CHAPTER 15

Propagation and Instrumental Insemination

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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

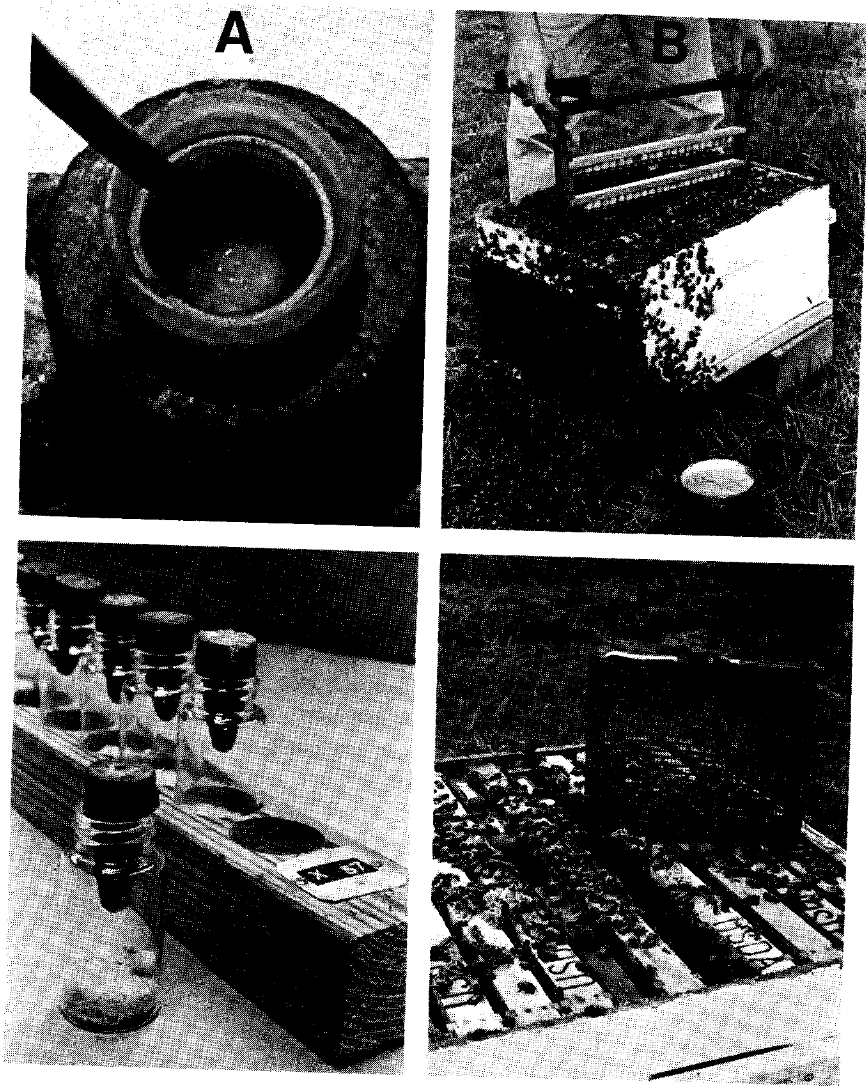

Fig. 1. Queen rearing and drone storage. (*a*) A queen cell cup with a drop of royal jelly and a larva floating on the jelly. The end of a wire grafting tool extends into the cup. (*b*) Cups are arranged on two bars that are made to slip in and out of a frame that goes into a cell builder. (*c*) Capped 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

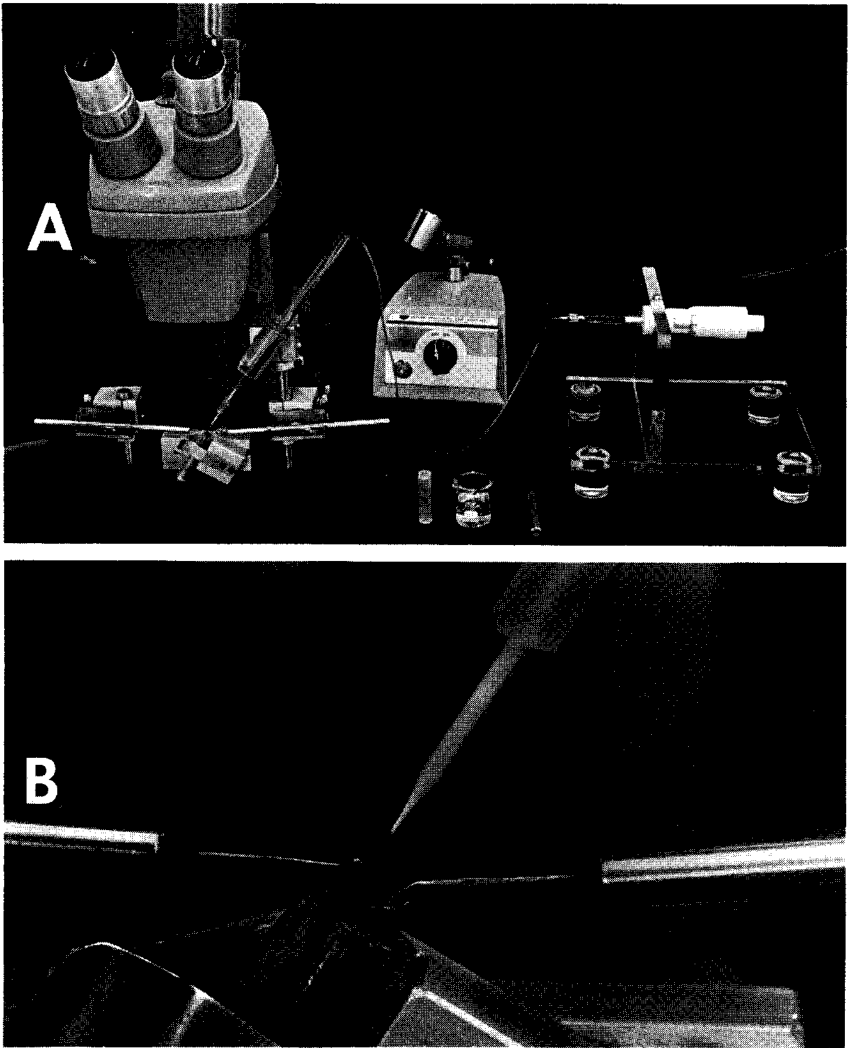

Fig. 2. Insemination equipment in use. (*a*) A Mackensen insemination stand (Mackensen and Tucker, 1970) holds the syringe shown in Fig. 3. The micrometer for the syringe (200 μl capacity) is mounted in a stand made of acrylic plastic. (*b*) Side view of a queen ready for insemination. The top of the 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.]

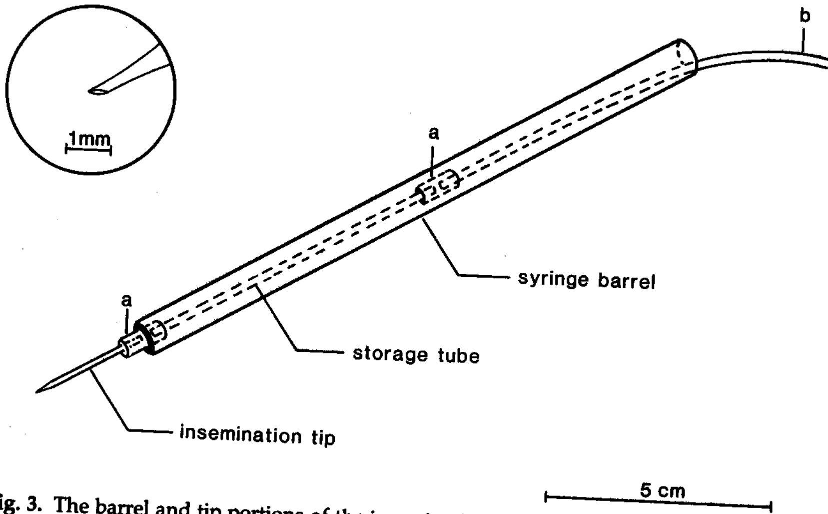

Fig. 3. The barrel and tip portions of the insemination syringe. The tip is a glass capillary tube that was drawn to a point with heat and polished to an angular point (see enlargement). The ID at the orifice of the point is 0.18 ± 0.03 mm; the OD at the orifice is 0.28 ± 0.03 mm. The storage tube is 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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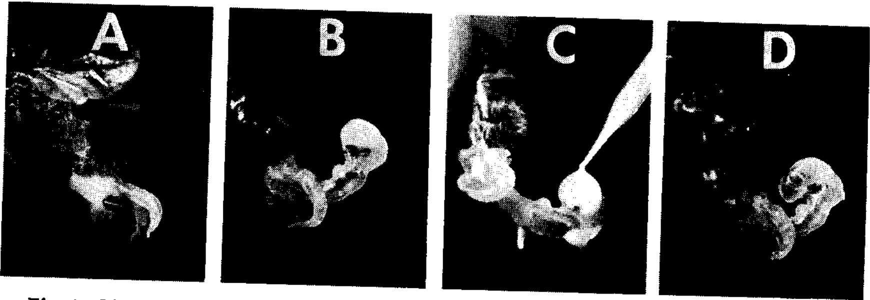

Fig. 4. Obtaining semen from mature drones. (*a*) The first stage of drone eversion caused by a dorso-ventral squeeze of the head and thorax. If the abdomen is turgid at this stage, the drone will likely yield semen. (*b*) The second stage of eversion. This is produced by a lateral squeezing of the turgid 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.