Instrumental Insemination: The Possibility of Semen Storage

Part IV — Conclusion

by SUSAN COBEY
Instrumental Insemination Service
6745 Bucktown Ln., Vacaville, California 95688

THE successful storage of mammalian semen has enabled the efficient maintenance of gene banks for breeding and stock improvement of various farm animals. In the future, hopefully this will be a reality of the beekeeping industry as well. There are many benefits to gain with the perfection of instrumental insemination and the practical storage of honey bee semen. Long-term storage would contribute significantly to the low cost maintenance of valuable lines, and material for genetic selection. Inbred lines are difficult and laborious to maintain and propagate. The number of colonies and labor now required to manage a breeding program would be reduced. Numerous hybrid combinations could be produced year-round.

The use of stored semen would make us less vulnerable to weather conditions and seasonal restrictions. Virgins could be inseminated when drones are unavailable. We could easily produce early queens for the northern beekeepers, and late queens for fall requeening. This could also eliminate the necessity of close coordination and timing of queen and drone rearing for I.I., especially during the busy package season. The maximum use of drones could be made when they naturally occur in large numbers and are easily reared.

Practical storage of honey bee semen may offer a solution to some of the potential problems facing the beekeeping industry. The use of stored semen would provide a method to import and export genetic material without the chance of spreading diseases and/or mite infestations. Importation of live bees into some countries is restricted for these reasons. If and when the Africanized bee reaches the United States, reselection programs may be necessary. Gene banks could be utilized to supply African gene-free stock.

Honey bee spermatozoa have a remarkable longevity of three or more years within the spermatheca of the queen. For successful semen storage, we need to know the factors responsible for this. The development of I.I. raised the question; how long can honey bee semen survive after being removed from the drone? In 1929, at the International Apicultural Congress in Berlin, Germany Dr. Rosch demonstrated the life of sperm in various media. She was able to keep spermatozoa alive for at least 24 hours at room temperature (Nolan, 1929). Honey bee semen is sensitive to drying. Undiluted semen placed on a slide protected with a glass cover slip was reported to survive 2 or 3 days at room temperature (Woyke, et al, 1966).

The need to develop a technique to collect, store and test honey bee semen was recognized. Jaycox (1960) experimented with the storage of semen in glass capillary tubes held at 95°F. He was able to store undiluted semen for 22 days. Success of storage was based on sperm motility, which is used to measure viability of stored mammalian sperm. Jaycox states that this criterion cannot be accurately used to determine viability of honey bee spermatozoa, which becomes inactive after ejaculation. Stored semen in a passive condition will become motile when diluted with saline solution and a small amount of honey. Taber and Blum (1960) found fructose present in fresh honey bee semen which is rapidly metabolized. They suggest sperm stored in the spermatheca may have a low metabolic rate and little or no nourishment.

To test stored semen for its fertilizing capacity, it had to be used to inseminate virgins. Taber and Blum (1960) were the first to report semen could be stored at room temperature for up to 68 days and successfully used for insemination. Storage was considered a success if 50% of the resulting eggs were fertilized. Of the 105 queens inseminated with stored semen, 31 produced fertilized eggs.

The partial success of honey bee semen storage encouraged the possible international exchange of genetic material without the need to import or export live bees. Shipments of semen were made by airmail to Europe and Brazil (Taber, 1961). Virgins inseminated with shipped semen were reported to produce some worker progeny. Not all shipments were satisfactory and not all queens produced fertilized eggs.

Storage of semen was complicated by the adverse effects of bacterial growth from contamination. The addition of antibiotics was found to prolong the viability of stored semen. Semen treated with streptomycin or tetracycline retained at least a portion of its fertilizing capacity for 16 to 18 weeks at room temperature. Queens inseminated with untreated semen stored beyond 12 to 13 weeks did not produce fertilized eggs (Poole and Taber, 1969). Storage at cooler temperatures was also found to prolong viability of stored semen. Streptomycin treated semen could be stored up to 35 weeks at 55°F to 59°F (Poole and Taber, 1970). Previous experiments with cold storage at freezing temperatures were found to be detrimental resulting in no fertilized eggs being produced.

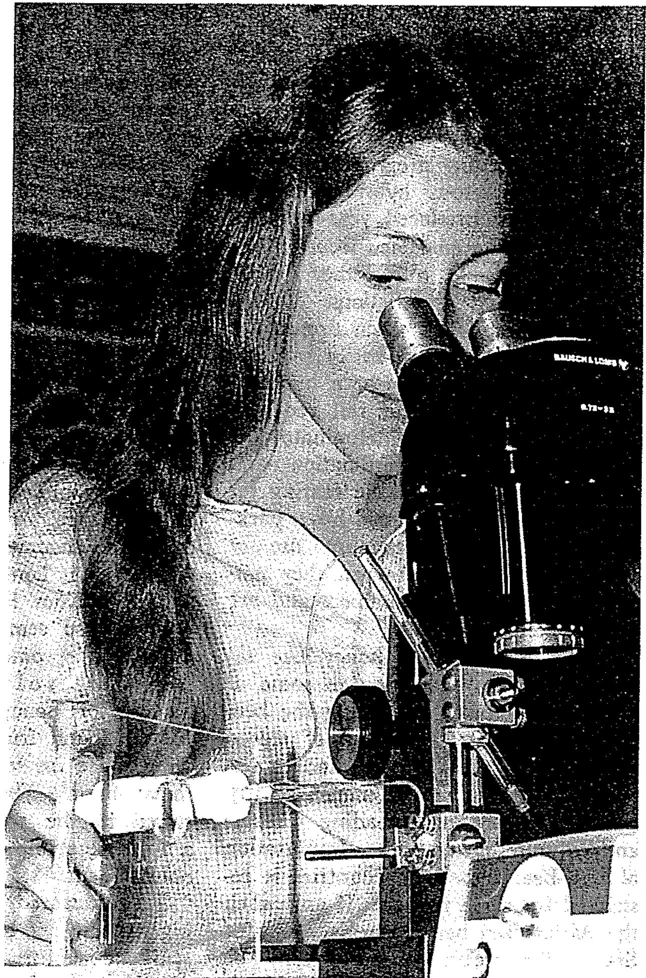
There are many kinds of environmental stress on sperm that are known to cause mutations. The aging of sperm in some animals is known to increase the frequency of lethal mutations. The decreased fertility of stored honey bee semen raised these questions. Storage at 55°F for 35 weeks was tested to determine the occurrence of mutations.

No mutations were found in the 17,503 drones reared from queens inseminated with stored semen. This test did not eliminate the possibility of embryonic lethals or the possible damage to sperm that would prevent egg fertilization (Laidlaw et al, 1977).

Semen prepared for storage by the Poole and Taber method was collected in a Mackensen syringe which has a limited capacity of 10 microliters (*ul*). Several syringe loads had to be collected and transferred into a glass capillary tube, centrifuged to remove air bubbles, and heat sealed for storage. The stored semen had to be transferred back into the syringe to be used for insemination. The transfer of semen was a time consuming job with a high risk of contamination. The need to design a syringe that would simplify this procedure was recognized.

Dr. John Harbo (1974) developed a new method of collection, handling, and storage of semen which eliminated the transfer and centrifuge steps, and made the insemination of a large number of virgins more efficient. A modified capillary tube was designed to serve as a syringe tip, collection chamber, and storage container. The tube holds a large volume of semen so that reloading was no longer necessary, and it could be connected and disconnected without disturbing the column of semen. Strong suction and pressure permit precise movement of semen during collection and insemination. Harbo (1979) has improved and refined this technique. The amount of semen collected is metered with the use of a microburette or gilmont syringe, eliminating the need to calibrate the top or storage tube. Semen could now be accurately measured, diluted to a desired ratio, and delivered. More efficient use of semen was possible with a petrolatum plug to separate the semen and saline. This reduces the loss of semen left on the side walls of the capillary tube and prevents mixing of semen with saline. It also provides an air tight seal for storage. A larger volume of semen could now be collected with the shortened and detachable tips. Interchangeable tips are connected with rubber tubing, which gives flexibility to the syringe and prevents breakage. Glass tips are cheaper and easier to clean compared to the plastic Mackensen tips. The tapered and angled glass tip, which has a smaller outside diameter, is more easily slipped past the valve fold to inject semen into the oviduct of the queen. Semen collected with a glass tip may be more difficult for a person just learning the technique, because the opening of the tip is smaller, and therefore more easily plugged with mucus. However, I feel this is good training and will make the inseminator more careful, considering the fact mucus will also plug the queen.

A new large capacity syringe was also designed by Kaftanoglu and Peng (1980a). The Mackensen tip was modified to accommodate a capillary tube at its base. The syringe barrel has a screw type plunger for precision movement of semen, and allows easy removal and replacement of tubes by equalizing pressure in the barrel. The Harbo syringe and the Kaftanoglu-



Susan Cobey inseminating a virgin using the Mackensen instrument and Harbo syringe. A. photo by D. Lawrence, B. photo by K. Lorenzen.

Peng syringe were designed for semen storage as well as routine inseminations. The large capacity syringes allow many queens to be inseminated with a single semen load. Varying amounts of semen may be collected depending on the size of the capillary tube used. The maximum capacity of the Harbo syringe is 200 *ul* (enough to inseminate 25 virgins with 8 *ul* each), and the Kaftanoglu-Peng syringe holds up to 150 *ul*. The process of semen collection and insertion can by separated with the use of a large capacity syringe. Semen may be collected and conveniently held overnight or for several days. When collecting a large quantity of semen, the inseminator must be extra careful to avoid contamination with fecal material. This can cause considerable loss of queens.

Collecting a large amount of semen is a tedious and time consuming job. The washing technique, developed by Kaftanoglu and Peng (1980b), is a simple fast method of semen collection without the use of a microscope. Semen and mucus are scraped into a funnel containing diluent. The mixture is centrifuged to separate the semen from the mucus in a capillary tube, which is then placed in a large capacity syringe to inseminate virgins. The washing technique was reported not to affect the quality of brood, though it was found to delay onset of oviposition (egg laying). A portion of the seminal plasma is removed during the washing process, possibly this is responsible for the delayed oviposition. This question is presently being investigated. The perfection of this technique offers practical application in bee breeding programs. Semen of different sources can be mixed homogenously (uniform dispersal) or at a desired ratio to produce consistent and improved quality stock.

Dr. Christine Peng, University of California, Davis. photo by K. Lorenzen.

The improved design of equipment and handling techniques for semen storage make it seem practical. A method of long-term storage in which honey bee spermatozoa retain high viability has proved a more difficult and complex task, compared to mammalian semen storage. A mammal is inseminated with a large number of sperm in which only one or a few viable sperm are necessary to immediately fertilize one or a few eggs. A queen bee must store 3 to 6 million sperm to continuously fertilize many eggs over several years. During the peak season, a queen will lay between 1500 and 2000 eggs per day. Therefore, the number of sperm that survive storage is much more critical compared to mammalian sperm. A queen inseminated with stored semen must receive a very high percentage of viable sperm to be able to head a productive colony.

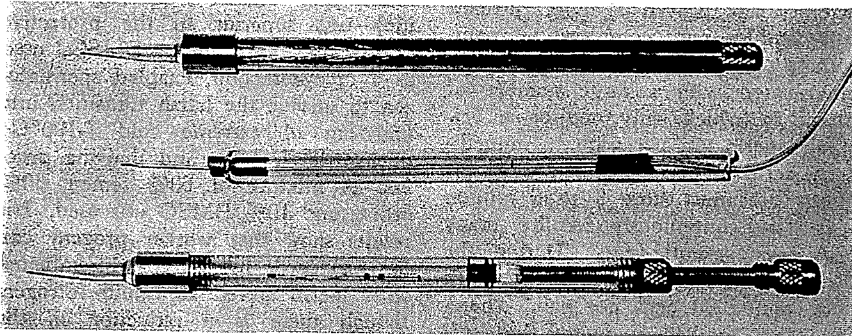
A practical method of storing cattle semen in liquid nitrogen has been used for over 20 years. Several researchers have attempted to apply similar methods to the storage of honey bee semen. Development of a suitable diluent to protect honey bee spermatozoa against the harmful effects of freezing has become a frustrating and complicated task. Millions of sperm must remain viable for several years in the queen's spermatheca after it has been recovered from storage. Glycerol has been used as a protective agent for storage of mammalian sperm at low temperatures. Swada and Chang (1964) reported some success with diluted semen containing glycerol stored at -79°C. They were also able to store semen in the seminal vesicles and in the spermatheca at -79°C. Melnickenko and Vavilov (1975) used drone haemolymph as a protective diluent for storage of semen in liquid nitrogen at -196°C. Verma (1973, 1978) investigated the storage of sperm in different diluents, and showed that osmotic pressure plays a role in control of sperm motility and longevity. He inseminated queens with stored semen which showed no reduction in motility and reported that few sperm reached the spermatheca.

Queens inseminated with semen stored in liquid nitrogen produced fewer eggs, and laid unfertilized eggs in worker cells. Unmated queens and queens inseminated with dead sperm are also known for reduced and nonuniform egg laying (Harbo, 1976). The queen controls fertilization by release of sperm from the spermatheca. Possibly the effects of storage cause a disruption in the egg fertilization process and result in irregular laying behavior.

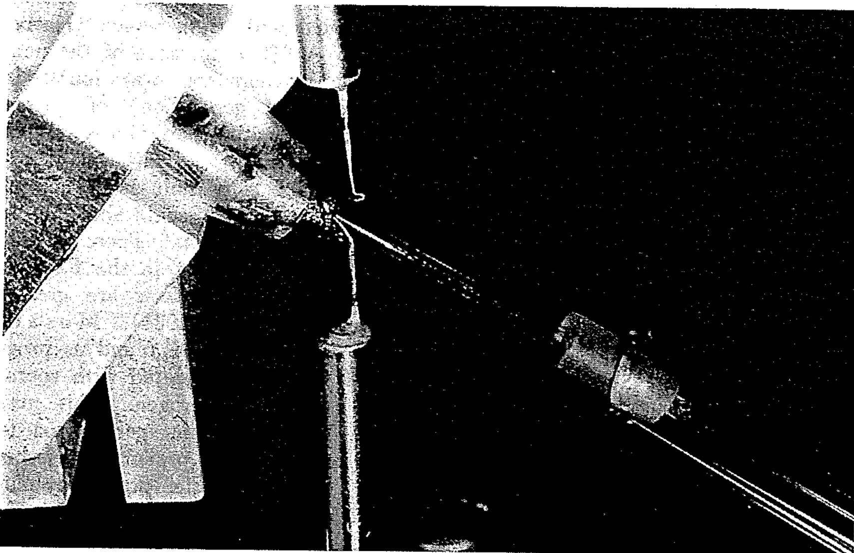
A technique to preserve honey bee semen in liquid nitrogen was developed by Dr. John Harbo (1977). He found that DMSO (dimethyl sulfoxide) was superior to the protective agents previously used. DMSO, used as a cryoprotectant, enabled spermatozoa to survive the harsh transition into and out of extreme cold (-196°C) during the freezing and thawing process. A mixture of 60% semen, 30% saline, and 10% DMSO was used. His results show that worker progeny can be produced by queens inseminated with sperm stored in liquid nitrogen, though there is a reduction in the number of sperm that reach the spermatheca (Harbo, 1977). Queens produced a high proportion of unfertilized eggs (drones), and had a lower egg hatch rate than control queens inseminated with fresh semen (Harbo, 1979a). A 3% occurrence of the nonhatching phenomenon was found in daughters (F1 generation) of queens inseminated with spermatozoa stored in liquid nitrogen (Harbo, 1979b). This may be a possible indication of genetic damage that could show up in subsequent generations. There is evidence this may persist in the F2 generation, if so the effects are greatly diminished (Harbo, 1981). Mosaic drone honey bees were found as progeny of queens inseminated with sperm stored in liquid nitrogen. This may be an inability of the spermatozoa to unite with the egg pronuclei (Harbo, 1980). Both pronuclei produced haploid tissue independently resulting in 2 types of sperm being produced by a drone which normally produces only one type of sperm. There are various factors that may cause mutations and egg death. Harbo suggests further research and progeny testing are needed before storage of honey bee semen in liquid nitrogen can be safely used.

Variations of the freezing procedure, diluents, and the chemical treatments used need to be evaluated in terms of viability and fertility of stored semen. Kaftanoglu and Peng (1983), using DMSO as a cryoprotectant, tested 11 different diluents for semen storage. They were able to store semen up to a year in liquid nitrogen. Queens inseminated with stored semen were reported to produce an average of 47%

Syringe tip comparison. Top — Glass tip. The tip is tapered and angled with a smaller outside diameter which makes it easier to bypass the valve fold. Bottom — Mackensen plastic tip. photo by K. Lorenzen.



Different syringe designs for I.I. Top — Mackensen syringe with plastic tip. Syringe must be reloaded between each insemination. Middle — Harbo large capacity syringe with glass tip. Bottom — Kaftanoglu-Peng large capacity syringe with adapted plastic tip. photo by K. Lorenzen.



Insemination of a virgin using the glass tip. photo by K. Lorenzen.

worker progeny, with some queens producing as much as 75% worker brood. Several queens inseminated with stored semen were superseded by their daughters, which naturally mated and built up strong colonies. Harbo, Kaftanoglu and Peng agree that we now have the capability to store genetic material in liquid nitrogen. Queens can be reared from progeny of frozen sperm, though their brood production is not sufficient to maintain a productive colony.

Sperm stored in liquid nitrogen has a low migration efficiency. Bolten and Harbo (1982) reported a significant increase in the number of sperm reaching the spermatheca when queens were given multiple inseminations with small volumes of diluted semen. They suggest queens I.I. with stored semen should be given this treatment until an improved or better technique is developed for long term semen storage.

An alternative method of semen storage is being studied by Dr. Christine Peng and her graduate student, John White. They are exploring the possibility of developing an artificial spermatheca for long term sperm storage. An attempt to determine what conditions are required for the longevity of spermatozoa in the spermatheca are being investigated. It may be possible to duplicate or simulate the environment of the spermatheca for practical semen storage.

A method of long-term honey bee semen storage, which results in high brood viability and is practical and usable by the commercial beekeeper, has yet to be developed. If this is pursued with as much persistence and determination as was required to develop a technique for I.I., it will be accomplished. When we look at the history of instrumental insemination, we certainly had many frustrations and misconceptions to overcome. The direction of apiculture research today is very important to the future of our beekeeping industry. We must look at the potential problems facing us as a challenge, and find solutions. We need to use the knowledge that is now available, and support further research. This means taking the scientific theory and current research developments out into the bee yards.

Acknowledgement: Special thanks to Ken Lorenzen for his photography and encouragement, and to Timothy Lawrence (my partner and husband) for his support, criticism, and suggestions in this series of articles.

LITERATURE CITED

Bolten, A. B. and J. R. Harbo 1982. Numbers of spermatozoa in the spermatheca of the queen honey bee after multiple inseminations with small volumes of semen. J. Apic. Res. 21(1): 7-10.

Harbo, J. R. 1974. A technique for handling stored semen of honey bees. Ann. Entomol. Soc. Amer. 67:191-194.

.... **1976.** The effects of insemination on the egg-laying behavior of honey bees. Ann. Entomol. Soc. Amer. 69:1036-1038.

.... **1977.** Survival of honey bee spermatozoa in liquid nitrogen. Ann. Entomol. Soc. Amer. 70:257-258.

.... **1979a.** Egg hatch of honey bees fertilized with frozen spermatozoa. Ann. Entomol. Soc. Amer. 72:516-518.

.... **1979b.** Storage of honey bee spermatozoa at -196°C. J. Api. Res. 18: 57-63.

.... **1980.** Mosaic male honey bees produced by queens inseminated with frozen spermatozoa. J. Hered. 71: 435-436.

.... **1981.** Viability of honey bee eggs from progeny of frozen spermatozoa. Ann. Entomol. Soc. Amer. 74: 482-486.

Jaycox, E. R. 1960. The effect of drying and various diluents on spermatozoa of the honey bee. J. Econ. Entomol. 53 (2):266-269.

Kaftanoglu, O. and Y. S. Peng 1980a. A new syringe for semen storage and instrumental insemination of queen honey bees. J. Apic. Res. 19(1):73-76

.... **1980b.** A washing technique for collection of honey bee semen. J. Apic. Res. 19(3):205-211.

.... **1983.** Preservation of honey bee spermatozoa in liquid nitrogen. submitted for publication.

Laidlaw, H. H., J. K. Poole and S. Taber III 1977. Test for mutation possibly induced by in vitro storage of honey bee sperm. J. Hered. 68:199-201.

Melnichenki, A. N. and Y. L. Vavilov 1975. Long term storage of drone sperm by freezing in liquid nitrogen. Proc. 25th International Congress of Apimondia, Grenoble, France. 311-314.

Nolan, W. J. 1929. Beekeepers of many nations meet. Glean. Bee Cult. (57): 640-642.

Poole, K. and S. Taber III 1969. A method of in vitro storage of honey bee semen. Amer. Bee J. 109:420-421.

.... **1970.** In vitro preservation of honey bee semen enhanced by storage at 13-15°C. Ann. Entomol. Soc. Amer. 63:1673-1674.

Sawada, Y. and C. M. Chang. 1964. Tolerance of honey bee sperm to deep freezing. J. Econ. Entomol. 57(6):891-892.

Taber, S. III and M. S. Blum 1960. Preservation of honey bee semen. Science 131(3415):1734-1735.

Taber, S. III 1961. Successful shipment of honey bee semen. Bee World 42: 173-176.

Verma, L. R. 1973. Osmotic analysis of honey bee semen and haemolymph. Amer. Bee Jour. 113(11):412.

.... **1978.** Biology of honey bee spermatozoa. Apidologie (93):167-174.

Woyke, J., F. Ruttner and V. Vesely 1966. Manual on artificial insemination. Preliminary ed. International course on A.I. Dol., CSSR.

Reprinted from May, 1983, American Bee Journal
Vol. 123 (5): 389 - 395