CHAPTER 15

Propagation and Instrumental Insemination

JOHN R. HARBO

I. INTRODUCTION

A. Genetic Control of a Colony

The ability to control mating is basic to any breeding program. However, 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 first became available in the mid nineteenth century, and shortly thereafter (about 1870) there was a sudden interest in controlled mating.

1. *Basic Reproductive Biology*

Work by Dzierzon and Langstroth led to early attempts to control mating. First came Dzierzon's theories. Dzierzon (1845) stated that female bees develop from fertilized eggs and males develop from unfertilized eggs. There is more to sex determination (Woyke, Chapter 4), but for practical bee breeding Dzierzon's model is adequate. Dzierzon 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 1850s 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.

2. *Queen Rearing*

Queen rearing became a prime area of interest as Dzierzon's and Langstroth's discoveries became known to beekeepers. Doolittle (1889) developed a method of queen rearing that involved the transfer of young larvae from worker cells into beeswax cups that were made about the size of natural queen cups. Doolittle's methods, with little or no modification, are still used in the beekeeping industry.

3. *Controlled Mating*

Natural mating (NM) of the queen (Koeniger, Chapter 10) can be controlled. However, one needs to exclude 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 in obtaining pure matings. Numerous attempts to mate queens in cages or other confined areas have failed or have not been repeatable (Harbo, 1971).

Instrumental insemination (II) is an alternative to island mating and requires much less space. II permits controlled matings in a place that is not geographically isolated, and it 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.

II 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).

B. History of Instrumental Insemination

The basic principles of II were developed between 1926 and 1947. Lloyd Watson first demonstrated a successful technique in 1926 (Cale, 1926). The success of Watson's technique was confirmed by Nolan (1929), who also developed holding hooks and an insemination stand that are similar to those presently in use (Nolan, 1932). Laidlaw (1944) vastly improved the success rate of II by learning to insert the insemination tip past a flaplike structure (the valvefold) that covers the entrance of the median oviduct. He depressed the valvefold and injected the semen directly into the median oviduct. Mackensen (1947) used carbon dioxide to immobilize queens during insemination. This made it easier to insert the tip into the median oviduct. Moreover, he found that CO_2 narcosis caused queens to begin laying eggs sooner after insemination. The first comprehensive manual for II of queens was written by Mackensen and Roberts (1948).

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. II has been used very little in commercial breeding programs, and attempts to market II queens for use in field colonies have ended in failure.

C. Chapter Objective

For instrumental insemination, this chapter describes only the equipment and procedures that I now use. The techniques are similar to those of most other workers with two exceptions. First, I collect large quantities of semen before beginning inseminations; others collect semen between inseminations. Second, I use glass rather than plastic insemination tips. The techniques of other workers are described in Mackensen and Ţucker (1970), Ruttner (1976), and Laidlaw (1977). The procedure begins with queen and drone production and ends with laying queens.

II. QUEEN PRODUCTION

A. Natural Queen Production

Queen and worker honey bees are genetically identical; both are females. They differ only in the way workers feed and care for the larvae. Queens can be produced from worker larvae that are 3½ days old or less (6½ days from egg laying) (Becker, 1925), so any colony with young worker brood has the potential to produce queens. However, most often they choose not to do so.

The reason a colony does not constantly produce queens can be traced to the resident queen. A mated queen inhibits the workers from producing queen cells, and this inhibition is effective only if the queen is able to travel freely over the brood area (i.e., not caged or restricted to a portion of the broodnest) (Butler, 1957). Lensky and Slabezki (1981) found that a pheromone is produced on the tarsi (feet) of a queen and deposited on the comb surface by her foot pads. When applied to the lower edges of a brood comb in an overcrowded colony, this chemical, in combination with secretions from the mandibular gland, inhibits the production of queen cups. When used alone, neither of these secretions inhibits construction of cups. However, if queen cells have already been started, a laying queen apparently does not suppress the rearing of queens (Lensky, 1971).

The natural periods for a colony to rear queens are when they are about to swarm, when they are replacing a failing or poor queen, and when they are replacing a queen that has been removed or accidentally killed. All these conditions could be attributed to inadequate queen movement over the

brood area, for a crippled, old, or poorly laying queen might be unable to get to all parts of the broodnest as the colony demands. Those queens therefore fail to inhibit cell production, and new queens are produced. Queen rearing under crowded, swarm conditions may also be caused by the immobility of the queen. According to Lensky and Slabezki (1981), the crowding of worker bees at more than 2000 bees per liter of actual space (hive volume minus comb volume) restricts the movement of a queen so that queen cells are produced.

Under conditions of swarming and supersedure, a queen usually will lay eggs in queen cups. Therefore, their restricted movement does not completely restrict them from where the queen cups are produced.

In the case of sudden queen loss or emergency supersedure, eggs are not laid into queen cups but queen cells are produced from larvae in worker cells. Örösi Pál (1957) found that emergency queen cells are started on cells with larvae, not on cells with eggs.

B. Management for Queen Rearing

To produce queens, a beekeeper must manage a colony of bees so that the workers will rear certain larvae, chosen by the beekeeper, into queens. There are many ways to do this (Laidlaw, 1979; Morse, 1979), but most methods rely on putting a colony into a natural queen-rearing state and then adding the young larvae that are to be reared into queens. These larvae are taken from worker cells and put into human-made beeswax cups that simulate natural queen cups (Fig. 1, *a* and *b*).

1. *Cell-Building Colony*

A colony that is managed to rear queen cells is called a cell builder. Sometimes these colonies have a laying queen (usually confined to a section of the colony away from the cells), sometimes they are queenless, and sometimes a queen producer uses two cell builders, one to start the cells and one to finish them. I have used both queenright and queenless cell builders, and recommend queenless cell builders for all but those who produce thousands of queens per year. Thus I will discuss only the management of a queenless cell builder.

There are some general qualities that a cell-building colony should have. It should have a larger rather than a small population of worker bees (>20,000). The bees should be crowded into a colony with 10, or at the most 20, frames (Fig. 1*b*). The colony should be fed sugar syrup, and also pollen if no natural pollen is available (feeding pollen often causes disease, so beware of the pollen source). Finally, young brood in the colony should be arranged

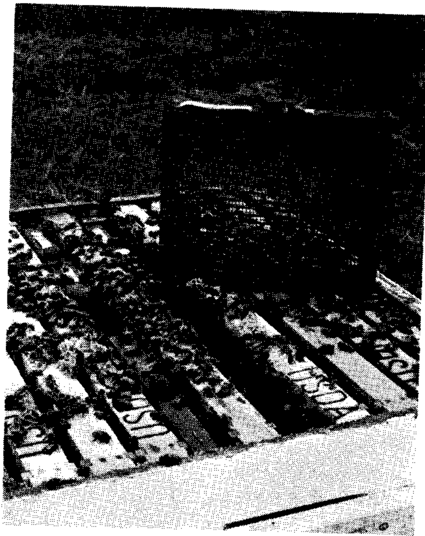

Fig. 1. Queen rearing and drone storage. (*a*) A queen cell cup with a drop of royal jelly and a larva floating on the jelly. The end of a wire grafting tool extends into the cup. (*b*) Cups are arranged on two bars that are made to slip in and out of a frame that goes into a cell builder. (*c*) Capped queen cells put into vials that are arranged on a wooden rack that goes into an incubator (35 °C). Each vial has artificial sponge material and a small amount of queen candy (made from honey and powdered sugar) at the bottom of the vial. A newly emerged queen can live about 2 days on the candy, and the sponge gives her a dry, nonslippery walking surface. (*d*) Drones stored in cages. The cages have excluder material on one side and screen (8 mesh per 25 mm) on the other. Cage size and shape can vary, but cages should not be high and narrow because the drones tend to gather at the bottom. If the drones become more than 3 or 4 cm deep at the bottom, some may die. Therefore, these cages measuring (16 × 19 × 2 cm) are given no more than 125 drones.

next to where the queen cups are to be placed, because young brood attracts nurse bees.

The cell-building colony must be managed on a regular schedule. If properly managed, the same colony can be used to rear cells for an entire season. A weekly schedule is the easiest to keep. Begin the weekly management by harvesting the queen cells (now capped) and put them into an incubator (Fig. 1*c*) or into colonies. Since the colony is queenless, it needs to be given brood or bees to maintain its population. About two good frames of brood (one with uncapped cells) usually fill this need and can replace two broodless combs in the cell builder. Finally, destroy any queen cells that may be reared on the brood comb, because any virgin queen that emerges in the cell builder will destroy the other cells.

2. *Breeder Queens*

A breeder queen is the mother of the queens to be reared in the cell builder. A breeder queen need not be kept in a large colony, yet she needs to be laying in a colony that will adequately feed the newly hatched larvae.

3. *Basic Grafting*

The process of taking larvae from the worker cells to queen cups is called grafting (Fig. 1*a*). Of course, the larvae come from the breeder queen and are put into the cell-building colony after being transferred to the cups. Grafting should be done after rather than before the weekly management of the cell builder.

A grafting tool is used to transfer a larva from the bottom of a worker cell to a queen cup. This tool can be purchased but is often homemade by bending and filing a wire or carving a green twig. A moistened (chewed) toothpick is often used because it is convenient, not because it is best.

Grafting is best done inside a building under a bright light. Four items are needed: the bars of cell cups (detached from the frames), royal jelly (diluted 1 : 1 with water), a grafting tool, and a frame of young brood from the breeder queen. Before grafting, place a drop of royal jelly into each cup on a bar of cups. Royal jelly is not necessary, but it is easier to get the larvae off the grafting tool if jelly is present. (Royal jelly is collected from queen cells before they are capped. Remove the queen from a colony and return in 3 days to collect jelly, or graft as usual and then harvest royal jelly 3 days later. Royal jelly stores well in the freezer.)

Choose larvae for grafting that are as young as possible, for the youngest larvae produce the best queens (Woyke, 1971). Weiss (1974a) found that the major decline in queen quality comes when larvae are over 48 hr old at the time of grafting and that there is very little difference in queens when the grafted larvae are 0–36 hr old.

4. *Double Grafting*

This is a grafting technique that some queen producers use in an effort to produce larger and presumably better queens. Graft as described above, then after 24 hr remove the cells from the cell builder and prepare to graft again. Discard the larvae from the cells (retain the jelly), graft new larvae into those cells, and then return the cells to the cell builder.

There are conflicting opinions as to the value of double grafting. Weiss (1974b) found that when grafting larvae 24 hr old, there was no difference in queen quality when the larvae were double or single grafted. However, when grafting larvae that were 36–48 hr old, the double-grafted larvae produced superior queens.

5. *Grafting Eggs*

This is a technique that was developed by Örösi Pál (1960). It is a form of double grafting, but instead of putting another larva into a cell, a 2- to 3-day-old egg together with a 3-mm disk of beeswax at its base is set into the queen cup. The jelly surrounds the beeswax base but does not touch the egg.

To graft eggs, one needs eggs of known age and a way to transfer a wax disk and egg to the cell cup. Örösi Pál (1958) recommends a simple punch made by wrapping a thin piece of metal (10 × 60 mm) around a 3-mm nail. The circle of the punch does not close completely, so that after the disk and egg are in the punch, a pin can reach through the side of the punch onto the disk to remove the disk and egg from the punch. An opening 2 × 5 mm is filed about 3 mm from one end and opposite the slit so that one can see inside.

6. *Seasonal Effects*

The best time to produce queens is when nutritious pollen is available to the bees. This usually corresponds to the natural growth period of a colony and will vary among different parts of the world. In general, if drone brood is being reared, queens can be produced. Queens can be produced during suboptimal periods if one is willing to feed large quantities of pollen (Taber and Poole, 1974).

III. DRONE PRODUCTION

A. Rearing Drones

Drones are usually reared in their home colony. Thus, there are no cell builders for drones as there are for queens.

However, as with cell-building colonies, a good drone-producing colony

has certain requirements. Probably the most basic need is a good pollen supply. Also, a large population is more apt to produce drones than a smaller population, and of course more drones will be produced if a colony has some drone comb available.

If one needs drones from a particular queen that is not producing any or is producing too few, the queen can be moved to a colony that does produce drones. Find a colony that is actively producing drones, remove the queen from the latter colony, kill the capped drone cells by scraping them with a hive tool, and introduce the queen from which drones are desired. Ten days later, kill the capped drone cells again (these are still drones from the previous queen). The workers will usually rear drones for the new queen as well as they did for their mother.

One can force a young queen to produce drones by not allowing her to mate. Such a queen is induced to lay eggs by giving her 3 min of CO_2 narcosis on each of 3 consecutive days or simply by letting her age (caged) for 5 or 6 weeks. The unfertilized eggs produced by these queens develop into small drones if the eggs are laid in worker-sized cells. These small drones produce viable semen that can be collected and used in instrumental insemination. However, if drone-sized cells are available in the colony, the unmated queens seem to prefer to lay their fertilized eggs in them, and the result will be normal-sized drones.

B. Storing Drones

Drones will not yield semen until they are 6 – 12 days old. Therefore, they must be aged by storing them somewhere. Moreover, drones are often stored for longer than 6 – 12 days simply for the convenience of the operator or to coordinate semen collection with queen maturity. The problem is to age the drones while keeping the group free of unwanted drones that may mingle with the desired drones.

One way to do this is to mark the drones. Individual drones can have a dot of paint put on their thorax, or they can carry a visible genetic marker. Of course the genetic marker should not be present in adjacent colonies, and if the marker is an eye marker, the drones must be kept from flying so that they will not be lost. A group of drones can be marked with spray paint if the nozzle has its orifice enlarged to deliver droplets that are slightly larger than used in painting. These painted drones can then be released into a colony and collected as needed.

A second technique is to confine the drones to a single brood chamber. W. C. Roberts used this technique when continuously rearing and storing many drones from a single queen. The drone-rearing colony serves as the drone storage colony. An empty super and two excluders are brought to the colony

that has drone brood of the desired stock. All the bees are brushed from the frames of drone brood as well as the other frames that are put into the new super (uncapped worker brood, pollen, etc.). When full of combs, this super containing no adult bees is placed above the brood chamber with an excluder above and below. Of course, adult workers quickly move into this super (drone chamber), so it is free of adults for a very short time. These colonies can be managed on a weekly schedule and should be opened only in mornings so that the drones do not fly out and unwanted drones do not fly in. During management, the empty drone and worker combs are moved down to the brood chamber, and combs of drone brood, worker brood, and pollen are brushed free of bees and moved into the drone chamber.

A third method of storing drones is to put them into cages (Fig. 1*d*). The cages should have excluder material on one side so that workers can pass into the cage to feed the drones. Drones survive best in populous colonies between frames of brood.

IV. INSEMINATION PROCEDURE

Simply stated, II is the instrumental transfer of semen from one or more drones to the oviducts of a queen (see Fig. 6). In both NM and II, the semen is deposited in the lateral oviducts. Once in the oviducts, 4–25% of the spermatozoa migrate to the spermatheca. The percentage of migration depends on the amount of semen given (discussed later). Sperm migration is usually complete within 24 hr (Bishop, 1920; Woyke 1983b), and the insemination process is complete at that point.

A. Equipment

The complete apparatus, except for the CO_2 tank and regulator, is pictured in Fig. 2. The insemination stand and hooks are described in detail by Mackensen and Tucker (1970). 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. 2). 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. 3. The design in Fig. 3 is an improved version of a syringe designed for use in semen storage (Harbo, 1979). 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

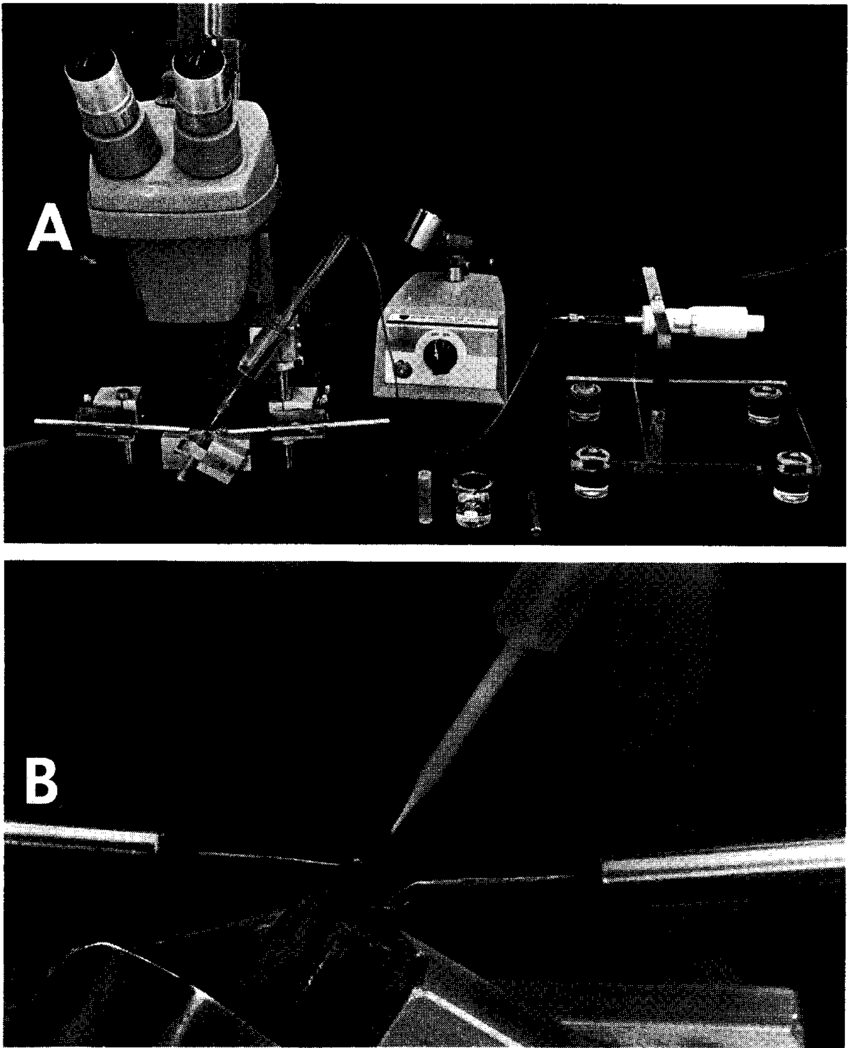

Fig. 2. Insemination equipment in use. (*a*) A Mackensen insemination stand (Mackensen and Tucker, 1970) holds the syringe shown in Fig. 3. The micrometer for the syringe (200 μl capacity) is mounted in a stand made of acrylic plastic. (*b*) Side view of a queen ready for insemination. The top of the queen holder is aligned with the top of the block. About $2\frac{1}{2}$ abdominal segments protrude beyond the end of the holder (hind legs should not protrude. [From Harbo (1985). Copyright in public domain.]

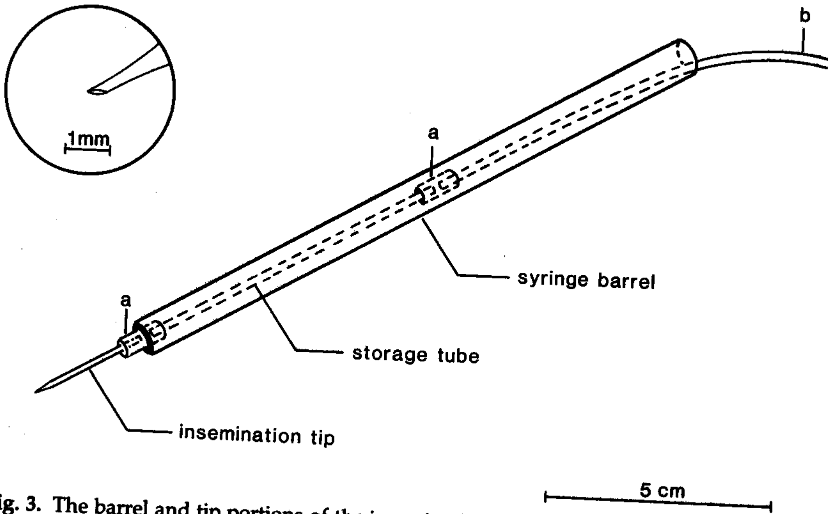

Fig. 3. The barrel and tip portions of the insemination syringe. The tip is a glass capillary tube that was drawn to a point with heat and polished to an angular point (see enlargement). The ID at the orifice of the point is 0.18 ± 0.03 mm; the OD at the orifice is 0.28 ± 0.03 mm. The storage tube is 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 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 syring micrometer shown in Fig. 2*a*. 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 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. [From Harbo (1985). Copyright in public domain.]

movements of the micrometer dial (semen is metered with an accuracy of $\pm 0.1\ \mu l$).

B. Semen

1. *Physical Properties*

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 plasma fraction of the semen, because the spermatozoa appear white when the

plasma is removed. Each drone produces about 10 million spermatozoa (Mackensen, 1955), and there are about 7.5 million spermatozoa per microliter of semen (Woyke, 1960; Mackensen, 1964). Spermatozoa are filamentous, without a distinct head, and 221–270 micrometers long (mean length = 242 μm) (Woyke, 1983a). Each cell has a volume of about 21 cubic micrometers (measured with a Coulter Counter®*). The specific gravity of semen is 1.077 g/ml (based on five samples totaling 260 μl). Verma (1973) reported osmolarity of honey bee semen to be 467 milliosmolar. See Taber (1977) for an extensive review of the chemical composition of semen and for metabolism of spermatozoa.

2. *Obtaining Semen from Drones*

Drones are not sexually mature when they emerge as adults. Kurennoi (1953) found that spermatozoa begin to move from the testes to the seminal vesicles when a drone is about 3 days old. 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 (Mackensen and Tucker, 1970) or leave a residue of semen in the oviducts (Woyke and Jasinski, 1978). Both conditions will kill a queen before she begins to lay eggs. The disease problem comes only from caged drones (Mackensen and Tucker, 1970), and the semen residue affects only caged queens that receive an insemination dose >4 μl (Veselý, 1970). Although there seems to be an ideal age for drones, a breeder can still use older drones if a few precautions are taken: antibiotic in the semen, reasonable sanitation, and smaller insemination doses 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 drone flight and hinged at the top so that a cage of drones can be easily placed inside. My cage is 32 × 28 × 22 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. It is easier to see inside the cage if the screen is painted

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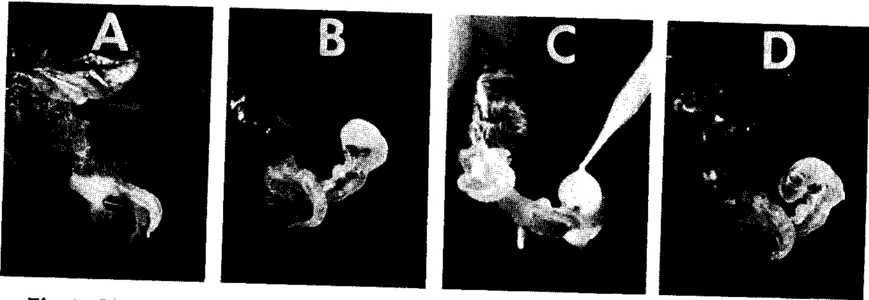

Fig. 4. Obtaining semen from mature drones. (*a*) The first stage of drone eversion caused by a dorso-ventral squeeze of the head and thorax. If the abdomen is turgid at this stage, the drone will likely yield semen. (*b*) The second stage of eversion. This is produced by a lateral squeezing of the turgid abdomen. Semen and mucus are discharged at this stage, making it ideal for semen collection. (*c*) Semen collection from a second stage eversion. (*d*) Very active drones may evert to this third stage when handled or after squeezing the thorax or abdomen. Semen can also be collected from the third stage, but the semen is sometimes discharged onto the abdomen or wings of the drone. [From Harbo (1985). Copyright in public domain.]

black. Release ca. 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. 4.

3. *Collecting Semen into the Syringe*

Assembly of the syringe is described 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 as often, since it does not come in contact with semen. I use 0.85% NaCl and 0.25% dihydrostreptomycin sulfate (Mackensen and Tucker, 1970). This saline solution is simple and adequate. However, Ruttner and Tryasko (1976) describe other salines ("Kiev" and "Hyer") that give excellent results. Between uses, the tips are stored in a 5% solution of sodium hypochlorite (common laundry bleach). The bleach is rinsed from the tip by forcing water through it with a plastic squeeze bottle.

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. 3). 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 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. 4*c*). Draw the semen into the tip. The cohesiveness of the semen will help separate it from 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 crusty material accumulates on the outside.

C. Queen Preparation

The storage of queens from emergence through insemination can be done in three 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 clipped (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. 5. 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. A third technique, described by Woyke and Jasinski (1979), eliminates the colony. The queens are kept in an incubator in separate cages with each queen attended by 150 workers. I have never used this last method, so further discussion will involve only the first two.

Queens should be inseminated when they are young. They should be at least 24 hr but not more than 5 or 6 weeks old. However, older queens can be inseminated (Section IV,F). When inseminating with 8 μl of semen, Woyke and Jasinski (1976) had the most spermatozoa enter the spermatheca when queens were 4 – 8 days old. They recommended that queens be inseminated at age 5 – 14 days.

A virgin queen can be inseminated after she has laid eggs (Section IV,F). 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.

D. Insemination

Semen does not need to be used immediately after collection. It can be stored in the syringe at room temperature (20 – 25 °C) for as long as 2 days with little or no loss of viability.

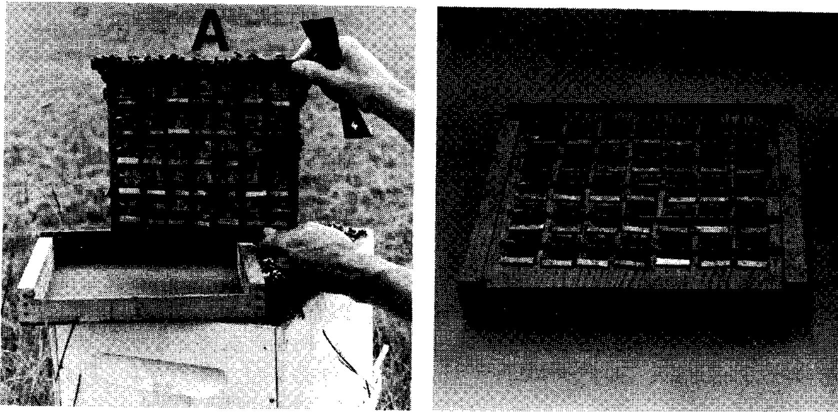

Fig. 5. A system for storing queens together in a colony. (*a*) The board used for holding queens in a colony is made of plywood (13 mm thick) that was cut to be the same size as a frame in the colony. Queens are stored in holes measuring 24 mm in diameter and covered with a permanent screen (10 mesh per 25 mm) on the back side and with hinged screens (8 mesh per 25 mm hardware cloth) on the front. The two hinges on each screen consist of 10 mm staples driven about 8 mm into the wood. Rigid horizontal wires (also hinged with staples) keep the screens from opening. Metal tags glued 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 (shown on the colony) 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 hr if necessary.

The flow rate of carbon dioxide should be adjusted before queens are put into the queen holder. The rate (ca. 35 ml/min works well if one has a flow meter) does not need to be too precise. The flow should be barely detectable with moistened lips. Increase the rate slightly if the queens are not completely motionless 15 sec after entering the queen holder. The purposes of CO_2 are (1) to immobilize the queens to make inseminations easier and (2) to cause the queens to begin egg laying earlier. Something in the natural mating process stimulates egg laying, but II queens without CO_2 treatment will not begin laying eggs any sooner than virgin queens.

I collect 0.5 – 1.0 μl of saline into the insemination tip between inseminations, 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 place the queen holder in the insemination stand. Force the queen to crawl into a dead-

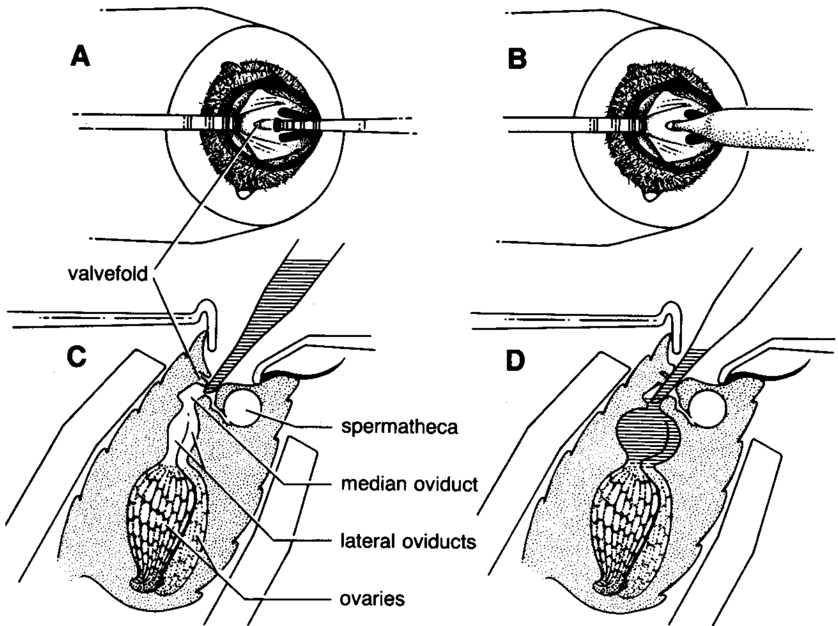

Fig. 6. The insertion of the insemination tip. (*a*) The operator's view of a queen ready to be inseminated. (*b*) The same view as (*a*) but showing the first stage of tip insertion. The tip has been inserted about 0.5 mm (about twice as far as the tip is wide). (*c*) A side view of (*b*). (*d*) Final 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 0.5 mm to bypass the valvefold, and then inserted another 0.75 mm. The total insertion depth is about 1.25 mm. Semen that was discharged from the tip has caused the lateral oviducts to swell. [From Harbo (1985). Copyright in public domain.]

ended tube (shown on end next to the saline in Fig. 2*a*). Then place an open tube (the queen holder shown in Fig. 2, *a* and *b*) 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. 2*b*). The plug at the end of the CO_2 hose is inserted into the tube to hold the queen in position. See Fig. 2 for queen adjustment.

The sting hook and ventral hook separate the sting apparatus from the ventral plate to expose the vagina (Fig. 6). 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 base of the sting. While the sting is pulled dorsally, 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. 6. 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 will be discussed in Section VI,A. Equalize pressure before removing the tip by moving the micrometer about 0.5 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.

Tips sometimes get plugged during semen discharge or between inseminations. If it plugs during semen collection or between inseminations, the operator has usually forgotten to retract the semen from the point or to collect saline into the tip after the last insemination. If this happens, soak the tip in saline, wipe it with tissue, and apply normal suction with the syringe. If the tip plugs during semen discharge, a mucus plug is usually the cause. This is caused by careless semen collection, for mucus is more apt to cause a plug when it is discharged than when it is collected. One can often see the mucus plug in the neck of the tip. This may require tip removal and cleaning.

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 min, so one can collect two or three queens per trip to save time; they can be returned narcose. Caged queens can stay out of the colony for many hours as long as worker bees are in the box below (Fig. 5).

E. Oviposition

To stimulate egg laying, queens are given three treatments of CO_2. Carbon dioxide 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. When the queens are motionless, turn off the CO_2 and leave the queens narcose in the sealed bag for about 3 min.

Queens free in the colony should be treated with CO_2 once per day on the 2 days after insemination. Expect egg laying to begin about 4 days after the last CO_2 treatment if the queens were 1 week old 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 small colony to lay eggs. For best success, keep the queens in the storage colony until they are at least 2–3 weeks old. Queens that are

over 2 months old when they are put into colonies to lay eggs do not need CO_2 treatments.

Acceptance of an II queen by worker bees is sometimes a problem. To reduce the frequency of queen death, have the II queens lay in a small colony for at least 4 days before putting them into large field colonies. Place each caged queen into a small colony consisting of 2000 – 5000 worker bees. These colonies may be newly made without brood. Release the queens after 3 days if the workers are not biting the cage. About 10 min after release, check some of the queens to see if any are being chased or bitten by one 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.

F. Special Techniques

1. *Single-Drone Insemination*

This is a common technique in research. Since all the gametes from one drone are identical, queens mated with semen from a single drone will produce daughters that are all more closely related than full sisters. These daughters, known to have a gamete in common, are called super sisters.

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 0.5 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.

2. *Inseminating Many Queens with Identical Spermatozoa*

This technique enables one to mate identical gametes to different queens. As with single-drone inseminations, each queen produces a family of super sisters. 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, in my experience, produced worker brood. I have inseminated as many as 14 queens from a single drone (ca. 0.5 million spermatozoa per queen), and they averaged 84% worker brood.

The second technique was demonstrated by Kubasek *et al.* (1980) and requires the use of queens that produce gynandromorph progeny. The advantage of this technique is that isogenic spermatozoa are produced in large quantities and can be collected throughout the season. The inseminations can be like multiple inseminations because the male tissues of many individuals produce identical gametes.

3. *Inseminating Queens with a Uniform Mixture of Spermatozoa*

Among other possibilities, this technique can be used to maintain genetic heterogeneity in a breeding program or to produce uniform, but genetically heterogeneous, inseminations in a 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. Kaftanoglu and Peng (1980), have shown that spermatozoa can be highly diluted, mixed, and then recovered by centrifugation. Queens have been successfully inseminated with this "washed spermatozoa" (Kaftanoglu and Peng, 1980; Williams and Harbo, 1982; Mortiz, 1983, 1984). Moritz (1983) showed that this technique produces uniform mixing of cells.

4. *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 organs in the abdomen of a drone. Mackensen and Ruttner (1976) describe a technique where they remove a seminal vesicle, pinch it with a forceps at the testes end, and thereby start peristaltic contractions which force the spermatozoa out the other end where they are collected with a syringe. 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.

5. *Using Spermatozoa from the Spermatheca of One Queen to Inseminate Another*

This technique is used to make gamete backcrosses (Cale and Gowen, 1964) or simply to 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.

6. *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 five to eight workers for about 3 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 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 >4 months lay a high proportion of unfertilized eggs.

7. *Inseminating a Queen with Semen from Her Own Drones*

This is a breeding scheme called self-fertilization that was first described by Mackensen (1951). 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 at this time. If some drone-sized cells are available, these "drone-laying" queens will usually lay eggs in them, and the resulting drones will be larger than if the queens are forced to lay 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).

V. EVALUATION

A. Spermatozoa in the Spermatheca

A necessary step in the mating of honey bees is to get spermatozoa into the spermatheca of a queen. A queen retaining more spermatozoa in her spermatheca is considered to be better mated than one with fewer.

Estimating the number of spermatozoa in the spermatheca enables quantification of success beyond simply mated or not mated. One method is to

count spermatozoa in a hemacytometer (a cell counting chamber). A spermatheca can contain as many as 7 million spermatozoa, so the contents should be dispersed in at least 10 ml of solution. More concentrated spermatozoa are difficult to count.

A much faster technique uses light absorbance to estimate numbers of spermatozoa. Harbo (1975) found a strong linear correlation ($r = 0.96$) between absorbance at 230 nm and density of spermatozoa in a 0.5 *M* NaCl solution. During the past 4 years, I have compiled a new correlation using a 10-ml rather than a 5-ml dilution (same diluent and wavelength) and including samples from a wider range (0–8 million cells/10 ml). Based on 194 samples, the regression formula was $Y = 0.034 + 0.061X$ (Y = absorbance units, X = millions of spermatozoa); $r = 0.95$. Rearrangement changed the formula to $X = 16.47Y - 0.56$, which converts absorbance (Y) to millions of spermatozoa (X). This formula was adjusted slightly because it consistently estimated high at the high end of the range (usually about 0.2 million higher than the counted mean) and slightly low at the low end. The line was adjusted to pass more accurately through the observed zero intercept but still through the mean coordinates. The adjusted line is within the 0.95 confidence interval for the line and the slope; it is $X = 15.72Y - 0.41$.

Figure 7 shows the correlation between the amount of semen given and the number of spermatozoa entering the spermatheca of young queens (queens <3 weeks old). Results are variable, but this represents an average expectation.

Even under seemingly uniform conditions, the number of spermatozoa entering the spermatheca is variable. Among sister queens inseminated in sequence with the same amount of semen, the coefficient of variation (CV) is 26% for 8-μl inseminations (Woyke *et al.*, 1974), 35% for 2.5-μl inseminations (Mackensen and Roberts, 1948), and 49% for inseminations with 2 million cells (Bolten and Harbo, 1982). With two inseminations of 2 million cells, the CV dropped to 31%, and for three inseminations of 2.5 μl the CV was 16%. In general, uniformity increased with larger inseminations and with multiple inseminations.

During natural mating, queens retain about 5.7 million spermatozoa in their spermatheca (CV = 18%). An average that approaches this (5.5 million) can be achieved with II, but it requires four inseminations with 2.5 μl (CV = 10%) (Mackensen and Roberts, 1948).

Both genetic and environmental factors affect the number of spermatozoa reaching the spermatheca. Woyke *et al.* (1974) showed that different geographic races differed in the number of spermatozoa entering the spermatheca after inseminations with 8 μl of semen. Woyke and Jasinski (1976) found that younger queens (<3 weeks old) retained more spermatozoa in their spermathecae after an insemination of 8 μl than did older queens.

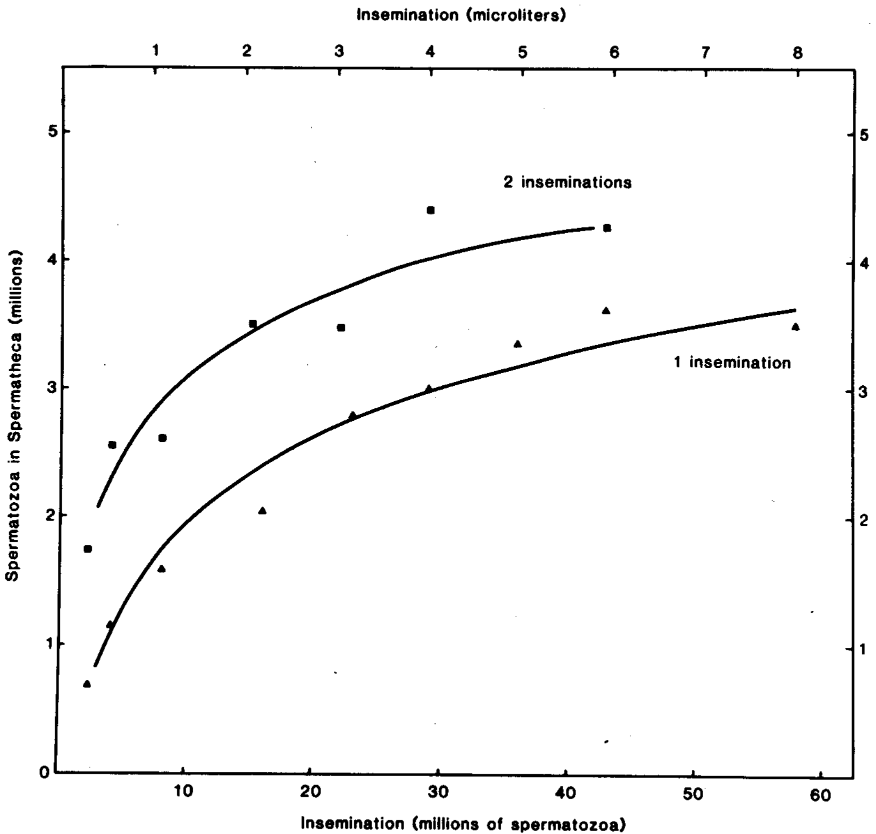

Fig. 7. The effect of insemination dosage on the number of spermatozoa entering the spermatheca. Data were compiled by Woyke (1960, 1971), Mackensen (1964), Veselý (1970), Woyke and Jasinski (1973), Bolten and Harbo (1982), and J. R. Harbo (unpublished). Regression formulas were derived from the means (plotted points) for each dose. Total *n* is 351 queens for one insemination and 94 queens for two. Regression formulas, $Y_1 = -0.24 + 0.95(\ln X_1)$ and $Y_2 = 1.1 + 0.86(\ln X_2)$, predict the number of spermatozoa entering the spermatheca (*Y*) from the number of spermatozoa given (*X*) with one and two inseminations, respectively ($r_1 = 0.98$, $r_2 = 0.96$). *X* and *Y* in the formulas are always in millions (e.g., 4,400,000 is entered and recalled as 4.4). [From Harbo (1985). Copyright in public domain.]

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 (Veselý, 1970). However, he found no significant difference between free and caged queens when insemination volumes were 2 or 4 μl.

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 two groups of unrelated queens. The first group (10 queens) was inseminated with 0.2 ± 0.1 μl of semen each. Five queens had 111,000–575,000 spermatozoa in their spermathecae, and those all produced >99% worker brood. The remaining five had 1000–39,000 in their spermathecae, and they produced 2–92% worker brood. The second group of queens 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 five had 6000–34,000, 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. Queens with no spermatozoa in the spermatheca do not lay as many eggs per day and do not position eggs as uniformly as do queens with spermatozoa in their spermathecae (Harbo, 1976). However, among queens that are producing worker brood, I failed to find a correlation between egg-laying rate and the number of spermatozoa in the spermatheca.

B. Queen Survival

Queen loss after insemination can be a serious problem. Probably the two most common causes are (1) a semen residue remaining in the oviducts for more than 1 or 2 days after insemination and (2) worker aggression when II queens are first introduced to colonies.

Insemination with large volumes of undiluted semen and keeping queens caged after insemination tend to cause semen residue in the oviducts. Normally, semen is discharged from the oviducts and some of the spermatozoa enter the spermatheca. If insemination doses are high (≥6 μl), a residue of semen is often left in the oviducts of caged queens (Veselý, 1970). This residue may serve as a medium for the growth of microorganisms, which may be the cause of death. Woyke and Jasinski (1978) found that after inseminating caged queens with 8 μl of semen, a residue of semen was found more often when semen came from older drones (those >4 weeks old) than when semen came from drones aged 2–3 weeks. Veselý (1970) concluded that queens free in a colony do not seem to have a problem with semen residue in the oviducts, nor do caged queens inseminated with <4 μl of semen.

Queen loss when first introducing an 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 is described in Section IV,E. 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.

C. 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 (1985). 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 1 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 cost of producing II queens is higher when each queen is running free in a small colony. About 2 min more per queen is required because three trips are necessary to find and bring the queens in for insemination or CO_2. Moreover, a producer needs to operate more of these small colonies when the young queens are allowed to run free because free-running queens spend about twice as much time in a colony as do queens that are only put into colonies when they are ready to lay eggs. A free queen needs a colony for 2 weeks; a 3-week-old banked queen can be put into a small colony, released on day 3, and be laying on day 4 or 5.

VI. RECOMMENDATIONS

A. Semen Dosage

Caged queens need multiple inseminations if they are to average more than 3 million spermatozoa in the spermatheca, because caged queens often cannot tolerate inseminations of undiluted semen that are >4 μl. I suggest two inseminations of 3 μl. If one wants to approach the sperm count of a naturally mated queen, three inseminations of 2 or 3 μl are needed. Multiple

TABLE 1. Time Required to Produce Instrumentally Inseminated Queens in a Group of 40 or More[a]

Step	Time required
Producing, caging, and storing drones of known parentage[b]	10 sec/drone
Collecting semen[c]	50 sec/μl
Total cost of semen (at 0.5 μl/drone)[d]	1.2 min/μl
Rearing queen cells[b]	4 min/cell
Marking and storing queens[b]	2 min/queen
Cost of a mature, virgin queen	6 min/queen
Insemination (includes transporting caged queens from and to the storage colony)[e]	3 min/queen
Additional CO_2 treatments	15 sec/queen
Total cost of queens inseminated once with:	
3 μl of semen[f]	13.1 min/queen
8 μl of semen[f,g]	19.1 min/queen
Total cost of queens inseminated twice with 3 μl of semen[f]	19.7 min/queen

[a] Queens produced in one cell builder and kept in one queen storage colony.

[b] K. Tucker, J. Harbo, and J. Bishop; unpublished data presented orally at the annual meeting of the American Bee Breeders Assn., 1974.

[c] Mackensen (1964) reported that collecting 1 μl of semen required 45 sec. J. Harbo (unpublished) 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.

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

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

[f] Cost does not include queen introduction and assumes no queen mortality after insemination.

[g] Doses of 8 μl are not recommended for caged queens (see semen dosage). Add ca. 2 min per queen to find and bring each queen in for insemination and CO_2 treatments.

inseminations of caged queens should each be 3 μl or less and should be spaced 2 or 3 days apart.

Woyke (1960) has long recommended one insemination of 8 μl or two inseminations of 4 μl for queens that are free in a small colony. Mackensen (1964) concluded that two inseminations of 3 μl may be an adequate minimum if queens are to be used in large field colonies. Multiple inseminations of free-running queens are given on consecutive days. Use Table I and Fig. 7 to decide on dosage, but consider (1) that a single insemination as large as 8 μl does not seem to be detrimental if the queen is running free in a small colony and (2) that multiple inseminations give more uniform results (lower

CV). There are practical limits where the cost of more inseminations or of using more semen yields diminishing returns in queen performance.

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, and some even survive more than a year.

B. Learning Insemination

When first learning to inseminate, use about 15 caged virgin queens. Two microliters 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, either the tip is 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 one or two abdominal segments. A heavy tracheal network makes the spermatheca look like a ball of string about 1 mm in diameter. The presence of spermatozoa does not seem to affect the size or the turgidity of the spermatheca. Remove the tracheae by rolling the spermatheca between thumb and forefinger. 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 small colonies. Inseminate each queen with at least 2 but no more than 8 μl of semen. Follow with CO_2 treatments on the next 2 days and watch for worker brood.

I suggest starting with queens free in colonies for two reasons. First, very good results can be obtained with only one large insemination, and fewer insertions reduce the probability of queen injury. Second, queen death from various causes can be expected, especially for beginners, and it is best for novice inseminators to take these losses (queen death in storage colonies and nonacceptance by workers when released in colonies) before the insemination step.

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