Anesthetization with carbon dioxide used initially for keeping the queen still during insemination, was found to have also another important effect which was an essential contribution to the success of the instrumental insemination (MACKENSEN, 1974). The queens undergoing two anesthe-

tizations with CO_2 on the same day will, as a rule, start oviposition one week later, even if they have not mated before. Virgin queens will start egglaying after being anesthetized with CO_2. Because these queens lay unfertilized eggs only, this is a proven method for producing drones even after the active season is over. Without CO_2 anesthetization, the queen will start egglaying after 4—5 weeks at the earliest. The start of oviposition depends on environmental factors which have a strong impact, and also considerably on the season. The queens inseminated in March start laying eggs after 5—6 days, while those in September on average after two weeks (MORITZ and KÜHNERT, 1984).

The practice of anesthetizing the queen for the first time one day before insemination is in wide use now. The caged queen usually defectes during this procedure. After a while, when insemination is made, the queens do not release more faeces, thus the hazard of infection is substantially reduced. The container in which the queens get anesthetized should be very hygienic. The first anesthetization with CO_2 of the 5—6 days old queen takes place in a air tight closed container filled with CO_2. After a 5—10 minutes' period the queen will be taken out, marked and one wing clipped off.

Recommandation is that each queen is separately marked, putiing a number on her back. Such plates with numbers are available in special shops. Utilization of such labels takes a little more extra time but avoids subsequent confusion. After marking the queens, one should wait until they recover from narcosis and only then they should be transferred back into the nurse colony.

Often, a single anesthetization with CO_2 is enough to induce oviposition after 8—12 days (WOYKE, 1963a). But in this case release of faeces during insemination may be a problem. Therefore, as two anesthetizations require only a little extra work as compared to the entire procedure of instrumental insemination, it is recommended to currently use this method as a routime in instrumental insemination.

Age of queens and drones

The best queens for instrumental insemination seem to be those of the natural mating age (6—13 days, OERTEL, 1940). But also queens of other ages can be successfully inseminated. Environmental factors are likely to have a greater

influence on the insemination results than the age of the queen. But in practice, there is a trend to shorten the period of time between emergence and insemination. The unmated queens require care which entails greater expenses and production losses. Primarily, the commercial queen breeder is interested in a fast production flow providing for the most efficient utilization of his queen production capacity.

Usually, availability of drones is the major problem in instrumental insemination. Drone rearing must be carefully planned and be a sustained activity (see the chapter "Rearing and Maintaining Drones") in order to be successful. When well cared for, drones are sexually mature when 8—days old at the earliest. Particularly in temperate climate sexual maturation of drones takes a little longer. In general, satisfactory amounts of semen are obtained from over 12—days old drones. For insemination drones are as important as queens and that they require the same intensive care. He who is sparing with the bee stock in drone rearing will be paid back later by insemination failures.

Amount of semen

The amount of semen injected depends primarily on the semen available. If enough semen is available, queens will be inseminated with 8—10 μl semen, to provide for approximately 3—5 million spermatozoa reaching the spermatheca of the queen (MACKENSEN, 1964; WOYKE, 1960). This ia almost the same number as in natural mating. By double insemination, the amount of semen in the spermatheca increases slightly, but this is not very important in practice. The minimum advantage of a slightly greater number of spermatozoa in the spermatheca is not worth performing an extra work and handling the queen once again for a second insemination.

Particularly, in selection programmes and genetic experiments such a great number of spermatozoa in the spermatheca is not always needed. Usually, one season is enough for obtaining another generation. In extreme conditions, queens may be inseminated with the semen obtained from one drone alone, but the amount must be at least 1μl. The best drones yield more than 1.25 μl of semen.

In special cases, it might be necessary to obtain semen directly from the seminal vesicles. This procedure may be use-

ful when a small number of sexually mature drones is available or when drones have been reared poorly. With very sharp fine-pointed scissors and forceps the seminal vesicles are exposed and clipped off at the point where they are in contact with the mucous glands. With great care they will be laid on a glass slide and pinched with forceps near the testis end. This will start peristaltic contraction forcing the semen out at the other end where it is taken up into the syringe. Often, the sperm collected by this method is not as effective as that collected after eversion and ejaculation (MACKENSEN, 1955).

A singular procedure is the insemination of a queen with the semen from the spermatheca of another queen. CALE and GOWEN (1964) used this technique for obtaining high inbreeding coefficient. They inseminated a daughter queen with semen from the spermatheca of the mother, which had been inseminated with the semen from a single drone. In 12 weeks only they obtained inbred lines with inbreeding coefficients of 95.5%. For this, the removed spermatheca was placed on a glass slide and covered with a thin film of sterile physiological saline solution. Then the tracheal network was removed and the spermatheca punctured with a pin, after which the syringe was inserted in the punctured area and the semen drawn in.

Another procedure of obtaining a high inbreeding coefficient in a short period of time is insemination of queens with the semen of her own drones.

By two anesthetizations with CO_2 the queen is induced to start oviposition. She will lay unfertilized eggs only, from which drones will emerge. When enough drones are sexually mature, the queen will be confined into a small cage inside the colony. She will stop egglaying and the ovaries will shrink. After 8 days she may be instrumentally inseminated.

Keeping record of inseminated queens

All inseminators working in a laboratory know very well that alongside with a devoted activity a rigorous record must also be kept. Each laboratory must keep a "record of the inseminated queens" to contain all data concerning each and every inseminated queen. This is necessary on the one hand for avoiding confusion with respect to individual queens, and on the other hand because it enables trouble shoothing of failures and eliminate them subsequently. So, it will be found out

if certain lineages give poor results or are easily infected. Also, such a record is important for assessing whether the operator's skill in performing the insemination has improved or not. With the availability of personal computers, keeping record by a computing procedure is quite attractive, as all data concerning any individual queen would be readily obtained. However, after 4-years experience at our insemination laboratory we found that it takes a very long time to enter the necessary data. With about 100 queens being inseminated weekly, 2 days are necessary for this purpose. So that this does not exactly mean readily obtainable information. Although sustained publicity is made for specific commercial software, it is unlikely that the queen breeder will increase his income by using a computer. But those who like to watch the display panel may use their personal computer also for other purposes than the pre-set video games. A precise and carefully kept record, which is always at hand, provides the sufficient information about the insemination activities. A model chart of which is now widely used, is given in Table 3.

Table 3

Data record sheet for instrumental insemination

Queen no.	No. of hive	Label.	Date	Mother	Drone		CO_2	Ins I	Ins II	Egg	Card no. / Observations
					planned	used					

With such record cards the genetic characteristics of individual queens are easily found even after many years. And this is essential in carrying out and planning breeding programmes. This method enables to know both the pedigree and the inbreeding coefficient of every individual queen.

Mixed semen procedure

In the last few years the "mixed semen" procedure has been used in instrumental insemination (developed by KAFTANOGLU and PENG, 1980). When a queen is inseminated by the classical procedure already described, the semen which reaches the spermatheca does not consist of equal amounts from all drones. Only a few drones are the fathers of most of the worker bees of a colony (LAIDLAW and PAGE, 1984; MORITZ, 1986). This is a disantvantage for selection, because the inbreeding coefficient increases faster than with a genetic balance (equal amounts of semen from each and every drone). In such a case, the assessment of the compatibility of crossing two lines is also more difficult. The contribution of drones with unequal amounts of semen can be technically corrected very easily, namely by inseminating the queen with homogenously mixed semen.

Semen from a great number of drones (depending on the number of queens to be inseminated) is collected with insemination syringe as already described. Use of high capacity capillaries is recommended. Especially with small numbers (20 drones) care should be taken to obtain approximately the same amount of semen from each and every one of them. When large amounts are collected, the semen may be stored in several capillaries until mixed. Mixing proceeds as follows: semen is diluted, mixed, and then is concentrated back in a centrifuge. In this way a homogenously mixed semen sample is obtained from a great number of drones (MORITZ, 1983).

A small sterile glass vial is used, with a capacity 20 times greater than the amount of semen to be mixed. The sterile diluent solution is poured in it (10 times the amount of semen = 1:10 dilution). Then the semen is added which deposits in threads on the bottom of the vial. Now, one carefully proceeds to homogenization. The best is to use a wide bore Pasteur pipette. Solution is drawn in and injected back into the

recipient; the operation is repeated until the solution turns into a uniform suspension of creamy colour. The homogenization should not take for more than 1—2 minutes.

The semen suspension is introduced into a centrifuge tube and is centrifuged for 5 minutes at 1000 g. The rotation per minute depends on the diameter of the rotor. In adjusting it one must follow the guide lines given in the centrifuge manual. When the semen sediments at the bottom, the supernatant liquid is carefully removed with a Pasteur pipette with narrow opening. Now semen is drawn from the centrifuge tube into the insemination syringe, and it should be injected into queens immediately. In this technique it is essential to keep the semen in the diluent solution as brief as possible — 15 minutes at the most to prevent possible damage of the semen, which will result in poor insemination.

In the "mixed semen" technique, the diluent solution for semen has a much greater influence on the success of insemination than in the classical method of insemination. The pH value and the concentration of salts (osmolarity) are absolutely essential: when the semen is diluted, each spermatozon is no longer surrounded by semen fluid, being exposed to the diluent solution. During centrifuging, the water-soluble proteins are removed from the surface of spermatozoa — which could reduce their viability. The Kiev solution, which gives good results in classical insemination, is hardly adequate for this technique. The inseminated queens have often become drone laying queens. Up to now the best results have been obtained with Tris buffer solution (Table 2) (MORITZ, 1984). But with this solution, too, the fertilizing capacity is reduced fast (FISCHER, 1987), when semen is stored for a longer period of time.

When using this technique, utmost care should be taken about sterile conditions. An infection of the semen can destroy several batches of queens. It is not recommended to remove the semen from the everted endophallus by rinsing it with diluent solution as suggested by KAFTANOGLU and PENG (1980). In doing so the threat of infection through the drone faeces is obvious.

Assessment of the results of insemination

The best evidence of a successful insemination is certainly the egglaying queen producing worker brood. But it takes at least two weeks before the brood is sealed and one can de-

termine that fertilized eggs have been laid. WILLIAMS and HARBO (1982) described an effective and fast test which enables to immediately assess whether insemination was successful or not: the spermatheca is examined to see if there are any spermatozoa in it. An experienced operator inserts a finger nail between the last two tergites and will uncover the spermatheca. But those who are not so skilful (as myself) will proceed as follows:

The queen is killed and then the abdomen is sectioned starting from the sting chamber on the left side. In the front end of the abdomen another cut is made along the first tergite, from left to right. Next, the queen is fixed with pins in a dish lined with beeswax, so that the abdomen is well streched — with a pin fixed into the last sternite. Then the entire dorsal part of the abdomen is lifted from the left and turned over to the right, and fixed with two pins. Now the internal organs are exposed. The intestine is carefully removed and the genital organs are exposed. The spermatheca can now be punctured and examined. If it contains semen, the insemination was successful because spermatozoa have reached the spermatheca. This procedure can be used by the beginner to check his skill, and is also an efficient biological test when one wishes to test the effect of diluent solutions. So, it is no longer necessary to wait for a long time, and queen maintenance capacities are used efficiently.

MAINTENANCE OF QUEENS BEFORE AND AFTER INSTRUMENTAL INSEMINATION

J. WOYKE

The conditions in which queens are kept before and after insemination play an important role for instrumental insemination.

To date mostly three methods are used to store queens before and after insemination:

1. storage of several queens in small cages in one bee colony,
2. maintaining individual queens in separate nuclei or bee colonies and
3. keeping individual queens, each in a small box with several hundreds of worker bees.

Maintaining queens in storage colonies

Storage of several queens in a colony before and after insemination is widely used. This method saves time in searching for queens in individual nuclei, and avoids losses of queens which try to escape through the queen excluder for natural mating. Sometimes nuclei can be avoided completely i.e. when inseminated queens are intended to be introduced directly into normal colonies, when the queens are to be sold, or when they are sent to the insemination station for insemination only.

Emerged queens are transferred to the storage colony which may be queenless or queenright. In the latter case, the queens are stored in the super which is separated from the nest with the queen, by a queen excluder. In the storage compartment, honey and pollen should be present as well as open brood. The brood attracts nurse bees which take care of the queens. The frame or frames with caged queens face the combs with open brood: When queens are stored for a longer period, combs with unsealed brood must be renewed each week. In absence of nectar and pollen flows, the nursery colony should be fed sugar syrup.

Two modifications of this method are used:

a) queens maintained in individual cages which can be handled separately, or

b) queens maintained in separated compartments of an especialy prepared frame in which they are handled collectively.

According to LAIDLAW (1954), LAIDLAW and ECKERT (1962) and MACKENSEN and TUCKER (1970), mature queen cells are put into individual cages. The cages are placed into holding frames in a strong queenless colony. The queens emerge here and are fed by worker bees through the mesh of the cages.

In Egypt, several mature queen cells are attached to comb with unsealed honey and then are covered each with a screenwire, halfsphere, push-in cage (diameter 5 cm) (personal observation). The comb is placed in a super of a queenright colony. The emerged queens can feed on the honey if they are neglected by workers.

The collective handling of queens is also practiced in China and in the USA. In China, a Langstroth frame is divided by vertical and horizontal bars into 28 compartments. One side of the frame is covered with unremovable wire mesh. The mesh on the other side is cut into pieces which enable separate acces to each compartment. Each mature queen cell is placed into one compartment and the frame is placed in the queenless part of a colony. Emerged queens remain here until instrumental insemination or introduction to bee colonies (personal observations).

An advanced method of collective handling of queens has been developed by HARBO (1985, 1986b). Instead of a frame, he uses a 13 mm thick plywood board, cut to the same size as a frame, in a colony. Queens are stored in 35 holes measuring 24 mm in diameter and covered with a permanent screen on the back side and with hinged screens on the front. For instrumental insemination, the whole board with all the queens is taken from the colony. To keep the queens in good condition, worker bees are brushed into a shallow box and the board with queens is placed on the top, to form a bee-tight seal. Workers trapped below, feed and warm the queens which can be kept outside the colony for over 8 hrs if necessary.

LAIDLAW (1954) treats the inseminated queens twice with CO_2 on succeeding days. Later they may be introduced to nuclei or further kept stored.

MACKENSEN and TUCKER (1970) cage each inseminated queen with few worker bees and keep them in another

nursery colony for at least 2 days. The inseminated queens are treated with CO_2 and introduced in a cage one day after nuclei have been made up. These queens should not be released before they are ready for egglaying.

To stimulate oviposition, Harbo (1985, 1986) treats the queens three times with CO_2 (insemination is counted as a treatment).

For convenience, he gives the last treatment on the day the queens are transferred from the queen bank to the bee nuclei. For best success, he recommends to keep the queens in the storage colony until they are 2 — 3 weeks old. Not only instrumentally inseminated queens are stored in one colony, but also naturally mated queens can be stored this way for a long period of time (REID, 1975; SZABO,1977; LEVINSOHN and LENSKY, 1981). LAIDLAW and ECKERT (1962) describe storage of 312 queens and DIERTZ and WILBANKS (1983) 360 queens in one colony.

FREE and BUTLER (1958) found that workers can thrust their tongues through wire-gauze with apertures of 1.5 mm and imbibe syrup placed 1 mm apart. However, workers will feed other workers only through wire-gauze with at least 2.5 mm apertures, which allows antenna contact between two bees. They concluded that apertures of at least 2.5 mm square are desirable in wire-gauze used for queen cages, and suggested that wire-gauze used for queen cages should have apertures as large as is possible without allowing bees to pass through. Consequently, screens with 2.5 mm apertures are mostly used now.

The method to store several queens in one colony has many advantages but it has also serious disadvantages such as:

a) injuries of queens,
b) retention of semen in the oviducts, and
c) low number of spermatozoa entering the spermatheca of caged queens.

WOYKE et al. (1956) found that worker bees injured the queens when several of them were stored in a colony. The experimental queens were kept in cages with apertures of 0.2 mm, 1.2 mm and 3 mm. The workers could not thrust their tongues through the smallest apertures, they could do that through the larger apertures and they could thrust part of head with the mandibles through the largest ones. After 8 days, 0.0,93.3 and 83.3% of the queens survived, respectively,

but the percentage of non injured alive queens was 0.0, 93.3 and 33.3% respectively. Thus queens died in cages with gauze which did not permit the contact with workers. The larger the mesh size the more queens were injured. Queens in cages with slots were injured even more. The workers bit claws, tarsi, antennae and even wings. According to JASINSKI (1987) 60% of 354 queens stored for 1 week in queenless colonies were injured. He detected 26 different types of injuries. Initial injuries occurred at the foot pad, which normally is white and soft. Black spots appear when it is injured only slightly. Heavily injured arolia are black, dry and shrinken (Fig. 28).

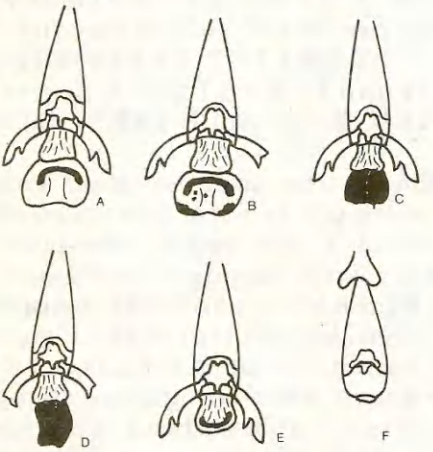


Fig. 28 — Injuries of pretarsus
A — normal; B — black spots on arolium and lack of one point of one claw, C — black arolium, D — black arolium and lack of one point on each claw, E — arolium dry and folded up, F — pretarsus bitten off

LENSKY and SLABEZKY (1981) recorded an oily colourless exudate deposited, from the foot-pad of a queen. This is the so called foot-print, which together with the mandibular gland secretion inhibits the construction of queen cell cups. WOYKE (1987, not published) found that queens with black ariola do not deposit the foot-print and probably are supersedured by worker bees. In contrast SZABO (1977) concludes, that there is no significant differences in loss and supersedure between queens overwintered in a storage colony or each in a small colony. However data show that from May to October 62% of the stored queens were lost, whereas only 33% died of those overwintered in separate colonies.

Injuries of queens seem to be common. JASINSKI found in 1977, 12.5% queens injured, which were imported to Norway from USA. Woyke found in 1986, 40% queens inju-

red stored in China and in 1987, 64% queens injured stored for 10 days in Egypt.

The second disadvantage of caging queens concerns the retention of semen in the lateral oviducts. VESELY (1970) found that queens caged for 24 h after insemination had oviducts filled with semen. Freely moving queens had empty oviducts.

The low number of sperm entering the spermatheca is another disadvantage of stored queens. Queens inseminated with 8 μl of semen and caged afterwards in a storage colony had 2.9 million spermatozoa, while those in an incubator had 4.2 million (WOYKE and JASINSKI, 1973). Queens stored in nursery cages had 2.7 million sperms while those kept on a whole comb under a queen excluder had 4.7 million (WOYKE, 1979). Queens caged in a storage colony had 2.5 million sperms, while those attended by 160 workers in an incubator had 4.4 million (WOYKE and JASINSKI, 1979).

Maintaining queens in nuclei and bee colonies

Queens may be maintained before and after instrumental insemination in nuclei or normal colonies. Mainly three types of colonies are used:

a) small baby nuclei, with combs smaller than those used in the apiary

b) nuclei with few combs of the size used in the apiary

c) normal colonies in one or two story hives.

There is a large variety of boxes for baby nuclei available. Some contain small frames and others only top bars. In recent years, the Kirchhain trapezoic nucleus with four top bars 17 cm long became very popular. The number of bees present in those small nuclei boxes has a significant influence on the results of instrumental insemination. WOYKE and JASINSKI (1982a) tested inseminated queens in Kirchhain nuclei with various numbers of attendant workers (from 50 — 1000). Clearance of oviducts, number of semen in the spermatheca and initiation of oviposition was only satisfactory if more than 350 workers were present in the nuclei (WOYKE and JASINSKI, 1982b; WOYKE, 1987). So this should be the minimum number of workers to attend queens in baby nuclei.

Larger nuclei contain 3 — 5 combs of the size used in the apiary and one frame feeder. One comb at least should

contain sealed brood with emerging workers. Queen excluders must be fixed to the entrances of the nuclei. They may be removed after the queens started to lay eggs.

The queens are maintained before and after instrumental insemination in different ways.

Some beekeepers store them till insemination in the so called queen banks. After insemination, the queens are introduced in cages into nuclei or normal colonies. Two days later the queens are treated the second time with CO_2 and are released. But the virgins are injured by worker bees during the storage, and a low number of spermatozoa enter the queen's spermatheca while they are caged in nuclei. Vesely (1970) found 3.9 million spermatozoa in the spermatheca of queens inseminated with 6 μl of semen and caged afterwards in mating nuclei, in contrast to 4.9 million in freely moving queens. Only 3.0 million spermatozoa were found in spermathecae of caged queens inseminated with 8 μl of semen whereas 5.1 million were in the spermatheca of queens moving on a whole comb (WOYKE 1979). Only 1.8 million entered spermatheca of queens caged under push-in cages in Egypt, but 5.2 million were found in the spermathecae of queens moving freely in three comb nuclei (Woyke, 1987, not published). So a 2.9-fold increase of the number of spermatozoa entering the spermatheca was achieved.

Since caging has so many negative effects, it seems to be better to introduce mature queen cells or young virgins to bee nuclei. After the queens are accepted, they are treated with CO_2 two days before insemination, and only than given into cages. After insemination they are released immediately or are introduced in cages plugged with very little candy.

If virgin queens are introduced into normal colonies, inseminated queens should be returned to the old colonies which already accepted them before insemination to avoid losses.

Mature queen cells or young virgins are introduced on one comb which is cavered by a queen excluder isolator. This isolator is placed in the centrum of a brood nest in a queenless colony.

It is easy to examine whether the queen in the isolator is accepted by the worker bees. Two days before insemination the queen is treated with CO_2. For the next two days, the queen may remain caged in the colony or can be released in the isolator. The queen must be released in the isolator

within half an hour after insemination. She remains in the isolator until she starts to lay eggs. There is no need to attach a queen excluder on the hive entrance with this technique.

Maintaining queens in small boxes

Often instrumentally inseminated queens do not remain in bee nuclei, but are sold, returned to the beekeepers who reared them, or are introduced to other queenless colonies. In a case like this, it is not practical to create bee nuclei to keep instrumentally inseminated queens just for few days. Usually such queens are caged and several of them are stored in queen banks. However, as mentioned above, such queens are injured, they do not clear their oviducts and a reduced number of sperms enter their spermatheca. It is advantageous to maintain the queens in small boxes about 12 x 12 x 6 cm. Each box should be supplied with a piece of comb, candy, water and worker bees. The workers are treated with CO_2, and the queen is introduced when the workers are still immobile. This method of introduction assures safe acceptance of queens. Conditions in which those boxes are kept, as well as the number of workers attending the queens, bear significant influence on the results of insemination. The number of workers attending queens before insemination, within the range of 20 — 500, did not significantly influence the results, when all queens were returned after insemination to the same number of 250 workers (WOYKE and JASINSKI, 1980b).

WOYKE and JASINSKI (1979) showed that insemination queens kept in nursery cages in a storage colony had 2.6 million spermatozoa in their spermatheca.

Queens in the incubator with 0, 50, 150, 250 and 350 attendants had 3.0, 3.2, 4.4, 4.5 and 4.1 million sperms respectively and those moving freely in bee colonies had 4.7 million in their spermatheca. Queens attended by 250 workers did not differ significantly from those moving freely in bee colonies.

Inseminated queens kept at room temperature (20°C) in cages with 20, 40, 80, 150, 250 and 350 attendants had 1.8, 2.2, 2.7, 3.2, 3.4 and 4.1 million sperms in their spermatheca, respectively (WOYKE and JASINSKI, 1980 a).

Thus, it is recommended to keep queens in cages in the incubator at least with 250 attendants and in room temperature with 350.

RESULTS OF INSTRUMENTAL INSEMINATION

J. WOYKE

The results of instrumental insemination depend on different characters like the process of entering of spermatozoa into the queen's spermatheca, the number of sperms in spermatheca, the duration of the beginning of oviposition, the longevity of queens, honey production and others. Different factors like rearing conditions of queens, temperature, age of queens and drones, race of bees and others, affect the various results.

Duration and dynamics of entering of spermatozoa into the spermatheca

During natural as well as instrumental insemination the semen is injected into the lateral oviducts of a queen, where from the spermatozoa pass into the spermatheca. In contrast to naturally mated queens, instrumentally inseminated queens do not clear the oviducts completly within 24 hrs (VESELY, 1970; WOYKE, 1960). The duration and dynamics of entering of spermatozoa into the spermatheca of instrumentally inseminated queens depends upon both the amount of injected semen and the conditions in which the queens are kept after insemination.

Experimental queens were inseminated with 1, 2, 4 or 8 μl of semen and either held in small boxes with 250 workers at 34 $^{\circ}$ C or caged without workers and stored in queenless colonies.

The queens were killed and examined at various times after insemination (WOYKE, 1983; WOYKE and JASINSKI, 1985; WOYKE, 1987b). The highest entry rate of spermatozoa into the spermatheca. Over time, the entry rate slows down and fits a logarithmic function y = a+bxlnt (t = time after insemination Table 4, Fig. 29).

Table 4

Parameters fitting the logarithmic regression function

Semen dose (μl)	Intercept "a"	Slope "b"	Semen entered spermatheca after (h)
1	508.9	251000	4
2	284.28	446000	8
4	185.73	692000	12
8	122.38	1178000	16

It can be assumed that most spermatozoa enter the spermatheca within 24hrs after insemination and the process is finished after 48 hrs. Semen which remains in the lateral oviducts after that period probably will not be cleared and may cause the death of the queen.

The presence or absence of attending workers has little or no influence on the final number of spermatozoa in the spermatheca when small amounts of 1 or2 μl of semen are injected. The presence of bees, however, clearly increases the final number when larger amounts of 4 or 8 μl are injected (Fig. 30).

Relation between the amount of semen injected and the number of spermatozoa in the queens spermatheca

The number of spermatozoa of naturally mated queens varies. MACKENSEN and ROBERTS (1984) found in 33 Italian queens *(A. m. ligustica)* an average of 5.73 million (3.34 — 7.36 mill.) spermatozoa and WOYKE (1960) in 102 European queens *(A. m. mellifera)* 5.34 million (0.69 — 7.92 mill). Queens mated during one, two, and three mating flights had on the average 5.06, 5.98 and 6.98 million spermatozoa, respectively. The number of spermatozoa entering the spermatheca after the first mating flight determines the subsequent behaviour of queens.

Queens with an average of 5.25 million spermatozoa in the spermatheca do not fly again, those with 4.62 million fly out but do not mate again and those with 3.46 million only, fly out and mate again on a second flight (WOYKE, 1958, 1964). It seems that about 4 million sperms in spermatheca is the lowest number for a sufficiently inseminated queen.

The number of spermatozoa in the spermatheca of instrumentally inseminated queens depends on the amount of injected semen. WOYKE (1960) inseminated queens with different doses of semen ranging from 1 up to 20 μl, and kept them afterwards with some attendants. MACKENSEN (1964) inseminated queens with doses ranging from 2 up to 36 μl and stored them without attendants in queenless colonies. Although MACKENSEN found fewer sperms in the spermatheca than WOYKE, the characters of the curves representing the results were very similar (Fig. 31). Any doubling of the amount of semen injected, resulted in an increase of about 1 million spermatozoa in the spermatheca.

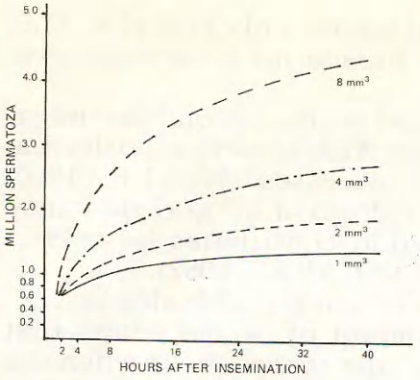


Fig. 29 — Dynamics of entry of spermatozoa into spermatheca of queens inseminated with different amount of semen.

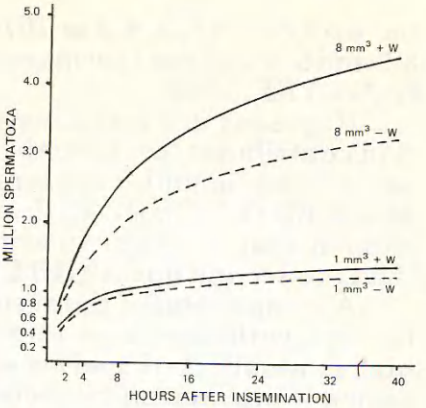


Fig. 30 — Dynamics of entry of spermatozoa into spermatheca of queens inseminated with 1 or 8 mm³ of semen and kept with (+W) or without (–W) worker bees.

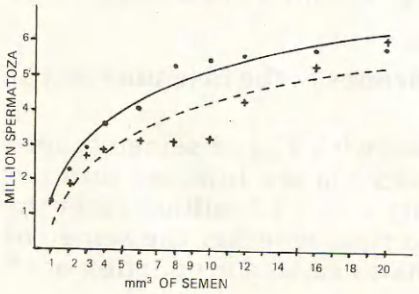


Fig. 31 — Relationship between the volume of semen injected into queens oviducts and the number of spermatozoa entering the spermatheca.
. . . . Woyke (1960) experimental data,
– – – – logarithmic regression curve y=1.33 + 1.65 ln X calculated upon above data,
++++ Mackensen (1964) experimental data,
– – – logarithmic regression curve y = 0.60 + 1.57 ln X calculated upon above data.

The concentration of spermatozoa in the semen is 7.5 — 7.6 million/μl (WOYKE, 1960; MACKENSEN, 1964). One drone produces about 1.5 μl semen with about 11 million spermatozoa. Thus one drone produces twice as many spermatozoa as a naturally mated queen has on the average in her spermatheca (5.3 million). However, if a queen is inseminated with semen of a single drone, only about 1.5 million sperms enter the spermatheca.

During instrumental insemination only 1.00 — 1.25 μl of semen is collected from one drone into the tip of the syringe. About 5% of the semen remains in the tip of the syringe and only about 7 million spermatozoa are injected into queen's oviducts from each μl of semen. After queens are insemina-

ted with either 1, 2, 4, 8 or 16 μl of semen, only 18.9, 17.6, 12.9, 8.5 and 5.3% of the sperms enter the spermatheca respectively (WOYKE, 1960).

If queens are inseminated twice, the second insemination contributes less than the first. This contribution decreases as the amount of semen increases (WOYKE, 1960; MACKENSEN, 1964). With small doses of 2.2 μl each, it may happen that the first insemination contributes less (39%) than the second one (BOLTEN and HARBO, 1982).

A comparison of the results of single and double inseminations with the same total amount of semen shows that higher numbers of sperms enter the spermatheca when the semen is injected on two separate doses. However, the differences between single and double inseminations up to a dose of 8 μl of semen were not statistically significant (WOYKE, 1960). The results suggest that no real advantage is gained by double inseminations with small doses up to 6 μl. The advantage of double insemination over single with 8 μl is discutable, but double insemination is recommended with 10 or more μl of semen.

Contribution of successive drones to the insemination of a queen

When a queen is inseminated with 8 μl of semen from 8 drones, about 55 million spermatozoa are injected into her oviducts. From those sperms only 4.0 — 4.5 million enter the spermatheca. Thus the question rises whether the semen of one drone only enters the spermatheca, or a proportion of all drones.

TABER (1955) mixed semen of wild and cordovan drones or injected two doses of semen of those drones. Queens inseminated this way produced both types of workers, but the proportion varied with time within a single colony. A study by MARTINHO and GONCALVES (1978) with Caucasian and Italian bees confirmed Taber's results. Also WOYKE (1936b) and LAIDLAW and PAGE (1984) showed that each drone inseminating the queen participates in the production of progeny. The phenotypic fluctuations were in some cases greater than that expected due to chance sampling alone, but did not result in an abnormally high representation of a single phenotype or the elimination of others.

When queens are inseminated several times, each time with small amounts of semen (1 drone), the order of insemi-

nation has only little effect upon progeny phenotypic frequencies (LAIDLAW and PAGE, 1984; MORITZ, 1986).

Daily fluctuations in progeny proportion may be observed even after queens are inseminated with mixed semen. But the fluctuation is so low that one has to conclude that the semen is mixed homogeneously (MORITZ, 1983; LAIDLAW and PAGE, 1984).

Rearing conditions of queens and the results of insemination

Rearing conditions of the queens significantly affect the results of instrumental insemination (Tab. 1). Queens reared from eggs or younger larvae are heavier, have more ovarioles in their oviducts (can produce more eggs) and have larger spermathecae which can store more spermatozoa (WOYKE, 1967, 1971).

However, the concentration of spermatozoa in the spermatheca of all queens was almost equal (3.3 — 3.5 million/μl), irrespective of their size. Table 5 shows that also naturally mated queens which were reared from older larvae had less spermatozoa in their spermatheca than those reared from younger larvae. Thus queens should be reared from the youngest brood.

Table 5

Volume of spermatheca and number of spermatozoa in spermathecae of naturally and instrumentally inseminated queens reared from brood of different age

Age of brood/used for rearing queens		Egg	1 day	2 days	3 days	4 days
No. queens		27	27	27	27	6
Mean volume of spermatheca	(μl)	1.182	1.093	0.936	0.821	0.586
Queens mated	No. queens	Mean no. spermatozoa/		in spermatheca	Millions	
Naturally	20+1	6.133	5.737	5.026	3.942	1.520
Instrumentally						
2x8 μl	20	6.145	5.820	5.269	4.630	—
8 μ	28+1	3.791	3.511	3.234	2.631	0.140
4 μl	20+1	2.746	2.440	2.335	1.955	1.288
1 μl	20	1.585	1.507	1.299	1.141	—

In column 2, + 1 indicates a mated queen reared from a larva 4 days old. Spermatozoa were counted in only 3 out of 6 queens; their mean spermathecal volume was 0.683 μl.

Temperature after insemination

The results of insemination depend very much upon the temperature in which the queens are kept after insemination.

Queens inseminated with 8 μl of semen and kept with 10 attendant workers in an incubator at 24°C and 34°C, had 3.1 and 4.0 million sperms respectively in the spermatheca (WOYKE and JASINSKI, 1973). Thus queens should be kept for two days after insemination at brood nest temperature. The storage of queens between two brood combs not necessarily assures the right conditions. Queens stored with 10 attendants in a queenless colony and in the incubator at 34°C had 3.0 and 4.3 million spermatozoa respectively (WOYKE and JASINSKI, 1973). The right conditions may be assured even at low outside temperatures, when the queens are released in boxes with at least 350 attendant workers (WOYKE and JASINSKI, 1980; WOYKE and JASINSKI, 1982a, b).

Age of queens

The age of the queens affects the number of spermatozoa entering the spermatheca as well as survival of queens and the onset of oviposition after instrumental insemination.

When queens were inseminated with semen from 1 drone only, the number of spermatozoa in the spermatheca did not vary among different ages, except at the first day of life when few sperms entered the spermatheca (MACKENSEN, 1955). The survival rate and the insemination success of young queens is poor (14 — 50% survival, FRESNAYE, 1966; MACKENSEN, 1976; WOYKE and JASINSKI, 1976). When 5 days old queens were inseminated, significantly more sperms entered the spermatheca (4.1million) and the survival rate was improved (75%). The best results were obtained (100% survival, 3.9 million sperms) with queens that are 7 — 8 days old. Older queens up to 47 days or more may be inseminated, but both the survival (73 — 100%) as well as number of sperms in spermatheca (2.1 — 2.4 million) are lower.

Age of drones

The results of instrumental insemination also depend on the age of drones. Very young drones(1 — 2 days) fail to pro-

duce semen or mucus. The colour of the cornua of the endophallus is white. Around the fifth or sixth day, only mucus comes out of the everted endophalus. The sticky substance on the cornua is yellow. From the eight to the tenth day, one can obtain light coloured semen, mostly mixed with fluid mucus. This mucus is easily drawn into the tip of the syringe, where it coagulates and plugs the tip. Drones older than 10 — 12 days, have creamy-yellow semen, which is easy to separate from the mucus. The colour of the cornua is now orange. Semen of drones older than 4 weeks is more difficultly drawn into the tip of the syringe (WOYKE and JASINSKI, 1978).

Experimental queens were inseminated with semen from drones 2 — 9 weeks old. As the age of drones increased, mostly a lower number of spermatozoa entered the spermatheca. With an increasing age of drones, the percentage of queens with residue of semen in their oviducts increased (0 — 14% if inseminated with four week old drones. WOYKE and JASINSKI, 1978). Therefore, queens should only be inseminated with semen of drones that are 2 to 3 weeks old.

Race of bees

The number of spermatozoa entering the spermatheca depends also on the race of the inseminated queen. WOYKE, JASINSKI and SMAGOWSKA (1974) inseminated queens of 6 races and 3 hybrids. The highest number of sperms was found in *A. m. mellifera* and *A. m. caucasica* queens, the lowest in *A. m. carnica,* and *A. m. ligustica.* The *A. m. scutellata* (adansonii) and *A. m. lamarckii* had intermediate numbers.

Hybrids of *A. m. mellifera x A. m. caucasica* and *A. m. mellifera x A. m. carnica* had more sperms than both paternal races, but hybrids *A. m. caucasica x A. m. carnica* had less than both original races.

Similar results were obtained when queens of different races and their hybrids were allowed to mate naturally. The differences were partly related to different sizes of spermathecae, but this was not the only factor involved. Some physiological differences must be typical for the various races tested.

Behaviour of queens after instrumental insemination

Queens which are released after instrumental insemination into nuclei try to fly out, and mate naturally. WOYKE

(1963a, 1966a) observed the behaviour of naturally mating queens, treated with CO_2 only, and inseminated with 1 — 16 μl of semen.

30% of the naturally mated queens, flew out and 18% mated in a second flight. Out of those queens treated with CO_2 only, or inseminated with 2 and 4 μl of semen respectively, 23, 29 and 24% flew out and 18, 14 and 10% mated naturally again. Only 4% of the queens inseminated with 8 μl flew out, but did not mate again. Queens inseminated with 12 and 16 μl of semen did not fly out. Thus, an increasing amount of semen decreased the percentage of queens flying out and mating naturally.

To determine the effect of the second insemination or the additional CO_2 treatment on the queen's behaviour, they were inseminated once or twice with 4 or 8 μl of semen. Some of these were treated additionally with CO_2 (WOYKE, 1963a, 1966b). A second insemination as well as an additional CO_2 treatment decreased the percentage of queens with multiple mating flights.

Nevertheless, some queens inseminated with 4 μ and CO_2 (13%) and those inseminated twice (7%) mated naturally again. Thus a queen excluder, attached to the entrance of hives with non laying, instrumentally inseminated queens, is indispensable.

Onset of oviposition

Naturally mated queens start to lay eggs 3 days after the last mating (OERTEL, 1940). But instrumental insemination without the use of CO_2 has little, if any, effect on initial oviposition (MACKENSEN, 1947). Queens not treated with CO_2 during insemination started to lay eggs after 40 days. One carbon dioxid treatment, applied during insemination, reduced the age at initial oviposition to 36 days and the second treatment, 24 — 48 hrs later, reduced it to 15.4 days. A third or fourth treatment did not further reduce the average initial oviposition age. Which was confirmed by PRABUCKI et al. (1987).

Thus two treatments with CO_2 are sufficient and laying starts at the average 5.3 days after the last treatment. MACKENSEN recommends two 10-minute exposures to CO_2 spaced 24 — 48 hours apart, but JANUSEK (1987) showed that shortening the second CO_2 treatment to 5, 3 or 1 min had

no influence on the initiation of egg laying. On the other hand, even a single treatment with CO_2 can affect the time of initial oviposition. Queens inseminated by WOYKE (1963a, 1966a) started to lay eggs 8 — 12 days after insemination, even when they were not treated a second time with CO_2.

WOYKE (1963b, 1966b) did not find that a second insemination or a second treatment with CO_2 had any effect on the interval between insemination and onset of oviposition. These differences may be explained partly by the younger age of queens inseminated by MACKENSEN (3 — 7 days), than by WOYKE (7 — 12 days). It is known that the age of queens at insemination determines the interval between insemination and onset oviposition.

Table 6 shows that young queens inseminated at the age of 2 — 4 days start to lay eggs mostly after a long period of 20 — 30 days. The shortest interval between insemination and onset of oviposition occurs when queens are inseminated at the age of 7, 10 or 12 days (MACKENSEN, 1974; KEPENA, 1985; JANUSEK, 1987; PRABUCKI et al., 1987).

In small nuclei, the number of bees in the colonies determine the beginning of oviposition (WOYKE and JASINSKI 1986a). Queens in 1 comb mating nuclei attended by 150, 350 and 750 workers started to lay eggs 15, 14 and 12 days after insemination and in 4 comb nuclei, after 13, 12 and 10 days respectively.

Table 6

Average interval in days between first insemination of queens and onset of oviposition

Queen age at first insemination	Mackensen 1947		Kepena 1985	Janušek 1987	Prabucki at al. 1987
	1 ins.	2 ins.	1 ins. + CO_2	1 ins. + CO_2	1 ins. + CO_2 and 2 ins. + CO_2
2		9.2	23		
3 — 7	31				
3		8.0	22	28	
4		7.2	22	19	
5		5.7		9 — 22	
6		6.7		10	11
7		6.1		5 — 12	11
8		6.0	6		13
9		7.0		6 — 10	13
10		4.5	9		8
11		3.0			7
12					8
13			8		

Queens in larger units with 19500 workers initiated egg laying 7 days after insemination. Thus, increasing the number of attendant bees, shortened the interval between insemination and oviposition. One of the factors involved may be the higher temperature maintained by the larger number of bees. An increase of temperature by 1°C, decreased the onset of oviposition by 1 — 2 days.

Do instrumentally inseminated queens mate naturally after they start to lay eggs ?

Instrumentally inseminated queens fly out and mate naturally before the onset oviposition if they are not prevented to do so by wing clipping or queen excluders fixed to the entrance.

Some beekeepers claim that queens fly out and mate naturally after they started to lay eggs. To investigate this, WOYKE and JASINSKI (1986b) instrumentally inseminated 31 cordovan queens with semen of cordovan drones. The queens were prevented from flying by queen excluders until they started to lay eggs. They were located in area with black bees. Black body colour is dominant to cordovan. Offspring of all queens was examined 7 times during two years. All 52,000 workers examined were cordovan. Thus no evidence was found that instrumentally inseminated queens naturally mate after they started to lay eggs.

Longevity of queens

According to HARBO and SZABO (1984) instrumentally inseminated (II) queens have a reduced longevity compared to naturally mated ones (NM). However, their queens were not treated identically before insemination. NM queens were kept in nuclei and II stored in queen banks and so the latter could be injured at a higher percentage. VESELY (1987, not published) made a mass comparison of longevity of naturally mated and instrumentally inseminated queens distributed in the years 1961 — 1980 to the experimental bee yards of the Bee Res. Inst. Dol in different bioclimatic regions of Czechoslovakia (Table 7). Queens failing to survive included those that died, were supersedured, were lost during swarming or had to be removed by the beekeeper due to drone produc-

tion, irregular laying and so on. Survival of instrumentally inseminated queens was slightly lower in all the three consecutive years. KONOPACKA (1986, 1987) also reported that instrumentally inseminated queens survived in a little lower percentage than the naturally mated ones, but also in this study the queens were not treated identically before the insemination.

Table 7

Survival of II and NM queens in Czechoslovakia from 1961 — 1980

Year	Number of surviving queens NM	II	x^2
0	1483 (100%)	672 (100%)	
1	860 (58%)	336 (50%)	4.72*
2	400 (27%)	101 (15%)	27.52**
3	89 (6%))	7 (1%)	25.23**

* P<.05
** p<.01

WILDE (1986) treated queens equally before II. He could not find any statistical significant difference in the survival of NM and II queens after two years.

Honey Production

Does instrumental insemination have any effect on honey production ? Of course, a good method of instrumental insemination must be used before one can compare instrumentally inseminated with naturally mated queens. Again, there is no point in comparing naturally mated with poorly artificially mated queens.

GENETIC ASPECTS OF INSTRUMENTAL INSEMINATION

J. WOYKE
E. HILLESHEIM

Thanks to instrumental insemination, tremendous progress in bee genetics and bee breeding has been made. Many mutants have been discovered and are maintained. Knowledge of their inheritance helps to solve many genetical and physiological problems.

The origin of unusual bees and sex determination have been clarified. Progress has been made in breeding bees that are resistant to diseases, better pollinators, and better honey producers. These studies can serve as guides in other fields of bee breeding.

Mutants

Many mutants were discovered and their genetics studied (Tab. 5). The glossary at the end of the book provides the reader with explanation of the scientific terms used in the table in the text of this chapter.

The eye colour mutants are the result of blocking some steps in the biosynthesis of ommochromes present in wild type eyes (DUSTMANN, 1968, 1969).

In historical order, the following eye color mutants were described: MIKHAILOFF (1930, 1931) described a white eye mutation and studied its inheritance; ROTHENBUHLER, GOWEN and PARK (1952b) described ivory (i), cream (cr), snow(s), and chartreuse (ch) eyes (See table 8). LAIDLAW, GREEN and KERR (1953) distinguished two chartreuse genes, $ch^1$and ch^2, and found red (ch^r), which is allelic to ch, and a non allelic gene, brick (bk). Interaction of bk and ch results in a buff phenotype. CALE, GOWEN and CARLILE (1963) found pink eye colour (p) which is also a viability gene. LAIDLAW, El BANBY and TUCKER (1964) described five new eye-colour mutants: Bensen green (ch^B), cherry (ch^c), garnet (g), pearl (pe) and tan/s^t Benson green and cherry are assignable to the chartreuse locus. Tan is allelic with s (snow); the s/s^t genotype results in a peculiar phenotype which is red. Tan is epistatic to chartreuse 2, and to brick, but

hypostatic to ivory and cream. LAIDLAW and TUCKER (1965b) described umber (i^u) which is an eye colour allele of ivory and partially dominant to it. WOYKE (1973b) found laranja (la) mutant in Africanized bees. SOARES and CHAUD-NETTO (1982) determined it to be an allele to snow (s^{la}). Laranja is epistatic to brick, s^{la}; ch interact in buff colour, s^{la}; i^u in white and s^{la}; g in ivory (WOYKE, 1973b).

The eye colour mutants are slightly different in haploid drones and diploid females. WOYKE (1973a) provided experimental evidence that this effect is not caused by gene differences as it had been assessed, because both haploid and diploid drones have mutant eyes of the same colour.

All these genes are recessive to wild type. Linkage was found between pearl and cream with a 0.33% crossing over value (LAIDLAW, El BANBY and TUCKER, 1965a). Linkage of eye-colour mutants and some other mutants were also found. MACKENSEN (1958) described the linkage between chartreuse and hairless.

LAIDLAW, El BANBY and TUCKER (1965b) mention the possibility of linkage between brick and reduced facet number. WITHEREL and LAIDLAW (1977) reported linkage between garnet and diminutive wing mutation.

Five eye shape mutants were described. Facetless (f; MIKHAILOFF 1930) and eyeless (e; LAIDLAW and TUCKER 1965a) mutants show pleiotropic effects. Drones of both mutants are sterile, but *f* have small testes, and no testes were found in *e* drones. LOTMAR (1936) described cyclops and KERR and LAIDLAW (1956) reduced facet number (rf). The entire absence of one of two compound eyes of drones was reported by DUSTMANN (1975).

Concerning body colour, an older view affirmed that the entire range of body colour variation from golden to black was the result of polygenes action without the participation of major factor genes (ROBERTS and MACKENSEN, 1951). LAIDLAW and El BANBY (1962) described an inhibitory gene, black (bl) which suprressed the yellow phenotype, and KERR(1969) discovered in African bees, abdomen costanho gene (ac) which supressed yellow colour in drones only. But the bimodal segregation in drone progenies of F_1 queens, led WOYKE (1977b) to the conclusion that those genes are major factor genes and are recessive to major yellow body colour allele (Y). The phenotype expression of all three alleles is modified by a series of poligenes. Because the character of both

dark major alleles appeared to be different from that originally described, symbols were changed into y_{bl} for black and y_{ac} for abdomen castanho.

Very useful as marker is the body colour mutation cordovan (cd). Here brown replaces all black pigment area. So it may be considered as epistatic to the black pigment within the expression of all three major body colour alleles Y, y^{bl} and y^{ac}. RUTTNER (1976) reported albino drones with unpigmented integument but with normal dark eyes.

Three body hair mutations, erbliche Schwarzsucht (S; DREHER 1940) hairless (h; MACKENSEN 1958) and HAARLOS (H; RUTTNER 1976) are described. Hairless is linked with ch (4.1% crossing over; MACKENSEN 1958).

Six wing mutations were found: Droopy (D; ROTHENBUHLER, GOWEN and PARK, 1952b), Rudimental wing (Rw; HACHINOHE and ONISHI, 1953), short wing (sh; KERR and LAIDLAW, 1956), truncated (tr) and wrinkled wing (wr; LAIDLAW, El BANBY and TUCKER, 1965 b) and diminutive (di; LAIDLAW 1966). These mutations are mostly linked with lethals, or are semilethal. Rw and i are linked with 31% crossing over (HACHINOHE and ONISHI, 1953). Several other mutants have been recorded but not yet published (See Table 8).

List of mutations and some other genes in the honeybee

Symbol	Name of mutation	Author	Aspect	Characteristics
1	2	3	4	5
	Eye colour			
—	white	1930 Mikhailoff	white	probably ivory
bk	brick	1953 Laidlaw, Green, Kerr	newly emerged bee (ne.) brick-red, later on (lo.) red brown	*bk;ch*^{2} interact buff, *bk;ch*^{r} and *bk;ch*^{c} interact pink (Laidlaw et al., 1953, 1964), *exist semilethal, hypostatic to i,* to *cr,* and *to s* (Mackensen, 1958) hypostatic to s^t (Laidlaw, et al, 1964)
by	bayer	Laidlaw (unpublished work)	ne. white, lo reddish-orange	no allele to *bk, ch, cr, g, i, pe, sp*
ch	chartreuse	1952 Rothenbuhler, *Gowen, Park*	ne. yellow green, lo. olive *green-reddish to reddish brown*	hypostatic to *i,* to *cr* and to *s* Rothenbuhler et al. 1952), *linked to h,* crossover *4.1 (Mackensen, 1958)*
ch^1	chartreuse-1	1953 Laidlaw, Green, Kerr	like *ch* — slightly darker *brown (variable)*	allele to *ch,* affected by *m* and interact *brown, ch*^{1}*/ch*^{r}intermediate, (Laidlaw et *al, 1953), recessive to ch*^{c}, dominant to *ch*^{B} *(Laidlaw et al., 1964)*
ch^2	chartreuse-2	1953 Laidlaw, Green, Kerr	like *ch,* greener, lo. reddish *to reddish brown*	allele to *ch, ch* ^{2}*/ch*^{r}intermediate, *ch*^{2}*; bk* interact buff, *hypostatic to i* and to s^t(Laidlaw et al, *1953, 1961)*
ch^B	Benson green	1964 Laidlaw, El Banby, Tucker	like *ch*^{2}, *but ne.* greener, lo. olive green to reddish	allele to*ch,*recessive to *ch*^{1}
ch^c	cherry	1964 Laidlaw, El Banby, Tucker	worker bees dark red, yellow to red brown *(very variable)*	allele to *ch,* dominant to *ch*^{1}, *bk; ch*^{c}, interact pink

1	2	3	4	5
ch^{li}	chartreuse *-limăc*	1981 Soares	ne. light yellow, lo. *reddish-brown*	allele to *ch, ch*^{li}*/ch* ^{r}light red *ch*^{li}*/ch*^{c} light *cherry, ch*^{li}*/ch*^{B} yellow green (Soares *1981)*
ch^r	red	1953 Laidlaw, Green, Kerr	ne. purple red, lo. red brown	allele to *ch, ch*^{1}*/ch*^{r} and *ch*^{2}*/ch*^{r} intermediate, *ch; bk* interact pink, hypostatic to *i (Laidlaw et al. 1953)*
cr	cream	1952 Rothenbuhler, Gowen, Park	white	epistatic to *ch* (Rothenbuhler et al. 1952), to *bk* (Mackensen 1958) and to *s* (Laidlaw *et al 1964),* linked to pe, *crossover 0.33 (Laidlaw et al. 1956).*
g	garnet	1964 Laidlaw, El Banby, *Tucker*	ne. garnet red, lo. dark even in wild type	
i	ivory	1952 Rothenbuhler, Gowen, Park	white	epistatic to *ch*/Rothenbuhler et al. 1952), to *ch*^{2} and *ch*^{r} (Laidlaw et al. 1953), *to bk* (Mackensen 1958), and to *s* (Laidlaw et al. 1964), partly recessive to i^u *(Laidlaw et al. 1965)*
i^{ro}	rose	Laidlaw (unpublished work)	ne. clear rose pink, lo. pink	homozygous do not fly to mate
i^u	umber	1965 Laidlaw, Tucker	ne. peach pink, lo. yellow-*reddish-brown*	allele to *i,* partly dominant to *i*
oc	ocelos claros	1977 Chaud-Netto	ne. compound eyes and ocelli rose lo. eyes brown or normal, ocelli glass white	

1	2	3	4	5
s^{la}	laranja	1973 Woyke, 1982 Soares, Chaud-Netto	ne. clear orange, lo. reddish brown	epistatic to brick s^{la}; *ch* interact buff s^{la}; i^{u} white and s^{la}; *g* ivory (Woyke 1973), s^{la}; ch^{r} reddish (Chaud-Netto 1975), s^{la}/s^{t} reddish (Soares, Chaud-Netto 1982)
m	modifier	1953 Laidlaw, Green, Kerr	brownish at ch^{1}-animals	affect ch^{1}, ch^{1}; m interact brown
p	pink	1963 Cale, Gowen, Carlile	rose pink	partly semilethal
pe	pearl	1964 Laidlaw, El Banby, Tucker	white	linked to *cr*, crossover 0.33 (Laidlaw et al. 1965)
s	snow	1952 Rothenbuhler, Gowen, Park	white (cannot be distinguished from ivory, cream and pearl)	epistatic to *ch*/Rothenbuhler et al. 1952), and to *bk*, exist semilethal (Mackensen 1958)
sp	spade	Laidlaw (unpublished work)	ne. rose pink, lo. red (similar to *bk*)	no allele to *bk, ch, cr, g, i, pe, by*
s^{t}	tan	1964 Laidlaw, El Banby, Tucker	ne. white, lo. bright yellow-brownish	allele to *s*, s/s^{t} is red, epistatic to ch^{2} and *bk, hypostatic to i* and *cr*
	Eye shape			
—	cyclops	1936 Lotmar	*cyklops eyes*	dominant, transmitted infrequently via eggs (Lotmar 1936, Kerr and Laidlaw 1956, Laidlaw et al. 1965).
—	einäugig	1975 Dustman	one compound eye missing	
e	eyeless	1965 Laidlaw, Tucker	no facets	males sterile, lack of testes, semilethal in hemizygote
f	facetless	1930 Mikhailoff	no facets	males have small sterile testes
rf	reduced facet	1956 Kerr, Laidlaw	atrophied eyes owing small number of facets	inherited complex in low frequency with *bk* or *g* (Laidlaw et al. 1965).

1	2	3	4	5
	Body pigment			
a	albino	Ruttner (unpublished work)	non-pigmented integument, non-sclerified, normal eye pigment (Fig. 56)	incomplete spermatogenesis, semilethal
cd	cordovan	1951 Mackensen, Nolan	colour similar to that of leather	epistatic to the black pigment within the expression of major body colour alleles *Y*, y^{bl} and y^{ac}
Y	yellow	1977 Woyke	major yellow body colour allele	dominant to y^{ac} and y^{bl}; modifiable by poligenes over wide range (Woyke 1977)
y^{ac}	abdomen castanho	1969 Kerr, 1977 Woyke	major body colour allele, sex limited, expressed as dark in haploid and diploid drones, and as yellow in females	y^{ac} *In* ♂♂ black y^{ac}/y^{ac} *2n* ♀♀ black, ♂♂ females yellow, y^{ac}/Y *2n* ♀♀ and females yellow, mofifiable by poligenes (Woyke 1977)
	Body haires			
S	schwarzuchtig	1940 Dreher	no down	dominant to wild type
h	hairless	1958 Mackensen	no down	recessive to wild type, semilethal in drones, linked to *ch*, crossover 4.1
H	Haarlos	Ruttner (unpublished work)	no hairs, hair on tomentum *exists (Fig. 58)*	bristles (pollen rake) are brittle, the *viable heterozygous workers produce small lellets. Hemizygotes (* ♀♀ *) are lethal*
	Wings			
D	Droppy	1952 Rothenbuhler, Gowen, Park	flat separated wings, *cannot fly*	dominant to wild type, lethal in hemi*zygote and homozygote* ♀♀
di	diminutive	1966 Laidlaw (unpublished)	small wing, normal venation	when flying workers and ☿ ♂ produce *a high tinkling sound, homozygous* ♀♀ *do not fly (Witherell and Laidlaw 1977)*

Rw	Rudimental wing	1953 Hachinohe, Onishi	atrophied wings	dominant to wild type, linked to *l*, *crossover 31*
sh	short	1956 Kerr, Laidlaw	small wing, bees cannot fly, *abnormal venation*	semilethal (Laidlaw et al. 1965)
tr	truncate	1965 Kerr, 1965 Laidlaw, El Banby, Tucker	the wing looks as if cut in the *middle, bees cannot fly, abnormal venation*	semilethal
wr	wrinkled	1965 Laidlaw, El Banby, Tucker	"rumpled" wing	incomplete penetrance; increase by *combination with bk*
	Sting			
sps	split sting	1977 Soares	lancets separated from stylet	low penetrance, frequency of 3.5% *raised to 62.0% after selection (Soares 1981)*
	Disease resistance			
r	removing	1964 Rothenbuhler	hygienic behaviour	♀♀ workers remove dead brood
u	uncapping	1964 Rothenbuhler	hygienic behaviour	♀♀ workers uncap cells with dead brood
	Viability			
l	lethal	1953, Hachinohe, Onishi	dead in early stage	linked with *Rw*, crossover 31
	Sex			
X^n	sex alleles	1951 Mackensen, 1963 Woyke	sex determination	hemizygous — haploid *drones heterozygous — females homozygous* — diploid *drones (Woyke 1962), 2n* ♂♂ larvae ♀♀ *eaten by workers (Woyke 1963)*

Unusual Bees (Mosaic and Gynandromorph Individuals)

Artificial insemination together with the mutant genes used as markers, has made it possible to determine the origin of unusual bees (Table 9).

Different unusual bees originating from unfertilized eggs have been described. Unfertilized binucleate eggs result in mosaic males (Table 9, 1a) (TUCKER, 1958; WOYKE, 1962a). Uniting of two pronuclei in unfertilized eggs produces parthenogenetic females (Table 9, 1b). Two haploid egg pronuclei can divide at least once before union. Next two haploid nuclei unite to form a diploid cleavage nucleus which develops into female tissues; the other haploid nuclei develop into mosaic male tissues (Table 9, 1c). Thus a gynandromorph is formed.

Different individuals originating from inseminated eggs have been described:

From fertilized eggs, not only females can develop, as it generally happens, but, when the sex alleles are in common, diploid males develop as well (WOYKE, 1963a). More details about these will be given in the next section.

If only one pronucleus of the two is fertilized in a binucleate egg, the developing gynandromorph possesses male tissue of matroclinous origin and the female tissues of biparental origin (Table 9, 2.1b). Polyspermy may be the cause of several types of unusual bees: Two sperms in an egg can unite, leaving the egg pronucleus unfertilized (Table 9, 2.2a). This results in a gynandromorph in which the diploid female tissues originate from two different fathers, without the participation of the mother. Polyspermy of an egg with one nucleus results in a fertilization of the nucleus but, sometimes, one or more accessory sperms do not degenerate and develop into male tissues. Thus a gynandromorph develops with the origin of tissues different from those mentioned above. Here the female diploid parts are originating from both parents but the haploid male parts develop from sperm only (Table 9, 2.b).

A mosaic female can result from polyspermy in a binucleate egg if sperms of different fathers unite with the two pronuclei (Table 9, 2.2c). The two haploid egg pronuclei can first divide and then two of them can be fertilized by different sperms and the other two can unite resulting in a female,

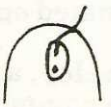
Origin of unusual honey bees hitherto described

1. From uninseminated egg

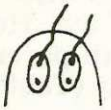
a		Mosaic male	Tucker 1958, Woyke 1962
b		Parthenogenetic female	Mackensen 1943, Tucker 1958, Woyke 1962, Tryasko 1965
c		Gynandromorph with mosaic male tissues	Tucker 1958

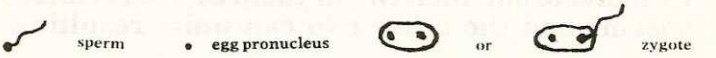
2. From inseminated egg

2.1. One sperm takes part in the origin of a bee.

a		Diploid male	Woyke 1963, 1965
b		Gynandromorph with matroclinous male tissues	Mackensen 1951, Woyke 1962, Drescher, Rothenbuhler 1963

2.2. More sperms take part in the origin of a bee.

a		Gynandromorph with diploid female patroclinous tissue	Laidlaw, Tucker 1964
b		Gynandromorph with patroclinous male tissue or Mosaic male with some diploid tissues	Rothenbuhler et al 1952, Rothenbuhler 1957 Drescher, Rothenbuhler 1964
c		Mosaic female	Taber 1955, Woyke 1962
d		Mosaic female with diploid parthenogenetic tissues	*Woyke*, 1962



that is partly parthenogenetic and partly has two fathers (Table 9, 2.2d).

Sex Determination

The problem of sex determination could be solved only thanks to artificial insemination. MACKENSEN (1951) found out that, after individual mother daughter matings one half of the queens produced brood in which only 50% of the individuals survived. This was confirmed by HACHINOHE and JIMBU (1958). Later MACKENSEN (1955) found at least 11 different alleles responsible for this effect. LAIDLAW, GOMEZ and KERR (1956) found 12.4±3.56 such alleles in a panmictic population in Brazil. ROTHENBUHLER (1957) reported patches of diploid male tissue in eyes of mosaic drones from related parents of a gynandromorph line. This was confirmed later by DRESCHER and ROTHENBUHLER (1964). But a drone of completely zygotic origin was not found. It was believed that the homozygous eggs for an "X" locus, obtained by inbreeding did not hatch, and that small areas of diploid male tissue could survive only by virtue of their association with normally viable haploid male tissue.

But WOYKE (1962b) showed in the meantime that all the eggs from inbred queens hatch. He proved that some drone larvae are of completely zygotic origin (1963b). The low survival rate is caused by the nurse bees eating newly hatched diploid drone larvae (WOYKE, 1963c), which produce a pheromon called cannibalism substance (WOYKE, 1967). The diploid drone larvae are viable (WOYKE 1963b; 1965b), and be reared to imagines (WOYKE, 1969). Their origin from fertilized eggs was proved cytologically (WOYKE et al, 1966; WOYKE and KNYTEL, 1966) as well as genetically (WOYKE, 1965a; WOYKE and ADAMSKA, 1972). Diploid drones are heavier and larger than haploids (WOYKE, 1977a, 1978a, b). They show some supermale as well as intersex characters (WOYKE, 1980). Their testes are smaller, and they may be only one-tenth of the testicular volume of haploids (WOYKE, 1973a). No reduction of the number of chromosomes occurs during spermatogenesis (WOYKE and SKOWRONEK, 1974). Consequently diploid drones produce diploid spermatozoa (WOYKE, 1975, 1984).

It can be stated that a series of sex alleles exists which in heterozygotes results in females, and in hemizygotes and homozygotes in males. The homozygotes are not inviable but are eaten by worker bees, and therefore were not observed.

Bee Diseases

Resistance of honey bees to disease had already been reported before artificial insemination was used in disease resistance studies (STURTEVANT, 1920; PARK, PELLET and PADDOCK, 1937). It was concluded that resistance to American foulbrood is heritable. Also, some information had been gained on the mechanism of resistance to European foulbrood (STURTEVANT, 1920) as well as American foulbrood (WOODROW, 1941; WOODROW and HOLST, 1942; STURTEVANT and REVEL, 1953).

Artificial insemination has made more detailed studies possible. ROTHENBUHLER and THOMPSON (1956) found highly significant differences between lines in survival of larvae treated with American foulbrood spores. This resistance is also heritable (LEWIS and ROTHENBUHLER, 1961). When the larvae of two lines had received spores at 21 hours of age, differences were found both in time of generation and in the number of bacteria observed (BAMRICK, 1964). But also the adult bees of different genetic lines protect the larvae to different degrees (THOMPSON and ROTHENBUHLER, 1957).

A strong difference in behaviour of four inbred lines toward dead brood of American foulbrood was demonstrated by ROTHENBUHLER (1964). Two inbred lines of bees showed a great difference in the time required to uncap and remove cyanide-killed brood (JONES and ROTHENBUHLER, 1964a). Colonies composed of young resistant bees will remove all larvae killed by foulbrood, whereas colonies made up of bees older than about 4 weeks remove the larvae only during a nectar flow. After genetic differences had been found, the heredity could be investigated. ROTHENBUHLER (1964b) made the necessary crosses and found, in the backcrosses, four types of colonies: 1 — uncappers of cells and removers of dead brood contained therein, 2 — uncappers only, 3 — removers only after human uncapping, 4 — neither uncappers nor removers. He developed a two locus hypothesis. It states that uncapping of a cell containing dead brood depends upon

homozygosity for a single recessive gene (designated u) and removing depends upon homozygosity for a second single recessive gene (designated r).

MORITZ (1988), however, showed that Rothenbuhler's model was based on a too small data set, and actually any other genetic model is as likely as the two locus hypothesis.

DRESCHER (1964) found that the tendency to paralysis (Schwarzucht) was irrespective to the mating drone. He suggested a matroclinous heredity.

Pollination

Differences in pollen collection of colonies have often been observed and studies have been made to determine if such differences are hereditary. NYE and MACKENSEN (1965) made studies to determine if the tendency to prefer alfalfa pollen is inherited. They showed that colonies headed by sister queens were more similar in the proportion of alfalfa pollen collected than those headed by unrelated queens.

Later MACKENSEN and NYE (1966, 1969) and NYE and MACKENSEN (1968) were able to select low and high preference lines, the selection being based on the percentage of pollen collectors that were collecting alfalfa pollen. In the high preference line, this percentage increased from 39.8 in the second generation to 86 in the sixth. In the line showing low preference the corresponding percentages were 26.2 and 8. From crosses and backcrosses they concluded that this characteristic is probably controlled by several genes that have a mainly additive effect. The selection also affected the percentage of pollen collected by the two lines from several different sources (MACKENSEN and TUCKER, 1973).

These results prove beyond doubt that the tendency to collect alfalfa pollen in preference to other pollens is heritable. They open the possibility of developing special high quality strains or hybrids of honey bees for commercial use in pollinating alfalfa. Probably it is also possible to breed strains of bees better capable of pollinating red clover and other crops.

Obtaining mutants by selection

Thanks to instrumental insemination a selected queen may be inseminated with the semen of selected drones, a ma-

ting station being no longer necessary for this purpose. This technique provides for a very rigorous selection, as queens may be inseminated with the semen collected either from one drone alone, or from several drones.

This technique also enables obtaining of mutants, which under natural conditions could not fertilize a queen, as, for example, the individuals with wing mutations. Mutations serve as gene markers and have helped to elucidate many problems regarding the bee biology as for example the mixing of semen in the spermatheca (MORITZ, 1983) and drifting (SAKOFSKI, 1986), and also to make new findings.

A number of eye colour mutations in bees have been described, as well as of the body colour, eye shape, wing, body hair and sting. All these mutations are recessive to wild type genes. Mutations occur spontaneously (mutation rate 10^6 or 10^7) (KERR et al., 1980; CHAUD-NETTO et al., 1983), but can also be obtained by means of mutagen agents (BRANDES and FRISCH, 1986). Usually, the mutation is obvious in the drone as it is a hemizygote and each gene will result in a phenotype.

The method of obtaining mutants will be described taking as an example the gene of leather body colour — cordovan (cd) (Fig. 32). A wild type queen is instrumentally inseminated with a mutant cordovan drone. The female offspring of the queen are heterozygous for cordovan locus (cd/+) and a wild type phenotype. From this F_1 generation a queen will be reared, which will become a drone layer following two anesthetizations with CO_2. Out of the drones obtained 50% will be wild type and 50% cordovan mutants. The queens of the F^1

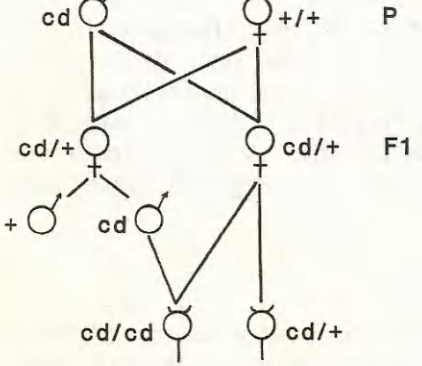


Fig. 32 — Crossing diagram to obtain a mutation.
cd=cordonan, t=wild type, F=first generation, p=parental generation

generation will be inseminated with the semen from the cordovan drones (cd/+). Half the female offspring of these queens are homozygous for cordovan (cd/cd) and half are heterozygous for this gene (cd/+) which means a wild type phenotype.

One of the difficulties faced in maintaining mutants is the capability of survival of a colony whose workers are carrying homozygous mutations. Because very often the mutation workers are no longer capable of carrying out their activities inside the colony, in a lot of eye colour mutations, as for example pearl = blind, orientation by means of sight is scarcely possible or impossible. Also, the hairless worker mutants are not able to properly perform their pollen collecting activity in terms of morphology (small pellets). Such circumstances could entail death of the entire colony if the beekeeper does not help now and then.

Mutants may be maintained without constant watching and help by the beekeeper, by colonies with heterozygous workers which have wild type phenotypes. A heterozygous queen inseminated with the semen of mutant drones is a simple solution for mutant maintenance. 50% of the descendants will be homozygotes and 50% heterozygotes. With this method, 100% homozygote workers can be obtained in the next generation following one crossing alone (homozygot queens x semen of mutant drones). But when several loci are involved selection will be much more intensive.

Of the 35 different morphological mutations described up to now, only four linkage groups are known (Del LAMA et al., 1985). This means that almost all markers are located on different chromosome pairs. The consequences for selection will be briefly illustrated by three mutant characteristics (Table 10).

A heterozygous queen was inseminated with semen which included all three mutations. As all characteristics are inherited independently, the descendants of the queen will be of eight different genotypes. In order to obtain ten homozygous queens for all three mutations an average of 80 queens must by produced, on condition that during the larval development no difference in terms of viability occurs between the 8 different genotypes.

Table 10

Scheme of the crossing of a heterozygous queen for 3 mutant characteristics with a drone with 3 mutant characteristics

Queen cd i^u di / + + + x Drone cd i^u di

	Queen gametes																						
	cd	i^u	di	cd	i^u	+	cd	+	di	+	i^u	di	cd	+	+	+	i^u	+	+	+	di	+	+	+
Worker genotypes	cd	i^u	di	cd	i^u	+	cd	+	di	+	i^u	di	cd	+	+	+	i^u	+	+	+	di	+	+	+
cd i^u di	cd	i^u	di	cd	i^u	di	cd	i^u	di	cd	i^u	di	cd	i^u	di	cd	i^u	di	cd	i^u	di	cd	i^u	di
pheno-types:	cd	i^u	di,	cd	i^u	+,	cd	+	di,	+	i^u	di,	cd	+	+,	+	i^u	+,	+	+	di,	+	+	+
		1					:							7										

cd = cordovan (body colour)
i^u = umber (eye colour)
di = diminutive (wing length)

In order to be able to store mutants in "banks" for many years, and to use them for experiments, the viability of the marker line must be ensured. To this end it is essential to avoid inbreeding depression as much as possible. The high homozygosity entailed by inbreeding reduces the intercolony variability (BRÜCKNER, 1976a), which results in a shorter life span and lower performance of different activities (PLASS, 1953; BRÜCKNER, 1976b, 1980).

Therefore, the marker line must be crossed with unrelated wild type genes after which the marker gene should be reselected. In this way, heterozygosis for all the other loci is maintained, which avoids inbreeding depression.

2. Genetics of behaviour

2.1. Behaviour of individuals

Honey bees are capable to distinguish precisely different scents. Individual bees may be trained to prefer certain scents, but differences are recorded between the results of the training in the different individual worker bees. BRANDES (1988) used for this purpose the proboscis extension reflex. A scent was provided to a hungry bee (conditioned stimulus) after which it was rewarded by syrup (unconditioned stimulus). The bees which learnt well responded to a conditioned stimulus which was subsequently rewarded by extending the proboscis; but they would not extend their proboscis when the conditioned stimulus was not followed by reward. The bees which learnt less well, were found to be significantly less capable of distinguishing the different stimuli, (both rewarded and unrewarded) than the bees which learnt well. By a selection experiment on workers and instrumentally inseminated queens, which lasted for several years, BRANDES (1988) obtained evidence that the difference in terms of learning capability between the individual worker bees are heritable.

But differences between worker bees also exist in terms of food, some would offer more while others less food (KORST an VELTHUIS, 1982). The tropholactic dominance is correlated with the reproductive dominance in worker bees. In a queenless colony, the predominant workers will develop their ovaries faster, lay more eggs and sinthetize more 9-oxo-2 decenoic acid, the major compound of the queen substance, than the other workers. A genetic quantita-

tive analysis is possible, thanks to instrumental insemination. It showed that all these characteristics are heritable (MORITZ and HILLESHEIM, 1985). The individual dominance of worker bees has a decisive influence on the behaviour of the colony (HILLESHEIM, 1986) and on its productivity (HILLESHEIM, 1987). The groups and colonies consisting of dominant workers alone are exceeded by subordinated groups in terms of hoarding behaviour, brood rearing, building of combs and supply of syrup. These results, namely selection advantage for the reproductive predominance in individuals and selection disadvantage for the entire colony, illustrate the genetic balance between the dominance and subordination genes in a population.

2.2 Behaviour of bee groups

Because of their eusociality, the honey bees require a completely different behavioural genetics approach than the insect classically used in genetic experiments, *Drosophila melanogaster.* In bees not only the behaviour of individual insects is studied but, above all, the behaviour of an entire group and of a colony (40.000 to 100.000 individuals).

The study of behaviour in honey bees, as group characteristics, covers a large range of patterns. Almost all activities are performed in the colony by many individuals together, such as guarding of the hive (STORT, 1980; COLLINS et al., 1984; MORITZ et al., 1987), brood rearing (HARBO, 1986 c), building of combs (LEE and WINSTON, 1985), honey- production (WINSTON and KATZ, 1982; WOYKE, 1984) and pollen collection (HELLMICH et al., 1985) to mention only some of these activities.

Using instrumental insemination, various experiments have been conducted on bees to elucidate the evolution of altruism and eusociality.

Use of genetic markers such as body colour is essential in the study of the kin recognition behaviour. Renouncing one's own descendants may pay off when the benefit-cost ratio between recipient and altruist is higher than the coefficient of kinship between both (HAMILTON, 1964a, b). If the coefficient of kinship (r) of brothers is relatively high, altruism may have a selective advantage. In Hymenoptera the coefficient of kinship of super sisters (the same mother and the same father) is 0.75 by haplo-diploidy, and the coefficient of kinship of the mother and her descendants is 0.5. Under

such conditions altruism may be advantageous. Because the queen mates several times, in a colony both super sisters and half sisters coexist (the same mother but different father). For an altruist it is more advantageous to prefer its super sisters. But in this case the premise is the recognition of the super sisters. Initial reports on the discrimination between kin were reported by BREED et al., (1984), PAGE and ERICKSON (1986) and VISSCHER (1986).

MORITZ made an interesting experiment on kin recognition in a colony by using the gene markers cordovan (cd — body colour), pearl (pe — eye colour) and diminutive (di — length of the wing) and the instrumental insemination technique. A homozygous queen for all three characteristics was inseminated with the semen collected from eight drones, each of them with a different combination of the characteristics of the three different gene markers. All daughters had the same phenotype as their father, and one could distinguish the super sisters from the half sisters.

Much of the new knowledge about honey bee behaviour would not have been possible without the instrumental insemination technique and use of previously selected mutants, so that both techniques should be further improved as they are indispensable in honey bee genetics.

SELECTION THEORY AND SELECTION PROGRAMMES

Jean-Marie CORNUET
Robin F. A. MORITZ

As compared to the selection of other live stock, selection of honey bees still lags behind. The causes include the complex biology of mating and the sex determination in honey bees. Bee colonies are readily affected by inbreeding, and inbreeding depression may easily make the selection work of many years useless. How could such failures of selection programmes be avoided? To answer this question we must firstly delve more thoroughly into the theory of genetic selection.

Involved in selection are both the genetical characteristics (which may be selected) and characteristics exclusively determined by environment factors (as nutrition, for example). The genetic influences are distinguished from the environmental influences by population variability. If all animals have the same characteristics, no selection of animals with special characteristics is possible. The essential parameter for selectivity is the genetic variation of the characteristic concerned. The greater the influence of environmental factors on the characteristic to be selected, the more difficult it is to achieve a shift of the characteristic towards the selection target. The heritability (h^2) is used in selective genetics as a measure of selectivity and therefore is an important parameter.

The characteristic of an animal is determined by its genome, but also by the environment.

Characteristic feature=genotype+environment

For a population of animals an average value (P) of the characteristic feature is obtained. Certainly, each individual of the population will differ from other individuals in terms of its genetic make-up (G) and its specific environment (E). None of the individuals is perfectly identical to another one. This means that there is variation for the characteristic feature in the population: a deviation from the average value determined for the entire population, statistically expressed as population variance. Just as the characteristic feature, the

variance (Vp) consists of the genetical variance (Vg) and the environmental variance by (V_E):

$$Vp=V_G+V_E$$

The genetical variance as part of the total variance is the heritability (in a wider sense). The genetic deviation of an individual animal from the average value determined for the population may result from additive genetic effects (as for example the intermediate heredity) or from dominance genetic effects. It is the additive genetic effects that are important for the selectivity of a characteristic feature. It is necessary to rigorously distinguish between the additive genetic effects and the dominance effects, since the genetical variance consists of these two components V_A and V_D:

$$V_G=V_A+V_D$$

Assessment of the additive genetic variance (V_A) is a central element in selection genetics. It helps to estimate selectability (heritability in a narrow sense): $h^2=V_A/V_P$. Once the selectability has been determined, also the efficiency of selection programmes may be anticipated. The genetic progress recorded per generation (r) is estimated (when the intensity of selection, i is known) as follows:

$$r=h^2i$$

This classical relation used in quantitative genetics must be modified when honey bees are involved; because of the fact that they live in a community. A complex characteristic feature such as honey production is the result of the interaction of thousands of individual worker bees, different from one another in terms of genotype. By the number of eggs laid, the queen also indirectly influences this characteristic feature. BIENEFELD (1986) was successful in distinguishing the worker components from the queen components in the characteristics of the entire colony, as well as in setting out their interactions. CORNUET (1987) suggested a different measure for the heritability of worker bee characteristics, also to be taken into account when assessing the performance of the colony. In assessing the kinship of different co-

lonies, new measures must be defined (MORITZ 1986) because the models of the classical quantitative genetics are insufficient for this purpose. The new theoretical developments certainly provide for the progress of practical selection work. In general, two selective systems are now in use with promising results.

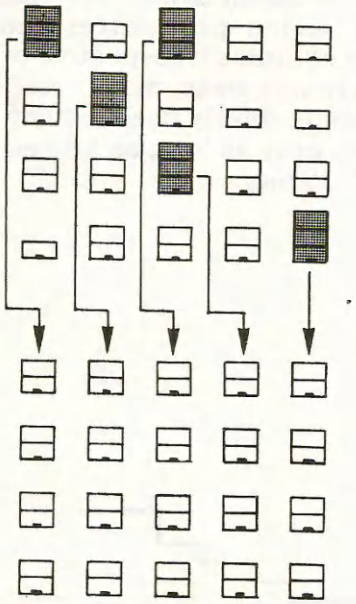


Fig. 33 — Diagram for a mass selection of a 20 colonies population
A number of five colonies (hatched) is selected after each season (A). These will be used for producing drones and queens for the next generation. Before a new step of the test (B) one has to remove the old unselectioned queens, the colonies have to be brought to the same strength and new queens will be introduced (four into a selectioned colony) Consequently, the test population is made up of 5 families of four colonies.

Selection in closed populations

Selection in closed populations is possible by instrumental insemination only. It may be compared to the selection of lines which certainly requires use of larger bee stocks (PAGE and LAIDLAW, 1982,a,b; PATE et al., 1983). After establishing the population to undergo selection, no new genetic stock must be added. The best colonies of the population are selected and used for obtaining the next generation (Fig. 33). So that the number (Q) of colonies to be selected/generation must be determined. These colonies will provide the

queens and drones necessary for obtaining the next generation. If a small number of colonies is selected, the selection intensity is high but the inbreeding coefficient also increases fast. If too many colonies are selected, inbreeding will be avoided but no genetic progress will be achieved. But because heritability, inbreeding depression and size of the population can be determined, an optimal selection programmes for a certain period of selection can be designed. In Fig. 34 the optimum number of colonies to be selected are given with instrumental insemination (mixed semen procedure) and mass selection (selection of the best colonies irrespective of their kinship) being performed. In honey bees, mass selection appears to be simplest, the most commonly used, and often the best method of selection, but only as long as inbreeding does not exceed 25% (MORITZ, 1986c).

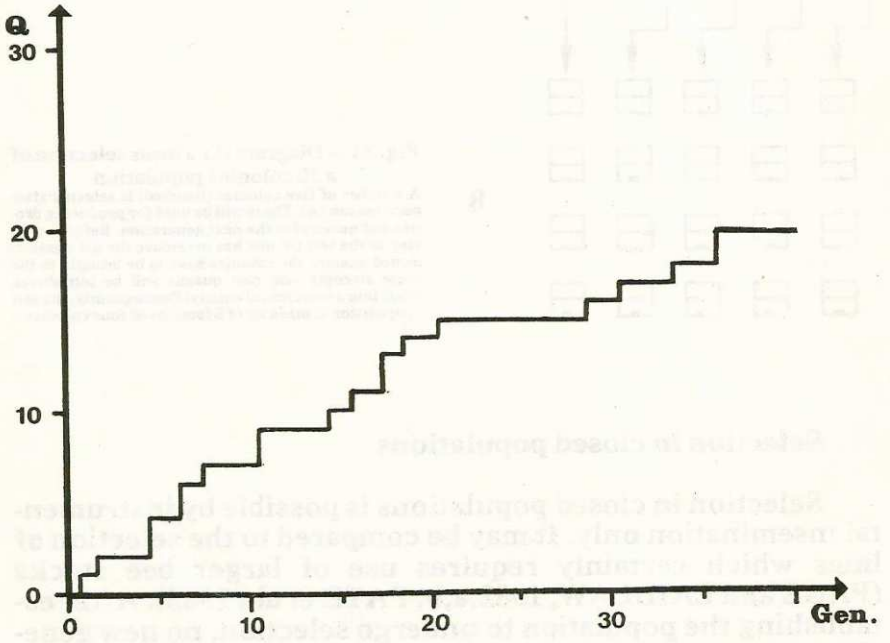


Fig. 34 — The best number of queens Q which should be selected in case of a mass selection/generation

A selection rate of 0.35 was chosen for honey output. At its base there is a testing capacity of 50 colonies. Selection's period (in generations on K coordinate) is of outmost importance. The shorter the selection experiment the smaller Q

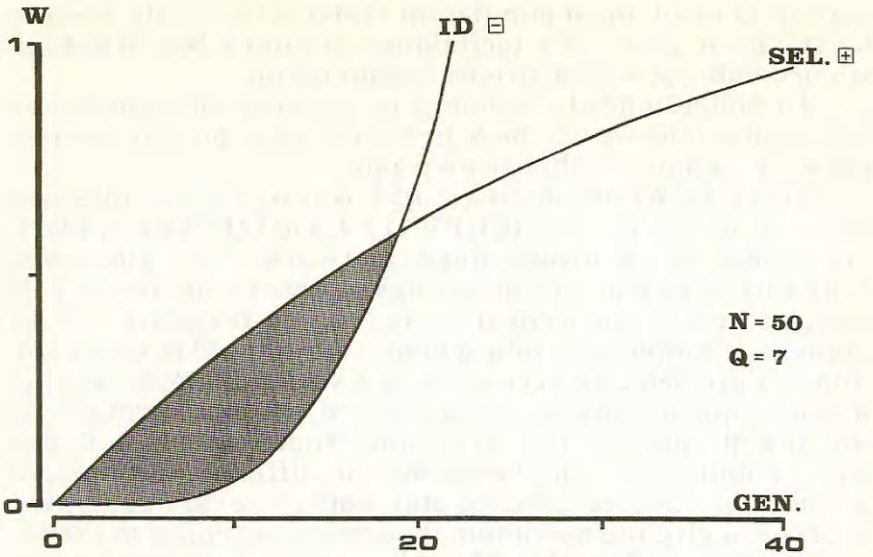


Fig. 35 — Interdependence between output (w: Y coordinate/inbreeding depression (iD), genetic progress (selection t) and selection period (Gen: X coordinate)
For each generation 7 colonies are selected out of a number of 50 colonies test populations. The hatched area points to the real improvement of the output. In spite of a selection after 20 generations, the output had reached again its initial value. In this selection diagrame (7 out of 50 colonies) the maximum peak of the output is reached almost on the tenth generation. This is when one has to cross a foreign genetic material which should prevent the output's decrease.

The advantage of closed populations is the high accuracy with which the genetic progress (selection efficiency) can be assessed. The breeding values of the queen can be determined precisely. Quite a big disadvantage is that every closed system of selection results in inbreeding. Because of the specific determination of the sex, the fact that diploid drones occur, and of the high sensitivity of honey bees to inbreeding depression, new genetic stock must be introduced at some time (Fig. 35). For a selection programme to be economically efficient, large populations and adequate testing facilities must be available. The closed population system of selection is efficient only when at least 50 colonies are used.

Open Population System

Open population systems allow the introduction of foreign genetic material into the population. Therefore such breeding systems are less susceptible to inbreeding. Under

careful control, open population systems currently seem to be the most promising techniques in honey bee breeding, when combined with artificial insemination.

To understand the genetics of breeding in open honey bee populations we will have to define the important genetic parameters more explicitly than above.

Recently, we proposed a model to describe the collective performance of a colony (CHEVALET and CORNUET, 1982). This model, which involves queen and workers contributions, allows us to give precise meanings to genetic parameters. It also provides a theoretical basis for bee breeding. As an example, a simple selection scheme is presented in which the colonies are selected according to a selection index. In this scheme, queens are naturally mated by local drones, of which a proportion β ($\beta<1$) come from selected colonies (open population). The values of the different parameters (number of selected colonies and family size) are computed in order to give the maximum theoretical response to the selection compatible with a tolerable increase in inbreeding.

Genetic and statistical model

Selective breeding is based on the fact that relatives are generally more alike than random individuals and on the assumption that this resemblance is (at least partly) due to a larger number of identical genes in two relatives than in two random individuals. Many of the traits that interest the breeders are measurements. For instance, if one measures the height of people, one simple way to show resemblance between relatives is to plot the size of sons versus the size of their fathers. Such a graph generally shows a tendency which statisticians summarize with a regression line (the straight line that best aproximates the trend of the set of points) or with one coefficient (either the liniar regression coefficient or the correlation coefficient). Another method, suited for species in which one sex can have a large number of progeny, is to rear families in the same environmental conditions and to see if the average measurements differ significantly between families. One measure of resemblance of family members is the intraclass correlation coefficient.

All the above coefficients rely on the computation of specific variances and covariances. Variances measure variations of individual data in one set, covariances measure covariations of pairs of data sets. There are simple classical relationships between these different coefficients and genetic

parameters such as heritability. However, these apply to traits measured in individuals, not to collective traits like those measured in honey bee colonies. So, in order to advance in this area, it is necessary to go back to the basis of the theory.

Definition

Let P be a collective trait measured in a colony (i. e. honey production). We consider that both the queen and the workers are responsible for this performance. So, we postulate the simplest following decomposition:

$$P=Q+W+E \qquad (1)$$

where: Q is the genotypic contribution of the queen,
W is the average of the genotypic contributions of the workers,
E is the sum of non-genetic effects.

Q, W and E are unmeasurable (and thus unknown) quantities. The genotypic contributions are decomposed (in a classic way) into additive and dominance effects, neglecting epistatic interactions for the sake of simplicity. This means that a genotypic contribution is considered as the sum of the effects of all loci involved with the trait under study. The effect of one locus is the sum of the effects of both alleles at this locus (additive effects) and a correcting factor (dominance effect) which comes from a possible interaction between these alleles. All these effects are unmeasurable but some breeding designs may provide estimates of their variances in the population under study. The variance of additive effects (V_A=additive variance) is especially interesting since the ratio V_A/V_P (V_P=phenotypic variance) is equal to the heritability of the trait in the population. But, in our case, we have not a single additive variance but at least two: one for the genes involved in the queen genotypic contribution (V_{Aq}) and one for the genes involved in the workers genotypic contribution (V_{AW}). Actually, as some genes may be common to both sets, a third additive variance (V_{Aqw}) has to be considered (CHEVALET and CORNUET, op. cit.).

What is the relationship between the usual coefficients estimated on honey bee colonies and these additive variances ? Is there a combination of these additive variances

which could be taken as the numerator of heritability ? In order to answer these questions, we have to apply the classic and very basic relationship:

$$Cov(X_1, X_2)=2\ \Phi_{1,2}V_A+d_{1,2}V_D \qquad (2)$$

where: Cov (X_1, X_2) is the covariance of trait X measured on two individuals 1 and 2

$\Phi_{1,2}$ is the coefficient of coancestry between 1 and 2 (probability that, at a given locus, one allele taken at random in 1 is identical to one allele taken at random in 2)

$d_{1,2}$ is a coefficient of genetic identity between 1 and 2 (probability that at a given locus, both genes of 1 are identical to both genes of 2)

V_A and V_D are respectively the additive and dominance variances of trait X the in population from which 1 and 2 are drawn.

Examples

Let us consider two breeding schemes generally used to provide estimates of heritability: a mother-daughter relationship design and a intra-family relationship design.

1. Mother-daughter relationship

Let us consider two sets of colonies. The first one is composed of colonies headed by unrelated, naturally fecundated queens. The colonies of the second set are headed by queens which are the daughters of the queens of the first set and have also been naturally mated. All colonies in the same set are assumed to have encountered the same environmental conditions. A collective trait is measured on every colony of both sets and data are normalized (divided by the corresponding standard deviation so that the phenotypic variance is equal to one). "Mothers" and "daughters" refer respectively to colonies of the first and the second set.

The mother-daughter covariance measures the degree of relationship between the "mothers" and the "daughters" performances. The pedigree in figure 36 shows the four different relationships to be considered:

— between queens (mother-daughter relationship: $\Phi = 1/4$, $d_1=0$)

— between the mother-queen and the workers of the

daughter colony (grand-mother-grand-daughter relationship: $\Phi_2=1/8$, $d_2=0$)

— between the workers of the mother colony and the daughter-queen ("mixed" sisters relationship: $\Phi_3=\Phi$ $[1/8<\bar{\Phi}<3/8]$, $\bar{d}_3=\bar{d}$ $[0<\bar{d}<1/2]$)

— between the workers of both colonies (aunt-niece relationship: $\Phi_4=\Phi_3/2$, $d4=0$).

Applying formula (2), we get the following mother-daughter covariance:

$$MDC=\tfrac{1}{2}V_{Aq}+\tfrac{1}{4}V_{Aqw}+(2\bar{\Phi}\ V_{Aqw}+\bar{d}V_{Dqw})+\bar{\Phi}\ V_{Aw} \quad (3)$$

2. Intra-family covariance

In this case, let us take a set of colonies with naturally mated, unrelated queens. In each colony of this set are reared queens which are also open mated. A "family" is considered as a group of colonies whose queens come from the same colony. All families are measured under the same environmental conditions. If the data are normalized, the resemblance between members of the same family can be measured by the average covariance of the performances of two colonies of the same family (called the intra-family covariance). Here again, the four relationships have to be considered (figure 37):

— between both queens (mixed sisters relationship: $\Phi_1=\bar{\Phi}$, $d_1=\bar{d}$)

— between the queen of colony 1 and the workers of the colony 2 (aunt niece relationship: $\Phi_2=\bar{\Phi}/2$, $d_2=0$)

— between the queen of colony 2 and the workers of the colony 1 (aunt niece relationship: $\Phi_2=\bar{\Phi}/2$, $d_2=0$)

— between the workers of both colonies (cousins-cousins relationship: $d_2=\bar{\Phi}$ 14

The intra-family covariance is then equal to:

$$IFC=(2\Phi V_{Aq}+dV_{Dq})+\bar{\Phi}\ V_{Aqw}+\bar{\Phi}\ V_{Aqw}+\tfrac{1}{2}\bar{\Phi}V_{A\,w} \quad (4)$$

Formulae (3) and (4) give the answer to the two questions addressed above:

1-here are the relationships between the covariances estimated on colony performances and the additive and dominance variances,

2-there is no common combination of additive variances in the two formulae. So, the two breedigns desings provide different estimates of heritability.

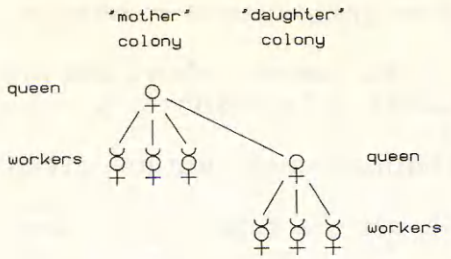


Fig. 36 — Pedigree showing the relationships between individuals of "mother" and "daughter" colonies

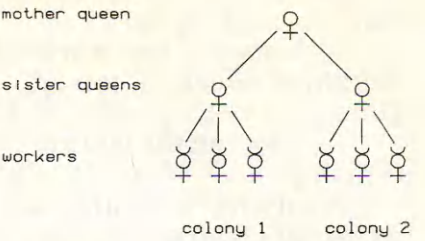


Fig. 37 — Pedigree showing the relationships between individuals of "sister" colonies.

Principles of open breeding schemes

In the proposed scheme, colonies are tested for one or more characters such as honey production, gentleness, etc... in every generation. Some are selected to produce the queens and the drones of the next generation. These queens are naturally mated. The queen containing nuclei are put in an apiary with selected colonies which have been previously induced for drone production. Therefore, only an unknown proportion (β) of the drones with which they mate come from the selected queens.

All the colonies whose queens come from the same selected colony form a family. Colonies are selected according to a selection index which takes into account their own value and the average value for the family.

Description of open breeding schemes

To demonstrate this principle in operation, let us study a specific numerical example. At the beginning, 10 colonies, headed with unrelated, open mated queens, are choosen from among the presumed "best" colonies. Queens are reared from these 10 colonies and then naturally mated. At the end of this step, a total of 120 young colonies (an average of 12 per family) is produced. These colonies are distributed among apiaries, trying to put equal numbers of each family in each apiary. Later, they are individually tested for the selection criteria.

An analysis of variance allows us to see it there are significant differences between families, apiaries, and possibly other factors (rearing colonies,...). A significant family effect means that the performance shows some "heritability" and that the selection should bring some visible results.

At the first generation, as there can be no estimate of the mother-daughter covariance, only a selection based on individual values for colonies is suggested. In the following generations, colonies are ranked according to a selection index (see below). The best 10 colonies are selected to produce drones and queens. The unselected colonies provide biological material to rear the new generation.

The young queens are brought to a fecundation apiary in which are installed the drone producing colonies (best 10 selected colonies). They are naturally fecundated by drones from selected colonies (proportion β, e. g. 70%) and surrounding unselected drones (proportion 1-β, i. e 30%).

The same cycle is repeated every generation. More complete information on the operation of the system can be found in CORNUET and CHEVALET (1987).

Definition of the selection index

The selection index of a colony is defined as the expectation of the performance of its "daughter" colonies. In these "daughter" colonies the queens are considered as having been open mated by a random set of drones (CHEVALET and CORNUET, 1982).

As a simple example, to demonstrate the operation of the index, we will consider a single parameter (e. g. honey production) and only two predictors– the value of the colony (m_{ij}) and the average value ($m_{i.}$) for the family to which it belongs. Figure 38 shows the relationships inside the i-th family. Let $_{oP}$be the variance of raw data. All data are divided by $_{oP}$ so that the variance of transformed data is equal to 1. Noting respectively T, C and A, the mother-daughter covariance, the

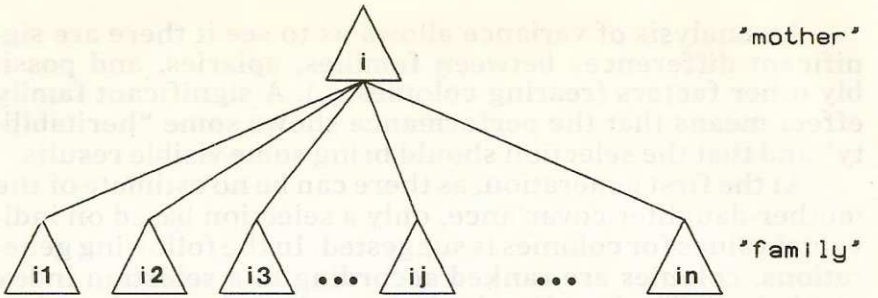


Fig. 38 — Schematic representation of the i-th family. Each triangle represents a colony.
A line joining two triangles indicates a mother-daughter relationship between the queens of the two colonies

between sisters covariance and the aunt-niece covariance, the formula for the index Jij may be written as follows:

$$J_{ij}=[(T-A)/(1-C)](m_{ij}-m_{i.})+[(T+11A)/(1+11C)]m_{i.} \quad (6)$$

With plausible values for covariances (T=0.200, C=0.129, A=0.058), this would give:

$$J_{ij}=0.163\ (m_{ij}-m_{i.})+0.348\ m_{i.}$$

Theoretical response to selection

Once a selection index has been defined, the theoretical response to selection can be predicted. The total genetic progress in one generation is the sum of the genetic progress via the female line plus that via the male line.

Female genetic progress

With the above definition of the selection index, the female genetic progress is simply the product of the intensity of selection times the standard deviation of the selection index:

$$\Delta G_{\varphi}=i\sigma_J$$

The intensity of selection is a function of n (family size=number of colonies per family), s (number of selected co-

lonies per generation) and t (correlation between indexes of sister colonies). It is approximately equal to:

$$i \approx i.-(n-1)/[2i.(ns-nst+st+1)] \qquad \text{(Hill, 1976)}$$

where $i. \approx 0.8+0.41 \ln(n-1)$ (Smith, 1969)

The covariance between indexes of sister colonies is equal to:

$$\text{Cov}(Jij,Jij_{\sigma'}) = \frac{C(T-A)^2+(A-TC)}{(1-C)^2} \frac{[2T+(n-2)(A+TC)-2(n-1)AC]}{(1-C)^2[1+(n-1)C]} \qquad (7)$$

The variance of the selection index can be expressed as follows:

$$(\sigma_J)_2 = \frac{(n-1)(T-A)_2}{n(1-C)} + \frac{[T+(n-1)A]^2}{n[1+(n-1)C]} \qquad (8)$$

So, the correlation "t" defined above is equal to Cov $(Jij,Jij')/(r_J)^2$.

Going on with our numeric example, we get:

$i=1.737$ $\sigma_J=0.213$ $\Delta G_{\varphi} = 0.371$

This means that the expected response to selection through the female line would be equal to $0.371\sigma_P$

Male genetic progress

Male genetic progress is made through the fraction β of males, coming from the 10 selected colonies, which mate the queens of the following generation. This progress is therefore proportional to β and to the product $i\sigma_J$. Also, as the participation of the selected males affects only the workers contribution, male genetic progress is proportional to the coefficient of regression $b_{W/J}$ on the selection index of the contribution of workers to the performance of a colony whose queen has been mated with drones from a selected colony. The male genetic progress is then equal to:

$$\Delta G_{\sigma'} = \beta \ i \ b_{W/J}\sigma_J \qquad (9)$$

The estimation of $b_{W/J}$ implies the estimation of a covariance unavailable from the breeding scheme itself. Furthermore, the estimation of β necessitates extra-experimentation. However, taking a plausible value of β (e. g. 0.7) and computing $b_{W/J}$ with the numerical values of our example (formulae are in CORNUET and CHEVALET, 1987), one can easily gain an idea of the possible contribution of the males to the overall genetic progress. In our example, we get:

$b_{W/J}=0.453$ $\Delta G_{\sigma'}=0.117$

Considering both the female and the male contributions, after one generation of selection, the total genetic progress in our example should be equal to $0.488\sigma_P$. If the standard deviation of honey production in the population under selection is e. g. 5 kg, this means that an average increase of 2.44 kg/colony could be expected after one generation of selection.

Long term progress

The above computations apply only to the first generation. As the population is open, there is a loss of progress in the selected population due to genes brought in by unselected drones. On the other hand, if the mating apiary remains in the same location over generations, the surrounding population is genetically improved through the outmating selected drones. This reciprocal influence on the genetic level of both populations has been modelled (CORNUET and CHEVALET, 1987). Figure 39 shows the increase in the genetic level of both selected and surrounding populations (with respectively 120 and 240 colonies) over 20 generations of selection for three different values (2, 5 and 10) of the number of selected colonies. The first number in the figure refers to the number of queen producing colonies and the second one refers to the number of drone producing colonies. It appears that, in our example, the best response is obtained with the lowest number of queen producing selected colonies and the highest number of drone producing selected colonies. This latter observation comes from the fact that, with a higher number of drone producing colonies, the proportion of selected drones (β) is higher and so is the male genetic progress, although the

corresponding intensity of selection is lower. Also, with a higher value of β, the benefit for the surrounding population is superior and this is profitable to the selected population since the external drones progress more rapidly.

The formula for β can be written as follows:

$$\beta = rD/(rD+E)=D(D+(E/r))$$

D is the number of drone producing colonies, E is the number of exterior colonies which drones may mate with queens from the selected population, and r is the ratio of the number of drones produced by a colony which is artificially induced to produce drones, compared to the number of drones naturally produced in a colony. In figure 39, a ratio of 30:1 has been assumed.

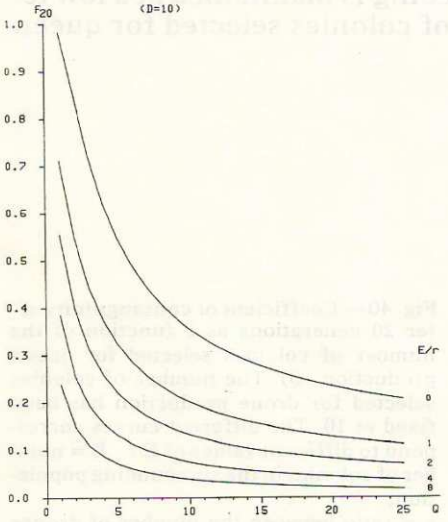


Fig. 39 — Genetic level of selected (upper curves) and surrounding (lower curves) populations as a function of generation.
The two numbers at the right correspond to the number of colonies selected respectively for queen and drone production

Influence of inbreeding

One way to study the influence of inbreeding in the selected population is to establish the recurrence equations for average inbreeding coefficients (coefficients of consanguinity and coancestry) from one generation to the next. This

has been done for the breeding scheme detailed above (CHEVALET and CORNUET, 1982; CORNUET and CHEVALET, in press). The parameters involved in the formulae are:

— the number of colonies selected for queen production (Q)

— the number of colonies selected for drone production (D)

— the average probability that two sisters have the same father

— the proportion β. As seen above, β is a function of D and E/r.

Figure 40 shows the value of the coefficient of consanguinity after 20 generations as a function of Q, for D=10 and different values of E/r. A null value for this ratio corresponds to a close breeding scheme. If the drones from the surrounding population are numerous compared to the drones from the selected population, inbreeding is maintained at a low level even with a small number of colonies selected for queen production.

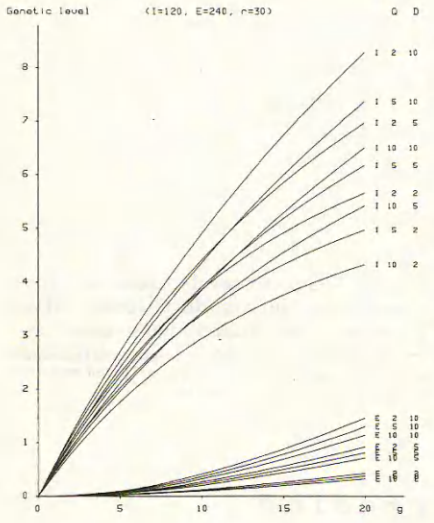


Fig. 40 — Coefficient of consanguinity after 20 generations as a function of the number of colonies selected for queen production (Q). The number of colonies selected for drone production has been fixed at 10. The different curves correspond to different values of E/r. E = number of colonies in the surrounding population,
r = ratio between the number of drones produced by a colony which has been artificially induced to produce drones, and the number of drones naturally produced by a colony).

Optimisation of sizes

For given genetic components of the variance, the total genetic progress is an increasing function of the family size

and of the number of selected colonies. In practice, the total number of hives is imposed by material constraints. For a given total, we need to know the number of selected colonies which maximises the genetic progress and keeps inbreeding below a tolerable threshold.

As the genetic progress and inbreeding coefficients are rather complex functions, we had to look for solutions using computer simulations. The general trends are as follows:

— if the unselected population surrounding the mating area is very small ($E<2r$), Q should not be less than 8 but D may be smaller;

— if the surrounding population is large enough ($E>10r$), Q may be smaller than 8 but D should be at least equal to 10;

— between the two situations, one can safely take values of Q and D under 8.

Taking values of 8 to 10 for Q and 10 to 12 for D is good enough for most situations, if not necessarily the most efficient combination.

Conclusion

This is a generalized genetic and statistical model for the description of a colony. Its use has been illustrated here through an open population selection program. It could equally well be used for a closed population selection program.

For whole colony characters there is no evident definition of heritability. Actually, the only pertinent elements necessary for the computation of the genetic progress are regressions or intra-class correlations.

The above breeding program looks very simple. However, it needs care at every step. As an example, in order to equalize possible effects over all families, each family should be equally represented in each queen cell bar in each rearing colony. Also, computations are complex (multifactorial analysis of variance, estimation of regression and intra-class correlation coefficients, computation of selection indices). However, corresponding computer programs exist.

SELECTIVE BREEDING IN CURRENT PRACTICE

V. MAUL

Breeding means gradually changing the characteristics or features of an organism for attaining a certain goal. It is accomplished by systematically comparing a number of individuals for obtaining descendants that are most adequate for the goal concerned. When the goal is attained all efforts are focused on maintaining the genetic progress obtained.

After the successful developments providing for controlled natural mating (mating stations on islands, zones for pure race mating), the instrumental insemination has been a revolutionary turn in bee breeding, as it enables as many as possible and also different combinations in mating in an apiary under most rigorous control. But with all the fascination exerted by the technical facilities, one must remember that instrumental insemination is an auxiliary agent only for the breeder. It requires a lot of work and is consequently expensive. It enables fast progress in breeding, but when improperly used pitfalls may occur (inbreeding following unilateral narrowing of the genetic make-up, see Chapter 10). That is the reason why successful selective breeding depends not only on the skill of the operator and technical advance but also on a rational and proper approach. In this section of the chapter the selective breeding work is discussed from the standpoint of the breeder, on the basis of the theoretic essentials in the preceding chapter.

Targets of selective breeding

The major target of interest for the breeder is the performance of the bee in terms of honey production and pollination activity. The performance substantially depends on the conditions available at the location of the apiary (climate, nectar sources). because of its dependency on the location the bee as subject of selective breeding is more closely related to cultivated plants than to live stock kept in stables.

The numerous geographical races of *Apis mellifera* species are a result of natural selection which provides for survival in a given environment. There are clear cut differences

between the bee races in different climate zones in terms of developmental cycle and the genetically encoded behavioural patterns. But natural survival is not equivalent to performance or optimum productivity. For increasing the performance in terms of honey production during precisely defined honey flow periods, types with large bee masses per colony are preferred at present with the slightest possible swarming impulse. The greater risk of survival in dearth periods is compensated by the measures taken by the breeder for maintenance. The productivity is also determined by the bees' behaviour. The breeder prefers the bees with a weaker defence behaviour, which stay quiet on combs during the various operations performed by him, and with a weak impulse for building additional combs or for filling all slits, so that all operations are readily performed and without any protection equipment. With beekeeping being practised in densely populated areas, gentleness of bees is now an essential requirement.

As attested to by many successful "transplantations", selective breeding in a certain zone does not necessarily imply the use for this purpose of the native bee race. Where such a native race is found, it should however be used as an initial basis. Because into the current practice breeding depends to a great extent on the natural mating of queens, the bee populations of pure races must be maintained in defined zones in order to avoid the undesirable effects of heterosis (RUTTNER, 1967). Irrespective of the origin of a population, constant selection for turning it into the most suitable to the conditions in the apiary concerned and to the specific management methods is indispensable. Account must also be taken of the experience gained with respect to inclement weather and honey flow conditions.

In addition to the major, general target, selection for special purposes — as for example for resistance to foulbrood (ROTHENBUHLER, 1964), for pollination of alfalfa (NYE and MACKENSEN, 1970) or for as long as possible proboscis to enable most efficient foraging of red clover (GOETZE, 1964) — may also be made. In this respect, the chief task of beekeeping is to try to cope with the damage caused by Varroa disease, by selective breeding (MORITZ, 1985; PENG et al., 1987). But in this section we shall not dwell on the methods of work of selection for such special purposes.

Assessment of bees' performance

The performance in terms of honey production of a bee colony is conditioned by both external and internal factors. The first include for example weather conditions and the nectar flow, the latter — the strength of the colony, the time when the colony is apt for foraging, the eagerness to forage, the capability of finding new honey sources, etc. The internal factors may in their turn depend on external factors (operations performed by the breeder, or genetic characteristics). The latter factors are of interest for the breeder because he seeks to improve the genetic characteristics of his breeding stock. In order to assess the genetically inherited characteristics of different colonies one must eliminate the external influences (figuratively speaking, all numerators (=productivity) must be reduced to the same denominator (=external factors).

Assessment of the performance requires the best possible uniformity of the external influences. When test colonies are formed, all queens of a test batch will be given the same chance from the very beginning. The test colonies will be either nuclei with brood combs of equal strength, or shoak swarms with the equal amount of bees. They shall always be kept in the same type of hives. After the colonies are formed, no further attempt of making them uniform or of adding extra bees must be made, but the colonies must be provided with the required conditions for their individual development following uniform principles. In the year when colonies are formed, assessment should be made when all initial bees have already been replaced by the daughters of the queen to be tested. Whether colonies are formed later, especially when they are transferred into normal colonies as an emergency measure, assessment of their performance after one overwintering is possible within certain limits only. All observations important for the assessment of performance and the operations performed by the breeder which influence the development of the colony must be recorded in the book of the colony. The principles of assessment must be as uniform as possible, to enable comparison of the results obtained in different bee breeding units. Further details in this respect are given in the "Technical Recommendation for Assessment of Performance of Colonies" (RUTTNER, F. and H., 1972).

The complex feature "honey productivity" is determined by several interrelated characteristics (as for example queen fertility, life span of worker bees, foraging activity, capability of detecting honey sources, capability of overwintering). Therefore, honey production as a criterion of selection usually has the highest selective value. However, also systematic, standardized recording is necessary of the other characteristics of a colony such as the strength of the colony throughout the year, the time when the colony is apt for foraging activity, amount of brood, and possibly also occurrence of scattered brood as a consequence of inbreeding.

The recorded data are necessary for assessment of performance and for determining its significance, account being also taken of the environmental conditions. If the performance in terms of honey production alone is assessed, errors may occur and the breeder will travel a round-about road to his target.

In each and every apiary weather and honey flow conditions have "typical" patterns (as for example predominance of early honey flows) every year. Extreme deviations will however occur from now and then. If, for example, there is no early honey flow in one year, then the colonies which will forage the normal flow must be selected instead of those which prefer an atypical late honey flow. The same rule must be followed when comparatively assessing the performance of bees of different origins bred in apiaries other than those of their origin, and when comparing the assessment data of different apiaries. For such comparative assessment, honey production is usually expressed in % of the apiary average. The average amount of the apiary indicates whether the honey flow was abundant, moderate or poor. But also the date when the flow starts and for how long it will last should be known, because they are decisive for the honey production. The colonies which need less food for maintenance because they have adapted themselves to the available honey sources, will give relatively good honey productions in years with moderate honey flows or in apiaries where only moderate honey flows are available. The colonies with intensive brood rearing activity and which are not too affected by weather conditions or by a less abundant honey flow will have a great honey production when the flow is abundant but will fail when the flow is moderate (BÖGER, 1969).

Finally, an important fact to be considered is the assessment of the performance of individual groups of colonies which, if separately taken could affect the overall assessment of performance. A group with average performance has relatively good results when compared to groups with preponderently low performance, while when compared to better performing groups, the results of the same group are relatively poor. When the colonies under the selective breeding programme are located in several apiaries, one must either have equal numbers of colonies in every group, or make up a standard group of colonies to be used as reference in assessing performance.

From the performance check we have to decide which colonies to select for the production of the next generation. In animal breeding the selection index is used for this purpose; it covers all selection characteristics which are assessed in marks (or points) according to an established system of recording. Attempts of a systematic record in marks or points of the performance in beekeeping have already been made (FALKENBERG 1972, KOBEL 1968). But predominant is the personal assessment of the breeder according to his own experience (BIENENFELD, 1987). Because the performance of bees is highly dependent on the environmental conditions specific for each and every region, a generally valid index of selection is more difficult to be established for them than in selective breeding of other animal species.

Planning of the breeding programme

Chapter 10, shows that successive selective bee breeding is like climbing on top of a mountain. Rigorous selection on the basis of objectively interpretable data recorded provides for a rapid genetic progress. But there is always a possible risk, namely the decrease in number of sex alleles and lower performance due to inbreeding. The smaller the number of the parent pairs chosen per generation, the earlier occurs the inbreeding depression. The possible duration of a selective breeding programme (the number of generations up to an obvious decline in performance) is determined by the testing capacity (number of colonies). For the evaluation of the possible variance, mating combinations must always be decided upon by considering a group of several individuals, irrespectively whether one or more groups of sisters are classically

combined with drones of different origins, or only the mother line is changed and the drone population is uniforme. In principle the following holds true: the larger the group, the greater is the certitude of good results. Groups of 4—10 colonies were found to be useful in practice.

Individual breeders who have a limited number of colonies will use closed populations for few successive generations only and if needed he will cross the stock with unrelated lines or different origins. Proper selection of the unrelated stock will diminish the inevitable discontinuity (break) during a long term selection work. It is better to have a greater number of test colonies for assessing performance, i.e. a larger population, by planned co-operation with other breeders. A good example in this respect is the co-operation in selective breeding in Northern Germany (TIESLER, 1983a, 1983b).

Testing a group of colonies in several apiaries enables a more precise assessment. The greater number of the colonies tested allows for sufficient genetic variability even under a most rigorous selection. The still widely used method of obtaining the new generation from "the best colony" is not recommended.

For one generation, the test period must last for at least one year, excluding the initial year and the first overwintering period. Before starting selection and breeding, the development of the colony after the second overwintering period will be assessed (its characteristics being now more obviously manifested). An even more precise assessment could be made in a full two years test. But it is known that the percentage of failure is higher in instrumentally inseminated queens after the third winter than in the naturally mated queens.

The procedure described above refers to the assessment of the performance of the queen and her own colony. But it may be combined with the assessment of the performance of the off spring or selection may be entirely based on the results of the tests of the descendants (recurrent selection). In this case the daughter generation must be prepared very early, so that after their performance testing the mother queen should be still available for the next descendants. Such programmes require a large number of test colonies and are not initiated too often because of the great difficulties in proper planning.

Taking out larvae for grafting during assessment of performance of the queen and of her own colony does interfere with the testing. Also, drone brood needed for insemination may be taken out to be introduced into (reserve) rearing colonies. It is convenient to provide all test colonies regularly with half frames for obtaining drone brood which will be taken out shortly before emergence and will be made use of or discarded depending on the selection decisions.

Assessment of the brood (sex alleles)

In Chapter 10 of the importance of the genes which determine the sex (sex alleles) and of inbreeding is pointed out. They determine the performance which in its turn depends on the development (strength) of the colony. A new method for reducing inbreeding is also described, namely mixed semen technique. But this technique is still little used in current practice, because not all inseminators have a centrifuge so that they collect the semen from each drone separately and use it as such.

In current practice queens are very often inseminated with the semen collected from drones originating from only one selected colony. In this case, the sister queens obtained may produce brood without gaps, but some of them could however have a 25% failure (WOYKE, 1972). The same situation may also occur, when queens are inseminated with semen from drones originating from unrelated colonies but this is very rare. When assessing the performance, the queens with brood gaps in the test colonies may be poor just because of the homozygosity at the sex locus, but not because of their actual breeding value. This makes it impossible to estimate a good performance (unfair comparative assessment). Before the development of the mixed semen technique, only two imperfect solutions were available:

1. Queens are inseminated with the "manually mixed" semen originating from several colonies. It is unimportant whether the semen is collected successively from the drones originating from different colonies or from "mixed" drones kept in nuclei. It is not certain that all amounts of semen injected will reach the spermatheca in the same proportions, and that the mixture will be uniform. Nevertheless in this way the percentage of failure is somehow equalized in the group of colonies concerned.

2.Queens are inseminated with semen collected from drones taken from one colony, but the percentage of brood gaps is previously determined and the colonies with brood gaps are eliminated, or their performance is assessed separately.

Brood gaps are measured in different ways. BÖGER (1969) uses a counting template covering 100 brood cells. This method is however relatively inaccurate because brood gaps may occur because of pollen or nectar storage and remain during next ovipositions. Therefore MAUL (1971) recommends use of special comb (test frames) after egglaying and sealing of the brood. This may be done very easily when establishing the nuclei. A very good opportunity for counting occurs when a swarm is transferred on comb foundation. After acquiring experience, the breeder can appreciate brood gaps, quite precisely, without a template. At the test apiaries of the beekeeping department in Kirchhain, the results of countings are recorded in the book (card) of the colony, in points (1—4) and are subsequently taken into account when assessing the performance.

Breeding record book (card)

All events related to breeding and insemination operations must be recorded. The record card for insemination used at the institute in Kirchhain is given in Table 2. The fact that it is a loose leaf is most adequate in the conditions of division of labour. The queen breeder records the data concerning the queen (breeding number of the queen — rearing record, date of emergence, identification sign number, and location of the mating nucleus). The breeder chooses the queens and drones he wishes for mating. The inseminator notes in the insemination record card the insemination procedure, insemination success and the purpose for which the queen will be used. The origin of drones may be indicated by their individual number in the rearing record book, or by the number in the rearing book indicating the grandmother (for example: daughters of 371/84).

Origin, terms, and kinship

A beginner in selective breeding faces difficulties in understanding the relations of kinship of queens and of bee co-

lonies. This is obvious when they prepare or consult the pedigree (record of ancestry), as well as during discussions about possible mating combinations between related colonies. This specific feature of bees is due to the parthenogenetic development of drones. They develop from unfertilized eggs, consequently have no father and can transmit only the characteristics genetically inherited from their mother.

The reduction division, which in diploid males is related to formation of spermatozoa, occurs in drones immediately after the unfertilized egg was laid. So that development starts from haploid eggs. The spermatozoa of the drone develop later by cell multiplication, being therefore genetically identical (but the spermatozoa of brother drones are not identical!). Consequently, the drone is only the multiplier of the characteristics genetically inherited from its mother. The true "diploid father" is the queen, the mother of the drone. This fact is taken into consideration at present when its origin is recorded, namely by indicating the number in the queen rearing book of the mother of the drone — for indicating the father or mating drone (Fig. 4). Consequently, the terms used to define kinship relations in terms of the diploid father must be interpreted specifically when bees are concerned. The table below is illustrative in this respect, by three examples.

Table 11

Terms used for kinship in bee breeding

Mating type			Kinship of drones and queens mated	
			term in general use	genetic term relationship (origin)
Drone's mother	=	mother queen	brother x sister	mother x daughter
Drone's mother	=	sister of the mother queen	cousin x cousin	aunt x niece
Drone's mother	=	daughter of the mother queen	nephew x aunt	brother x sister

When these terms of kinship are used in a selection programme, one must specify whether they are the terms in general use or the genetic terms. Graphic representations of the ancestry (pedigree) can be interpreted more precisely so that confusion is avoided.

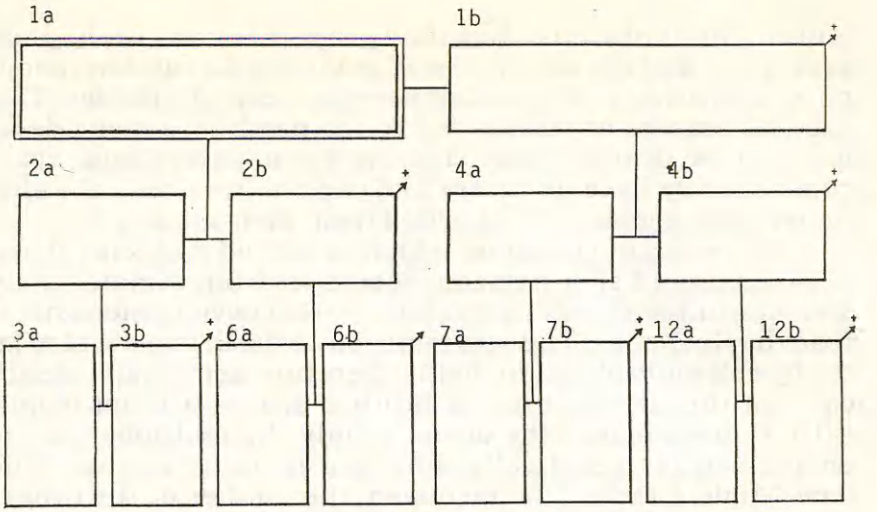


Fig 41 — Model for an apicultural pedigree
1 a: The number of queens for which we want to determine the pedigree from the selection note-book; 1 b — Number of mother (mothers), of drones from the selection note-book with which 1 a mated; 2 a/2 b — parents for 1 a etc. Other additional data on origin might also be mentioned in the breeding note-book.

Inbreeding systems

For scientific investigators, fast achievement of hereditarily highly pure stock by mating of very closely related individuals might be of interest. But vitality will also rapidly decrease fast (inbreeding depression). Inbreeding depression is prejudicial to selective breeding whose target is high performance (honey productivity). Breeders therefore prefer to mate less closely related individuals, accepting the fact that in this way hereditary purity is less rigorous and is obtained more slowly. Recommended to this end is to mate related individuals whose common ancestor is as far back as at least 2—3 generations. RUTTNER (1973) gives an example for obtaining a line by combined matings of related individuals. WOYKE (1972) reported on the effects of the combined matings of related individuals resulting in diploid drone brood (gaps in brood comb).

But now, with the new trends of breeding in closed or open populations using the mixed semen technique, such recommendations for specific combinations of related individuals are no longer operational; now, semen is always obtained from the entire population available, so that definite recording of the kinship of every individual is impossible.

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GLOSSARY

Abdomen — Abdomen

Allele — One of a pair or series of hereditary factors (genes that occur at corresponding loci on chromosome pairs)

Bulb — An enlargement of the endophallus

Bursa copulatrix — The cavity at anterior end of the sting chamber

Cervix — A narrow of the endophallus

Cornu (plural: *cornua)* — Horns of the endophallus

Diploid — Containing two sets of chromosomes

Dominant — A gene that expresses itself to the exclusion of the other member of the pair

Dorsal — On the back side

Ductus ejaculatorius — Ejaculatory duct, leading from the seminal vesicles to the endophallus

Ductus spermaticus — Duct of spermatheca (between vagina and spermatheca)

Ectophallus — Exterior portion of the penis

Ejaculation — Release of semen and mucus

Endophallus — Interior portion of the penis

Epistatic — A term describing genes of one locus that suppress the expression at another locus

Epithelium — Sheet-like tissue on the outer and inner body surfaces and gland passages, always consisting of a single layer of cells in insects

Eversion — Drawing out of the interior portion of the penis

Genotype — The genetic makeup. This term may refer to genes at a single locus or all the genes of the organism

Glandula mucosa — Mucus gland

Gynandromorph (gynander) — An individual with a body that is part male and part female

Haploid — Containing only one set of chromosomes

Hemizygous — The condition in which only one member of a pair of alleles is present. An organism in this condition is a hemizygote

Hemocytometer — A device for counting blood cells

Heterozygous — The condition in which corresponding genes (alleles) in a chromosome pair are different. An individual in this condition is a heterozygote

Homozygous — The condition in which the two corresponding genes of a chromosome pair are similar. An individual in this condition is a homozygote

Hypostatic — A term describing genes of a locus whose expression is suppressed by genes at another locus

Inbreeding coefficient — A measure of the degree of inbreeding of an individual (probability that 2 genes of a locus are from the same ancestor)

Interaction — The effect of genes on each other

Lethal factor — Inherited factor that leads to the premature death of the inndividual containing it

Linkage — Association of characters in inheration due to the location of the con trolling genes on the same chromosome

Locus (plural: loci) — point on the chromosome where a particular gene is located

Microliter (ul) — Cubie millimeter

Milliter — Cubic cm

Mosaic bee — One having genetically different body tissues side by side

Mucus — Clearly explained in the text

Mutant — An individual showing a mutation

Mutation — A sudden change that is inherited

Orificium vaginae — The orifice of the vagina

Ovary — Duct where eggs develop

Oviduct, lateral — Duct leading from each ovary to the median oviduct, expandable to receive semen during mating

Oviduct, median — The single duct leading from lateral oviducts to the vaginal chamber

Parthenogenesis — Development of an individual from unfertilized egg

Phenotype — The character or characters of an individual determined by its genotype

Polyspermy — Penetration of an egg by more than one spermatozon

Recessive — A member of an allelic pair of genes whose expression is suppressed by the other member

Sclerite — A chitinous plate of the exoskeleton of insects

Semen — Male sexual fluid or ejaculate

Spermatheca — Organ for the storage of spermatozoa in the queen

Spermatocyte — Immature spermatozon

Spermatozoa — Mature male sex cells

Testioles — Semen ducts (in tests)

Vaginal chamber — Enlarged inner portion of vagina

Vas deferens — The coiled tube leading from testis to seminal vesicle

Ventral — Toward or on the lower side of body

Vesicula seminalis — Seminal vesicle — sperm storage organ of the drone

Vestibulum — Enlarged base of the everted endophallus

Viscosity — measure for the flowing degree of a liquid

Zygote — A fertilized egg

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