Instrumental Insemination Of Queen Bees — 1985

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In cooperation with Louisiana Agricultural Experiment Station

Part 1

SELECTIVE BREEDING and the instrumental insemination of bees have not made headlines as have Africanized bees and acarine mites. Yet when searching for solutions to these and other problems, breeding is often involved. Honey bees respond well to selective breeding, and the breeding approach has served well in solving disease problems and improving yield in many plants and animals.

Instrumental insemination (II) is important in selective breeding because it is the only way that a breeder can control which drones mate with a queen. Bee breeders and beekeepers are well aware of the importance of II and controlled mating. However, one can also improve stock in a breeding program that uses only natural mating (NM). Instrumental insemination should not be seen as a replacement for NM, but as an additional tool that gives a breeder absolute control of matings.

This article is designed to describe the technique of instrumental insemination (II) that I now use without reviewing the techniques and equipment used in the past or used by others.[13,18,20,22] Although a number of slightly different manipulating devices and syringes are available, the same basic technique applies to all of them. Part 1 explains how to inseminate queens and describes the equipment that I use. Part 2 (Next issue) describes special techniques such as single drone inseminations and selfing, evaluates the success of II, reports the cost (in hours) of producing II queens, and recommends specific methods for different situations.

BACKGROUND

Before the mating of the honey bee could be controlled, it was necessary to learn a little about bee reproduction and colony manipulation. This knowledge came from Dzierzon and Langstroth in the mid-nineteenth century. In 1845 Dzierzon[4] reported that female bees develop from fertilized eggs and males develop from unfertilized eggs. There is more to sex determination, but for practical bee breeding Dzierzon's model is adequate. He also learned that a queen lays all the eggs in a colony and that once egg laying begins, a queen will not mate again. The discovery of bee space and the development of the movable frame hive by L. L. Langstroth in 1851 enabled beekeepers to examine their bees without seriously disrupting the colony. Thus, by the 1850's some beekeepers knew enough about bee reproduction to want to control the queen and with the movable frame hive they could replace queens with relative ease. Shortly thereafter (about 1870) there was a sudden interest in controlled mating.

Natural mating of the queen can be controlled to some extent by excluding all drones from an isolated area except those of the type desired to mate with the queens. The isolated area need not be an island, but islands have been successful. Numerous attempts to mate queens in cages or other confined areas have failed or have not been repeatable.[5]

Instrumental insemination is an alternative to island mating. It requires much less space, permits controlled matings in a place that is not geographically isolated, and allows the bee breeder to use many different drone types at one place on the same day. This feat would otherwise require a different isolated area for each drone type used.

Instrumental insemination also enables breeders to make matings that are impossible with natural mating. For example: (1) mating a queen to a single drone or to a few specific drones, (2) mating mutant queens and drones, and (3) mating a queen to her own male offspring (selfing).

The major use of II has been in research. It has been used to develop inbred lines, maintain mutant markers, and make specific matings for genetic research such as backcrosses and single drone inseminations. Very few commercial breeding programs have used II, and attempts to market II queens for use in field colonies have ended in failure.

The basic techniques of II were developed between 1926 and 1947. Techniques have been refined since then, but the quality of the final product (the instrumentally inseminated queen) has not changed.

PROCEDURE

Simply stated, II is the instrumental transfer of semen from one or more

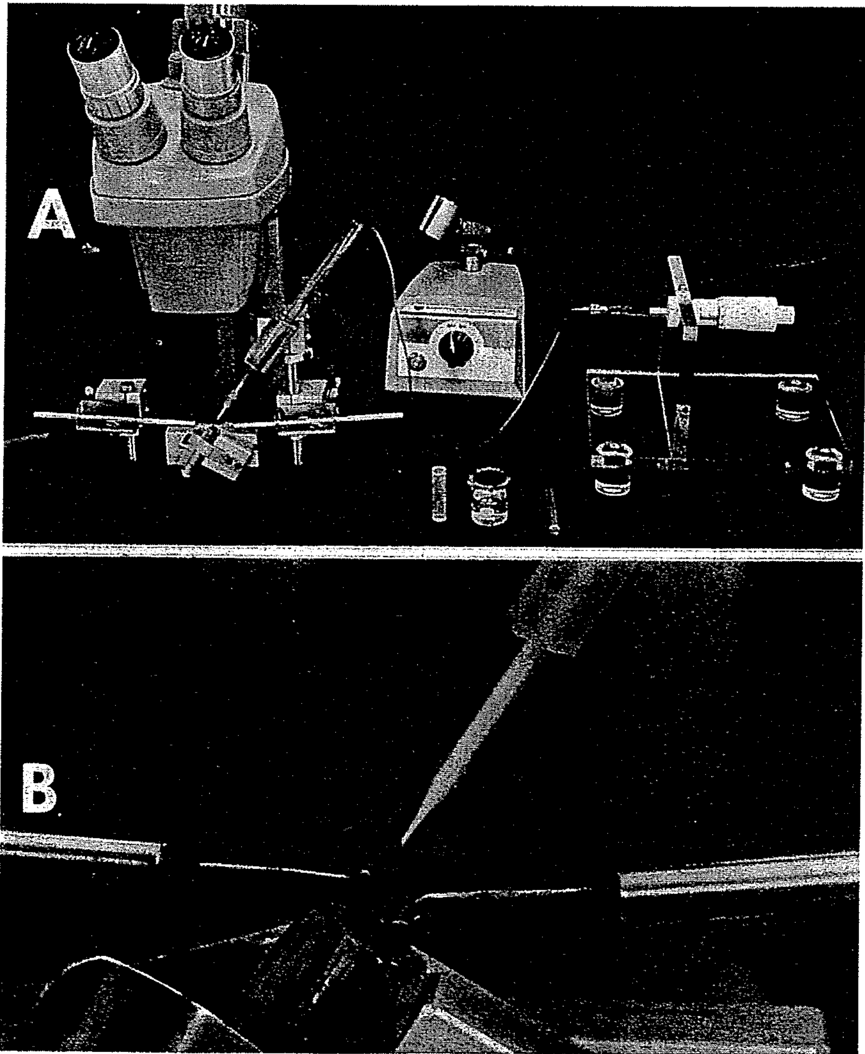

Fig. 1. Insemination equipment in use. A: A Mackensen insemination stand[20] holds the syringe shown in Fig. 2. The micrometer for the syringe (200 μl capacity) is mounted in a stand made of acrylic. B: Side view of a queen ready for insemination. The top of the queen holder is aligned with the top of the block. About 2½ abdominal segments protrude beyond the end of the holder (hind legs should not protrude). Notice that the sting chamber is opened toward the syringe. A common error for beginners is to open the sting chamber too far to the left making it seem straight as they view from the microscope.

drones to the oviducts of a queen (Fig. 5). In both NM and II, the semen is deposited in the lateral oviducts of the queen. Once in the oviducts, the spermatozoa migrate to the sperm storage organ, the spermatheca. This migration is usually complete 24 hours after insemination.[1]

The insemination process is complete when the spermatozoa are in the spermatheca. A newly mated queen has millions of spermatozoa in her spermatheca (see "Evaluating Inseminations" in Part 2). The spermatozoa are kept alive in the spermatheca and a queen fertilizes eggs by releasing some spermatozoa from her spermatheca just before an egg is laid.

Equipment

The complete apparatus, except for the carbon dioxide (CO_2) tank and regulator, is pictured in Fig. 1. The insemination stand and hooks are described in detail by Mackensen and Tucker.[20] They suggest that the queen holder block be adjusted so that it leans 30° from the vertical and makes a 10° angle with the syringe (the syringe being 10° more to the horizontal (Fig. 1). The adjustment of the stand varies with the operator, but the 10) angle between the queen and syringe should be maintained.

The syringe and tip are shown in Fig. 2. The design in Fig. 2 is an improved version of a syringe designed for use in semen storage.[7] The present version has a removable storage tube, but the storage tube is also used during routine inseminations when no semen storage is planned. Boiled saline within the tubing and micrometer produce an air-free hydraulic system throughout the syringe and make the system responsive to slight movements of the micrometer dial.

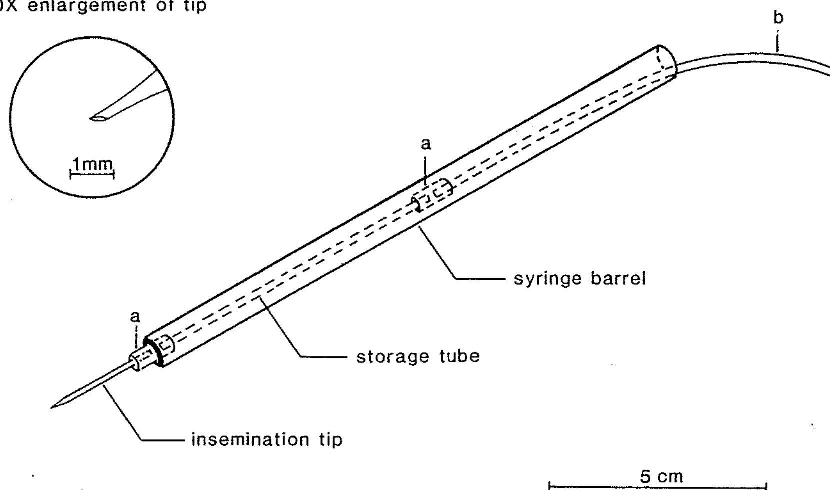

Fig. 2. Insemination syringe and its tip components. The tip is a glass capillary tube that was drawn to a point with heat and polished to an angular tip (see enlargement). The ID at the orifice of the point is 0.15 - 0.20 mm; the OD at the orifice is 0.26 - 0.32 mm. The storage tube is a glass or plastic tube (usually about 1 mm ID) that receives semen when large amounts are collected and which can be removed and stored, if desired. The syringe barrel is glass tubing with an ID 6 mm and an OD 8 mm. The opening at the tip end of the barrel is reduced to a diameter of about 4.2 mm. This reduced orifice fits tightly 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. 1A.

To assemble the syringe, 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 (a) 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 boiled saline. It is boiled to remove dissolved air, thus keeping the hydraulic system free of air and responsive. However, the saline in the storage tube and tip and that used during insemination is not boiled, for boiling may inactivate the antibiotic.

Semen Collection

The color of honey bee semen is light tan in young drones changing gradually to dark tan with age. This pigmentation is probably in the fluid surrounding the sperm cells (spermatozoa) because spermatozoa appear white when the fluid is removed. Each drone produces about 10 million spermatozoa[16] and there are about 7.5 million spermatozoa per microliter of semen.[17,25] One microliter (abbreviated μl) is a millionth of a liter or 1 cubic millimeter (a droplet about 1¼ mm in diameter; about the size of this o).

Drones are not sexually mature when they emerge as adults. Spermatozoa begin to move from the testes to the seminal vesicles when a drone is about 3 days old.[12] Transfer is complete in 3-6 days. The age at which drones become mature (when all the spermatozoa are in the seminal vesicles) varies from 6 to 12 days. Spermatozoa remain in the seminal vesicles until the time of mating.

As a source for semen, it is best to choose drones that have aged 10-21 days after emergence. Drones younger than 10 days are often not yet sexually mature, and those older than 21 days are more likely to cause disease in the queens[20] or leave a residue of semen in the oviducts.[29] Both conditions will kill a queen before she begins to lay eggs. The disease problem comes only from caged drones[2] and the semen residue affects only caged queens that receive an insemination dose greater than 4 μl.[23] Although there seems to be an ideal age for drones, a breeder can still use older drones if a few precautions are taken. Namely, antibiotic in the semen, reasonable sanitation, and smaller insemination doses (4 μl or less) should eliminate problems caused by older drones.

For semen collection, mature drones should be placed in a small cage that is next to the insemination device. The cage should be large enough for a hand to comfortably pick drones from any inside surface and hinged at the top so that a cage of drones can be easily placed inside. My cage is 12 x 11 x 9 inches (30 x 28 x 23 cm). It should be screened on the sides and top, and fitted with a cloth front so that one can insert a hand to collect the drones without letting them escape. It is easier to see inside the cage if the screen is painted black. Release about 30 drones at a time so that they do not exhaust themselves before they are ready to be used. The procedure for squeezing drones to yield semen is shown in Fig. 3.

The syringe tip and storage tube should be clean and should contain fresh saline before semen collection begins. Saline in the micrometer and plastic tube need not be replaced so often since it does not come in contact with semen. I use 0.85% NaCl and 0.25% dihydrostreptomycin sulfate (an antibiotic).[20] Between uses, the tips are stored in a 5% solution of sodium hypochlorite (undiluted laundry bleach). The bleach is rinsed from the tip by forcing water through it with a small plastic squeeze bottle. The storage tube is replaced after one day of use unless it is removed earlier for storing semen.

For those without a balance, an adequate saline (about 1% NaCl) can be made with salt tablets from a drug store. Each tablet is pure sodium chloride (NaCl) and weighs 1 gram (check the label). Measure 400 ml of water (8/9 pint), add 4 salt tablets and 1 gram of dihydrostreptomycin sulfate. This antibiotic is available at veterinary supply stores or from a veterinarian in premeasured capsules (1000 mg = 1 gram). It is often sold

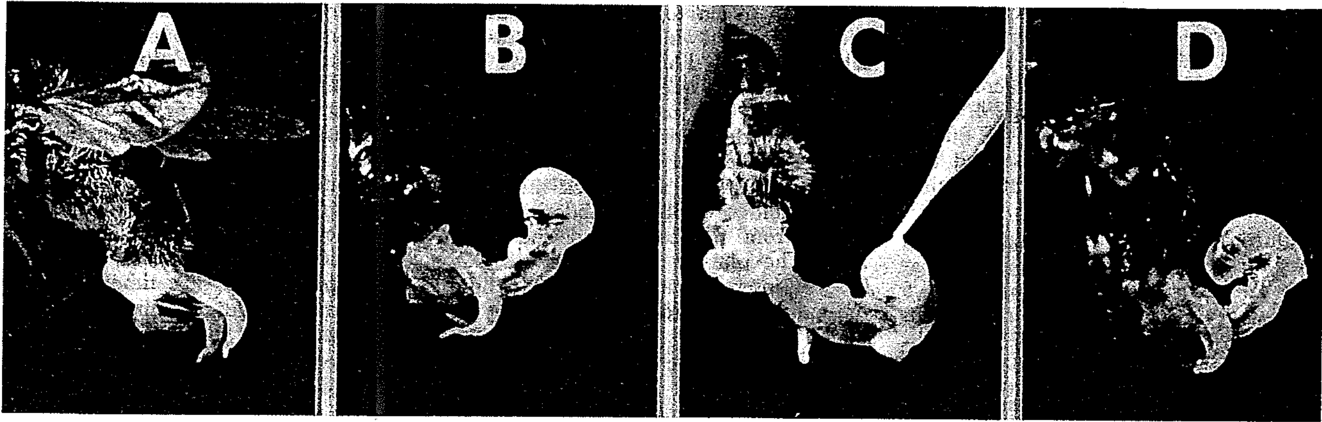

Fig. 3. Obtaining semen from mature drones. A: The first stage of drone eversion caused by a dorso-ventral (top to bottom) squeeze of the head and thorax. If the abdomen becomes stiff after this, the drone will likely yield semen. B: The second stage of eversion. This is produced by a lateral squeezing of the stiff abdomen. Semen and mucus are discharged at this stage, making it ideal for semen collection. The semen is light tan and it is usually on the surface of a large quantity of mucus (mucus is white). C: Semen collection from a second stage eversion. D: Very active drones may evert to a third stage when handled or after squeezing the thorax or abdomen. Semen can also be collected from this stage, but the semen is sometimes discharged onto the abdomen or wings of the drone.

in a mixture with penicillin; that is all right. Keep the saline refrigerated.

Before semen enters the syringe, an air-free column of saline should exist from the tip through the tubing and micrometer. Move this column back about 3 μl from the tip so that the incoming semen will be separated from the saline by an air bubble. Focus the microscope at the end of the tip and have the tip orifice facing downward (as in the tip enlargement in Fig. 2). With the drone held in such a way that the ejaculated semen is at the top of the everted genitalia, move the semen to the tip from below. Semen is the light tan material on the surface of a pearly white globe of mucus. Touch the tip with the semen and then pull the semen away slightly, but do not lose contact with the orifice of the tip (Fig. 3C). Draw the semen into the tip. The cohesiveness of the semen will help separate it from the white mucus which can plug the tip.

No air bubbles are put between semen from different drones. However, after collecting semen from a drone keep the semen column about 1-2 mm from the point to prevent drying and plugging at the orifice. As the next load of semen is brought toward the tip, move the column of semen to the tip and make direct contact between the semen in the tip and that to be collected. Dip the tip in saline and wipe with tissue if dried semen or mucus accumulate on the outside.

Queen Preparation

The storage of queens from emergence through insemination can be done in 3 ways. The simplest way is to have each queen free in a small nucleus colony that has a queen excluder over the entrance to prevent the queen from escaping. For insemination, the queens are found, caged, inseminated, returned to their colonies and released. Be sure that the wings on one side are

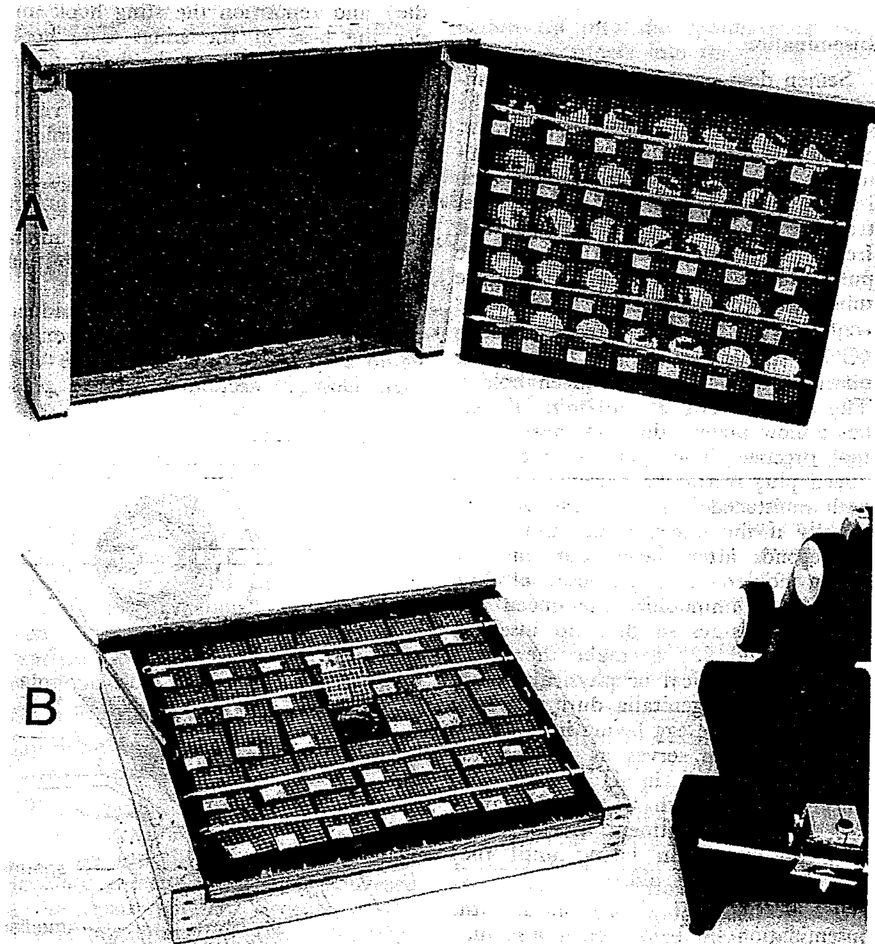

Fig. 4. A system for storing queens together in a colony. A: The board used for holding queens in a colony (right) is made of plywood (½ inch or 13 mm thick) that was cut to be the same size as a frame in the colony. Queens are stored in holes measuring 15/16 in. (24 mm) in diameter and covered with a permanent screen (10 mesh/inch) on the back side and with hinged screens (8 mesh/inch or 3 mesh/cm hardware cloth) on the front. The two hinges on each screen consist of 3/8 in. (10 mm) staples driven about 1/4 in. (7 mm) into the wood. Rigid horizontal wires (also hinged with staples) keep the screens from opening. Metal tags glued or soldered to each screen serve as labels and as weights to keep the screens closed when the horizontal wires are unlatched. Before a cage of queens is brought inside to be inseminated, the box at the left is taken to the colony, worker bees are brushed or shaken into the box, and the board of queens is placed on the box to form a bee-tight seal. B: Caged queens during insemination. Attendant workers trapped below enable queens to remain out of the colony for over 8 hours if necessary.

cut off (about half) so the queen does not fly away when the colony is opened. A second technique keeps up to 70 queens together in a larger colony in cages such as shown in Fig. 4. One trip to the colony brings all the queens in for insemination and a second trip returns them. This technique requires less time transporting queens.

Queens should be inseminated when they are young. They should be at least 24 hours, but not more than 5 or 6 weeks old. However, older queens can be inseminated (see "Special Techniques" in Part 2). For best results queens should be inseminated at age 5-14 days.[28]

A virgin queen can be inseminated after she has laid eggs (see "Special Techniques" in Part 2). However, if a queen has been previously mated (II or NM), allowed to lay eggs, and then reinseminated by II, my experience has been that the queen dies.

Insemination

Semen does not need to be used immediately after collection. It can be stored in the syringe at room temperature for a number of hours. If insemination needs to be delayed 1 or 2 days, it is best to keep the semen at a cooler temperature (5-15°C), but do not freeze it. The entire syringe can be put into the cool area, or the storage tube can be removed and stored.[7]

The flow rate of carbon dioxide (CO_2) should be adjusted before queens are put into the queen holder. The rate (about 35 ml/min. if one has a flow meter) does not need to be too precise. The flow through the queen plug should be barely detectable with moistened lips. Increase the rate slightly if the queen is not motionless 15 seconds after she is put into the queen holder. The purposes of CO_2 are (1) to immobilize the queen and relax her tissues so that tip insertion is easier and (2) to make the queen lay eggs. Chemical or physical stimuli from the male genitalia during natural mating stimulate egg laying in queens.[10] Carbon dioxide serves as a substitute for these stimuli in II queens, and queens treated with CO_2 begin laying 2-6 days after treatment; without CO_2, they do not begin laying until they are about 6 weeks old.[14]

I collect ½ to 1 μl of saline into the insemination tip before each insemination, leaving no air space between semen and saline. The saline precedes the column of semen into the oviducts of the queen. The functions of the saline are (1) to prevent the semen from drying out and plugging the tip, (2) to lubricate the tip for easier insertion, and (3) to add antibiotic for disease control.

The next step is to get the queen into the queen holder and clip the queen holder to the insemination stand. Force the queen to crawl into a dead-ended tube (shown on end next to the saline in Fig. 1A). Then place an open tube (the queen holder shown in 1A and 1B) end to end with the open end of the dead-ended tube. When the queen reaches the end of the dead-ended tube, she will back into the queen holder until her abdomen protrudes from the constricted end (Fig. 1B). The plug at the end of the CO_2 hose is inserted into the tube to hold the queen in position. See Fig. 1 for queen adjustment.

The sting hook and ventral hook separate the sting apparatus from the ventral plate to expose the vagina (Fig. 5). These hooks should be moved at the handles with the little finger edge of the operator's hands resting on the table. The first step is to separate the dorsal and ventral plates. Then hold the sting down with a sting depressor (a pin-sized wire with a handle) and reposition the sting hook under the base of the sting. The hook is shaped to fit under the hard, darkened parts at the base of the sting. While the sting is pulled to the right, the other hand is on the queen holder, turning it slightly to keep the sting shaft aligned with the sting hook.

Insertion of the tip into the oviduct is shown in Fig. 5. Just before insertion, lubricate the tip by dipping it in saline.

With the tip in the median oviduct, discharge the semen. The volume given to a queen is discussed in the "Semen Dosage" section. Equalize pressure before removing the tip by moving the micrometer about ½ μl beyond the intended mark then return to the mark. This prevents semen from running out of the tip as the syringe is withdrawn from the queen.

When inseminations are complete, return the queens to their colonies. Queens free in a nucleus colony can remain out of their colony for at least 20 minutes, so one can collect 2 or 3 queens per trip to save time; they can be returned while still immobilized by the CO_2. Caged queens can stay out of the colony for many hours as long as worker bees are in the box below (Fig. 4).

Egg Laying

To stimulate egg laying, queens are given 3 treatments of CO_2. CO_2 during insemination counts as a treatment, so if a queen was inseminated twice, she needs only one more treatment. The following is one of many ways to treat queens with CO_2. Put caged queens into a plastic bag and eliminate as much air as possible. Inflate the bag with CO_2 by running a CO_2 hose into the bag while the bag is held shut. Carbon dioxide is heavier than air, so keep the open end of the bag up. When the queens are motionless, turn off the CO_2 and leave the queens in the sealed bag for about 3 minutes.

Queens free in the colony should be treated with CO_2 once per day on the two days after insemination. Expect egg laying to begin about 4 days after the last CO_2 treatment if the queens

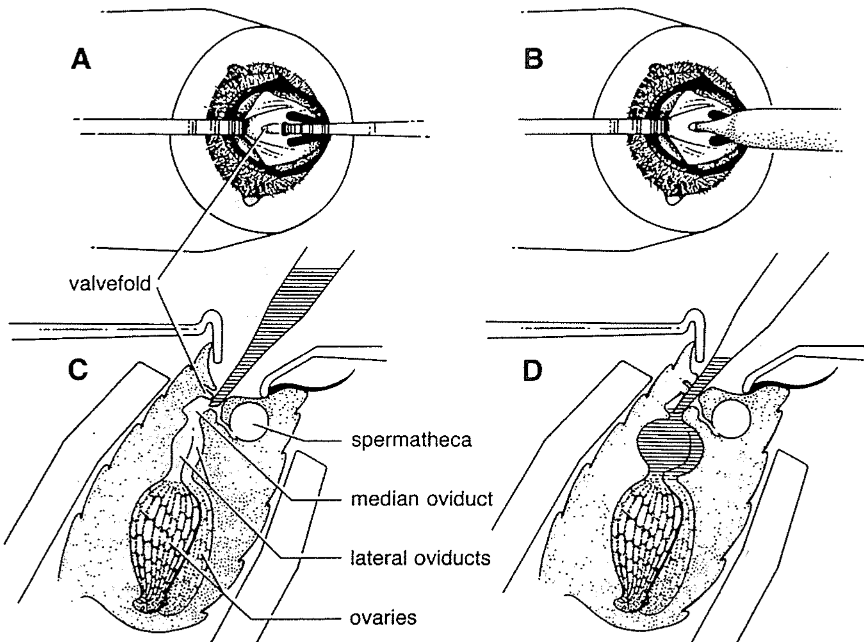

Fig. 5. The insertion of the insemination tip A: The operator's view of a queen ready to be inseminated. B: The same view as A, but showing the first stage of tip insertion. The tip has been inserted about ½ mm (about twice as far as the tip is wide). C: A side view of B. D: Final placement of the tip in the median oviduct. After the first stage of tip insertion (B and C) the tip is moved ventrally (left) about ½ mm to bypass the valvefold, and then inserted another ¾ mm. The total insertion depth is about 1¼ mm. Semen that was discharged from the tip has caused the lateral oviducts to swell.

were aged one week at the time of insemination.

Queens stored together in a colony do not need CO_2 treatments on the days immediately following insemination. For convenience, I often give queens their final treatment on the day that they are transferred from the queen bank to a nucleus colony to lay. For best success on introduction, leave the queens in the storage colony until they are 2-3 weeks old. Queens aged over 2 months at the time of introduction do not need any additional CO_2 treatment.

Introducing II queens into large colonies is sometimes a problem. To reduce queen loss, have the II queens lay in a nucleus colony for at least 4 days before introducing them to large field colonies. Place each caged queen into a nucleus colony consisting of about 2,000 bees. These little colonies may be newly made without brood. Release the queens after 3 days if the workers are not biting the cage. About 10 minutes after release, check some of the queens to see if any are being chased or bitten by 1 or more workers. Those that are should be recaged for 2 or 3 more days. If some of the queens in a group are being chased or bitten, all should be rechecked and perhaps recaged. Queens 3-4 weeks old usually begin to lay 1-2 days after release.

Part 2

SPECIAL TECHNIQUES

Single Drone Insemination

THIS IS a common technique in research. Since all the spermatozoa from one drone are genetically identical, a queen mated with semen from a single drone will produce daughters that are all more closely related than full sisters. These daughters (workers or queens) receive identical genetic material from their father and are called supersisters.

The procedure is similar to that of a regular insemination except one must rinse the tip between inseminations to ensure that the tip contains only semen from one drone. Before collecting semen, collect ½ microliter (μl) of saline just after the air space. This saline is discharged into the queen with the semen and reduces the amount of semen that is left coating the sides of the insemination tip. For best results, choose a drone that has a large semen load.

Inseminate Many Queens with Identical Spermatozoa

This technique enables one to give genetically identical spermatozoa to different queens. As with single drone inseminations, each queen produces a family of supersisters. Moreover, the families produced by the different queens have a unique relatedness to each other.

There are two ways to do this. The first way is simpler, but more limited since one merely chooses a drone that yields a large quantity of semen. Collect semen as for a single drone insemination and then inseminate each queen with 0.2 μl of semen. Before each insemination, collect 0.2 μl of saline which will be injected into the oviducts with the semen. Young queens inseminated in this manner have produced worker brood. I have inseminated as many as 14 queens from a single drone (about 0.5 million spermatozoa/queen), and they averaged 84% worker brood.

The second technique requires the use of queens that produce gynandromorph progeny.[11] The advantage of this technique is that genetically identical spermatozoa can be produced in large quantities and it can be collected throughout the season. The inseminations can be like multiple inseminations because the male tissues of many individuals produce genetically identical spermatozoa as long as the mother queen had only genetically identical spermatozoa in her spermatheca (*ie* sperm from 1 drone)

Inseminate Queens with a Uniform Mixture of Spermatozoa

Among other possibilities, this technique can be used to maintain genetic diversity in a breeding program and to produce uniform inseminations in a large group of queens. These inseminations include genetically diverse spermatozoa, but they are uniform because spermatozoa for each insemination are taken from the same large, thoroughly mixed pool of spermatozoa. Studies have shown that spermatozoa can be highly diluted, mixed and then recovered by centrifugation.[9] Queens have been successfully inseminated with this "washed spermatozoa"[9,21,24] and the technique does produce uniform mixing of cells.[21]

Collecting Spermatozoa from the Seminal Vesicles of a Drone

This technique is used when one desperately needs semen from a particular drone and the drone has failed to ejaculate semen after being squeezed in the usual manner. Because of the time of migration of spermatozoa from the testes to the seminal vesicles, drones must be at least 4 days old before there is much hope for success. Drones aged more than 1 week should have sufficient numbers in their seminal vesicles to perform a minimal insemination.

The seminal vesicles are the smaller of the paired, sausage shaped glands in the drone. One method is to remove a seminal vesicle, pinch it with a forceps at the testes end, and thereby start contractions which force the spermatozoa out the other end where they are collected with a syringe.[19] A less elegant technique of dropping the seminal vesicles into a small glass cone, adding a small amount of saline, and then partially macerating them to free the spermatozoa has also proved satisfactory.

Using Spermatozoa from the Spermatheca of One Queen To Inseminate Another

This technique is used to make gamete backcrosses[3] or to simply recover spermatozoa that have been stored in other queens. Thus, vigorous queens can be used as storage banks for spermatozoa.

Take the spermatheca from the donor queen(s) and remove the tracheal covering. Place the spermatheca on a smooth wax surface that has a slight depression and puncture it with a sharp needle. Then insert the insemination tip into the spermatheca, collect the contents into the syringe, and inseminate as usual.

Inseminating Very Old Queens

Queens more than 8 weeks old are considered old for insemination, but insemination is still possible for at least 5 months. There may be no age limit. An old queen should be placed in a mailing cage with queen candy and 5-8 workers for 3-5 days to reduce the size of her abdomen. The insemination volume should be 2 μl or less to enhance survival. After insemination, place the queen in a colony and release her in 2 or 3 days. No CO_2 treatments after insemination are necessary to induce egg laying. The percentage of spermatozoa entering the spermatheca is lower for old than for young queens and many queens inseminated when aged for more than 4 months are partial drone layers.

Inseminating a Queen with Semen from Her Own Drone

This is a breeding scheme called self-fertilization that was first described by Mackensen.[15] A virgin queen is treated with CO_2 to get her to lay unfertilized eggs that will develop into drones. The queen is not inseminated. If some drone sized cells are available, the queen will usually lay eggs in them, and the colony will produce larger drones in drone-sized cells than they will in worker-sized cells. When one or more of these drones are about 2 weeks old, their semen can be used to inseminate the queen. The procedure is identical to that of inseminating a very old queen (described above).

EVALUATING INSEMINATIONS

A queen retaining more spermatozoa in her spermatheca is considered to be better mated than one with

fewer. Therefore, estimating the number of spermatozoa in the spermatheca enables one to measure success beyond simply mated or not mated. Of course, the queen must be killed in order to make these measurements.

Fig. 6 shows the relationship between the amount of semen injected into the oviducts, and the number of spermatozoa entering the spermatheca of young queens (queens less than 3 weeks old). Results are variable, but this represents an average expectation. In general, smaller inseminations are more efficient (a higher percentage of the spermatozoa enter the spermatheca).

During natural mating (NM) about 5.7 million spermatozoa enter the spermatheca. An average that approaches this (5.5 million) can be achieved with II, but it requires 4 inseminations with 2½ μl.[18]

Both genetic and environmental factors affect the number of spermatozoa reaching the spermatheca. Geographic races differed in the number of spermatozoa entering the spermatheca after inseminations with 8 μl of semen.[30] Younger queens (less than 3 weeks old) retained more spermatozoa in their spermatheca after an insemination of 8 μl than did older queens.[28] Queens running free in a mating hive retained more spermatozoa in their spermathecae than did those caged in a mating hive when each was inseminated with 6 μl of semen.[23] However, there was no significant difference between free and caged queens when insemination volumes were 2 or 4 μl[23]

To produce 100% worker brood, a young queen needs to have about 100,000 spermatozoa in her spermatheca. The 100,000 estimate is based on 2 groups of unrelated queens. The first group (10 queens) was inseminated with about 0.2 μl of semen each. Five queens had 111,000-575,000 spermatozoa in their spermathecae, and those all produced more than 99% worker brood. The remaining 5 had 1,000-39,000 in their spermathecae, and they produced 2-92% worker brood. In a second group, each queen received about 0.05 μl of semen (about 250,000 cells). Five had 45,000-88,000 spermatozoa in their spermatheca and those produced 91-99% worker brood. The remaining 5 had 6,000-34,000 and those produced 12-54% worker brood. These results show that young queens produce some worker brood from even the smallest inseminations. Thus, if a young queen produces only drone brood, it is usually not because she was given an inadequate amount of semen during insemination; more likely the semen was not injected into the oviducts.

The presence or absence of spermatozoa in the spermatheca affects the egg laying behavior of a queen. When laying in worker-sized cells, queens with no spermatozoa in their spermatheca do not lay as many eggs per day and do not position eggs as uniformly as do queens with spermatozoa in their spermathecae.[6] However, among queens that are producing worker brood, I failed to find a relationship between egg laying rate and the number of spermatozoa in the spermatheca.

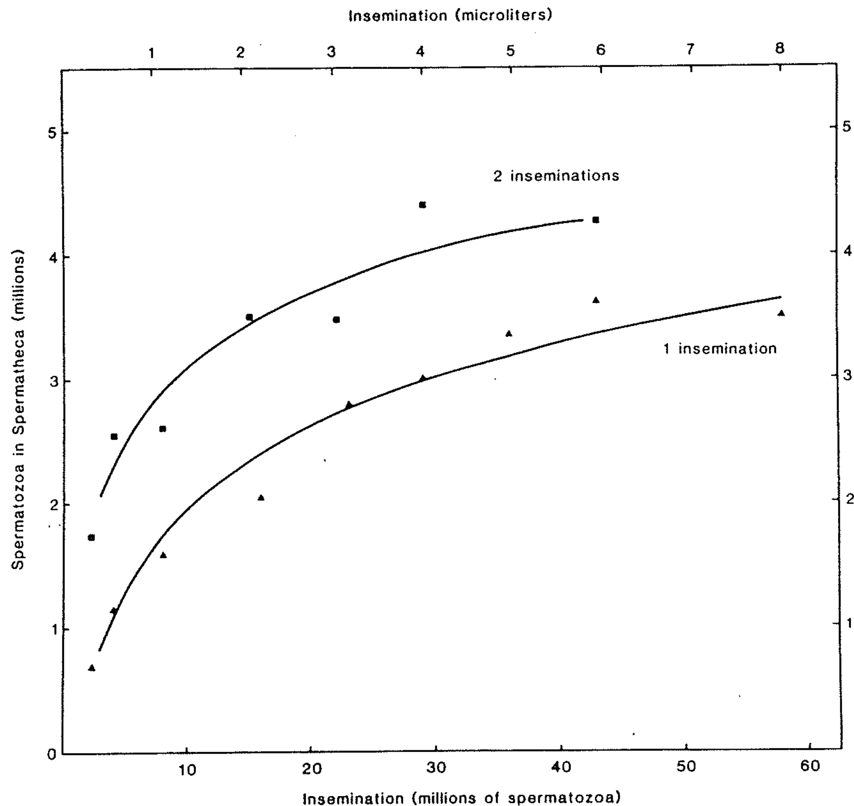

Fig. 6 The effect of insemination dosage on the number of spermatozoa entering the spermatheca. Data were compiled from my measurements and from those of others. 2,17,23,25,26,27 For 2 inseminations, each one is the size indicated on the horizontal line.

Queen Survival

Queen loss after insemination can be a serious problem. For beginners, physical injury to the queen during insemination is common, but queens often survive even when semen is injected into the body cavity. When the body cavity is punctured, a clear droplet of fluid (bee blood) usually appears. After a day or so, a black "scab" can often be seen at the site of the injury. Bacterial and viral infections can also cause queen death, but reasonable cleanliness and the use of antibiotic keep these disease losses minimal. After an inseminator has learned to insert the tip properly, the most common causes of queen loss are (1) queen death in storage, (2) a semen residue remaining in the oviducts for more than 1 or 2 days and (3) worker aggression when II queens are first introduced to colonies.

Semen residue in the oviducts is often the cause of queen death after insemination. Insemination with large volumes of semen and keeping queens caged after insemination tend to cause a residue. Normally, semen is discharged from the oviducts and some of the spermatozoa enter the spermatheca. If insemination doses are high (greater than 6 μl), a residue of semen is often left in the oviducts of caged queens.[23] This residue may serve as a medium for the growth of microorganisms, which may be the cause of death. More semen residue was left in the oviducts when the semen came from older drones (those more than 4 weeks old) than when the semen came from younger drones (2-3 weeks old) (8 μl inseminations).[29] Queens free in a colony do not seem to have a problem with semen residue in the oviducts, nor do caged queens inseminated with less than 4 μl of semen.[23]

Queen loss when first introducing a II queen to a colony is common because a young II queen that has never laid eggs is not accepted by workers as readily as a laying queen. A procedure for introducing II queens was described earlier under "Oviposition." In general, older II queens are easier to introduce than younger ones, and smaller colony populations seem to accept them more often than larger populations.

Queen Performance

A project conducted in 1981-82 compared 59 II and 59 NM queens in large, field colonies in Alberta, Canada and Louisiana, USA.[8] II queens had

a significantly higher supersedure rate and they produced less brood than NM queens. Only 31% of the II queens survived for 12 months (August 1981-August 1982), whereas 58% of the NM queens survived. In the spring of 1982, the amount of brood produced by the colonies with surviving II queens was significantly less than that produced by NM queens. Probably because of this difference in brood production, honey production for the colonies with II queens was also lower.

Thus II queens are not recommended for use in large, field colonies. If one uses II queens in a breeding program, the final product (the queens that go into production colonies) should be naturally mated. Of course, if one can use II to produce vastly superior stock, the quality of the stock might override the handicap of instrumental insemination.

PRODUCTION COSTS

The equipment needed to produce II queens is minimal. Excluding the beekeeping equipment, which is not included in this analysis, the one time cost of the insemination equipment, CO_2 regulator, binocular microscope, and light is about $1000 (1984). The only parts that need replacement are the CO_2 supply, the insemination tip, and the sting hook. Their costs are less than $40 per year unless the operator is careless.

Table I lists the labor costs of producing II queens when the queens are separately caged in a large colony. There are controllable variables in the procedure: the amount of semen given and the number of times a queen is inseminated. An uncontrollable variable exists in semen collection because of variability in drones. Sometimes a group of drones averages much less than 0.5 μl of semen per drone and at other times they may average as much as 0.9 μl.

The labor costs of producing II queens is higher when each queen is running free in a small nucleus colony. About 2 more minutes per queen is required because 3 trips are necessary to find and bring the queens in for insemination or CO_2. In addition, if many queens are stored (caged) in a colony before they are shipped, they require only half as many nuclei as do queens free in the nucleus. A free queen needs a nucleus for 2 weeks; a 3 week-old banked queen can be put into a nucleus, and laying in less than a week.

Table I. Time required to produce instrumentally inseminated queens in a group of 40 or more. Queens produced in 1 cell builder and kept in 1 queen storage colony.

Item	Time
Producing, caging and storing drones of known parentage[A]	10 sec/drone
Collecting semen[B]	50 sec/μl
Total cost of semen (@ 0.5 μl/drone)[C]	1.2 min/μl
Rearing queen cells[A]	4 min/cell
Marking and storing queens[A]	2 min/queen
Cost of a mature, virgin queen	6 min/queen
Insemination (includes transporting caged queens from and to the storage colony)[D]	3 min/queen
Additional CO_2 treatments	15 sec/queen
Total cost of queens inseminated once with:	
3 μl of semen[E]	13.1 min/queen
8 μl of semen[E,F]	19.1 min/queen
Total cost of queens inseminated twice with:	
3 μl of semen[E]	19.7 min/queen

A/ K. Tucker, J. Harbo, and J. Bishop: unpublished data presented orally at the annual meeting of the American Bee Breeders Assn., 1974.

B/ Mackensen [17] reported their collecting 1 μl of semen required 45 seconds. I measured 47 sec/μl. If drones are sluggish, too young, or for some other reason not prone to ejaculate, the time could easily be doubled.

C/ Drones that yield semen usually produce more than 0.5 μl. The 0.5 μl estimate included drones that produce no semen and was based on a typical group of 203 drones; 93 yielded no semen, 102 μl was collected from the others.

D/ Estimate came from 126 inseminations that were timed in groups of 4 to 28. The mean ± SD = 2.46 ± 0.4 min/queen. This included record keeping, but not semen collecting or transporting queens. Time was increased to 3 minutes to allow for transporting the group of queens from and to their storage colony.

E/ Cost does not include queen introduction and assumes no queen mortality after insemination.

F/ 8 μl doses are not recommended for caged queens (see semen dosage). Add about 2 min/queen to find and bring each queen in for insemination and CO_2 treatments.

RECOMMENDATIONS

Semen Dosage

Caged queens need multiple inseminations if they are to average more than 3 million spermatozoa in the spermatheca. Since caged queens cannot tolerate inseminations larger than 4 μl, I suggest 2 inseminations of 3 μl. If one wants to approach the sperm count of a naturally mated queen, 3 inseminations of 2 or 3 μl are needed. Multiple inseminations of caged queens should each be 3 μl or less and should be spaced 2 or 3 days apart.

Woyke[25] has long recommended 1 insemination of 8 μl or 2 inseminations of 4 μl for queens that are free in a nucleus colony. Mackensen[17] concluded that 2 inseminations of 3 μl may be an adequate minimum if queens are used in large field colonies. Multiple inseminations of uncaged (free) queens are given on consecutive days. Use Table I and Fig. 6 to decide on dosage, but keep in mind that (1) a single insemination as large as 8 μl does not seem to be detrimental if the queen is free in a nucleus colony, and (2) multiple inseminations give more uniform results. There are practical limits where the cost of more inseminations or of using more semen yields diminishing returns.

Sometimes one simply needs mated queens, and long-term egg laying is not important. In these cases, I simply inseminate the queen once with about 2 μl of semen. Such queens seem to perform well for many months, some even survive more than a year.

Learning Insemination

When first learning to inseminate, use about 15 caged virgin queens. Two μl of semen per queen is enough to practice the insertion and injection. If 2 μl is discharged into a queen without backing out around the tip during injection, the tip is either in the correct position or the operator has punctured the body cavity. Check for success on the following day by removing the spermatheca from each queen. No microscope or dissecting tools are needed. Use fingernails or forceps and pull away the last 1 or 2 abdominal segments. A heavy tracheal network makes the spermatheca look like a ball of string about 1 mm in diameter, and the presence or absence of spermatozoa does not seem to affect its size or firmness. Remove the tracheae by rolling the spermatheca between your fingers. If the spermatheca is crystal clear, the insemination failed; if it is white and opaque, it succeeded.

After having some success with practice queens, start serious inseminations with queens that are free in nucleus colonies. Inseminate each queen with at least 2 but with no more than 8 μl of semen. Follow with CO_2 treatments on the next two days and watch for worker brood.

I suggest starting with queens free in nucleus colonies for 2 reasons. First, very good results can be obtained with only 1 large insemination and fewer insertions reduce the probability of queen injury. Secondly, queen death from various causes can be expected, especially for beginners, and it is best for slow inseminators to take these losses (queen death in storage colonies and during queen introduction) before they inseminate the queens.

REFERENCES

1. **Bishop, G. H. (1920).** Fertilization in the honey-bee II. Disposal of the sexual fluids in the organs of the female. **J. Exp. Zool. 31,** 267-286.

2. **Bolten, A. B. and Harbo, J. R. (1982).** Numbers of spermatozoa in the spermatheca of the queen honeybee after multiple inseminations with small volumes of semen. **J. Apic. Res. 21,** 7-10.

3. **Cale, G. H., Jr. and Gowen, J. W. (1964).** Gamete backcross matings in the honey bee. **Genetics 50,** 1443-1446.

4. **Dzierzon, J. (1845).** Gutachten uber die von Hr. Direktor Stohr im ersten und zweiten Kapital des General-Gutachtens aufgestellten Fragen. **Bienen-Zeitung (Eichstatt) 1,** 109-113, 119-121.

5. **Harbo, J. R. (1971),** "Annotated Bibliography on Attempts at Mating Honey Bees in Confinement." Bibliography No. 12, Bee Research Assn., Gerrards Cross, Bucks., England.

6. **Harbo, J. R. (1976).** The effect of insemination on the egg-laying behavior of honey bees. **Ann. Entomol. Soc. Am. 69,** 1036-1038.

7. **Harbo, J. R. (1979).** Storage of honey bee spermatozoa at -196°C. **J. Apic. Res. 18,** 57-63.

8. **Harbo, J. R., and Szabo, T .I. (1984).** A comparison of instrumentally Inseminated and Naturally Mated Queens. **J. Apic. Res. 23:** 31-36.

9. **Kaftanoglu, O. and Peng, Y. (1980).** A washing technique for collection of honeybee semen. **J. Apic. Res. 19,** 205-211.

10. **Koeniger, G. (1981).** In which segment of the mating process of the queen does the induction of oviposition occur. **Apidologie** 12: 329-343. (In German).

11. **Kubasek, K. J., Rinderer, T. E., and Lee, W. R. (1980).** Isogenic sperm line maintenance in the honey bee. **J. Hered. 71,** 278-280.

12. **Kurennoi, N. M. (1953).** When are drones sexually mature? **Pchelovodstvo 30, 28-30.** (In Russian).

13. **Laidlaw, H. H., Jr. (1977),** "Instrumental Insemination of Honey Bee Queens, Pictorial Instructional Manual." Dadant and Sons, Inc., Hamilton, Ill.

14. **Mackensen, O. (1947).** Effect of carbon dioxide on initial oviposition of artificially inseminated and virgin queen bees. **J. Econ. Entomol. 40,** 344-349.

15. **Mackensen, O. (1951).** Self fertilization in the honey bee. **Glean. Bee Cult. 79,** 273-275.

16. **Mackensen, O. (1955).** Experiments in the technique of artificial insemination of queen bees. **J. Econ. Entomol. 48,** 418-421.

17. **Mackensen, O. (1964).** Relation of semen volume to success in artificial insemination of queen honey bees. **J. Econ. Entomol. 57,** 581-583.

18. **Mackensen, O., and Roberts, W. C. (1948),** "A Manual for the Artificial Insemination of Queen Bees." U.S.D.A. Bureau of Entomol. and Plant Quar. ET-250.

19. **Mackensen, O. and Ruttner, F. (1976).** The insemination procedure. **In** "The Instrumental Insemination of the Queen Bee" (F. Ruttner, ed.), pp. 69-86, Apimondia, Bucharest, Romania.

20. **Mackensen, O. and Tucker, K. W. 1970),** "Instrumental Insemination of Queen Bees." U.S.D.A. Agric. Handbook No. 390.

21. **Moritz, R. F. A. (1983).** Homogeneous mixing of honey bee semen by centrifugation. **J. Apic. Res. 22,** 249-255.

22. **Ruttner, F. (1976),** "The Instrumental Insemination of the Queen Bee," Apimondia, Bucharest, Romania. 123 pp.

23. **Vesely, V. (1970).** Retention of semen in the lateral oviducts of artificially inseminated honey-bee queens (**Apis mellifera** L.) **Acta Entomol. Bohemoslow, 67,** 83-92.

24. **Williams, J. L. and Harbo, J. R. (1982)** Bioassay for diluents of honey bee semen. **Ann. Entomol. Soc. Amer. 75,** 457-459.

25. **Woyke, J. (1960).** Natural and artificial insemination of queen honey bees. **Pszczelnicze Zesz. Nauk. 4,** 183-275. In Polish. English summary.

26. **Woyke, J. (1971).** Correlations between the age at which honeybee brood was grafted, characteristics of the resultant queens, and results of insemination. **J. Apic. Res. 10,** 45-55.

27. **Woyke, J., and Jasinski, Z. (1973).** Influence of external conditions on the number of spermatozoa entering the spermatheca of instrumentally inseminated honeybee queens. **J. Apic. Res. 12,** 145-151.

28. **Woyke, J., and Jasinski, Z. (1976).** The influence of age on the results of instrumental insemination of honeybee queens. **Apidologie 7,** 301-306.

29. **Woyke, J., and Jasinski, Z. (1978).** Influence of age of drones on the results of instrumental insemination of honeybee queens. **Apidologie 9,** 203-212.

30. **Woyke, J., Jasinski, Z., and Smagowska, B. (1974).** Comparison of reproductive organs and effects of artificial and natural insemination of honey bees of different races and their hybrids. **Pszczelnicze Zesz. Nauk. 18,** 53-75. In Polish. English summary.