SPERM STORAGE

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A. Natural Storage

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

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

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

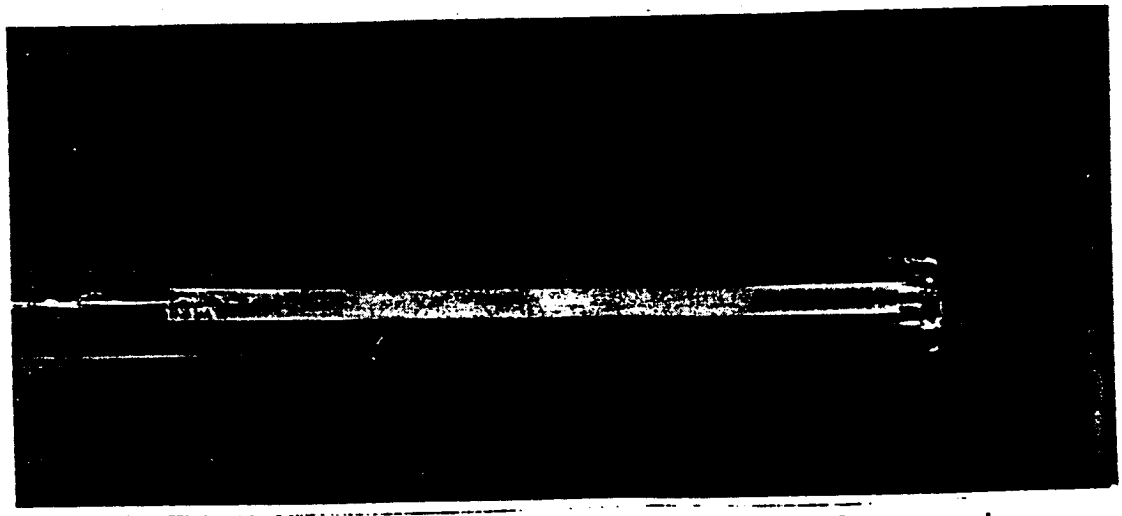

Fig. 24 — A glass capillary tube of semen that is ready for storage in liquid nitrogen.

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

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

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

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

B. *In Vitro* Storage

1. Storage methods

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

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

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

2. Storage at nonfreezing temperatures

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

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

3. Storage at subfreezing temperatures

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

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

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

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

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

4. Damage to sperm

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

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

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

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

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

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

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

5. Present Use of Sperm Storage

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

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