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STORAGE OF HONEYBEE SPERMATOZOA AT —196°C[1,2]

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Summary

A method for storing honeybee spermatozoa in liquid nitrogen is described. A hydraulic syringe attached to a meter accurately measures collection, dilution, and delivery of semen, and a thermocouple inserted into the semen records the rates of freezing and thawing. From collection to insemination the storage process lost only 7% of the semen mixture.

A mixture of 60% semen, 10% dimethyl sulphoxide (DMSO) and 30% saline was the most successful for storage. Glycerol gave some protection as a cryoprotectant, but was inferior to DMSO. After semen was stored with 10% DMSO and 30% water, more spermatozoa moved to the spermatheca than after storage with 10% DMSO and 10% or 20% water.

Introduction

Spermatozoa of the honeybee *Apis mellifera* survived storage at – 196°C, the temperature of liquid nitrogen (Melnichenko & Vavilov, 1976; Harbo, 1977). I have used such frozen spermatozoa for over 50 inseminations that produced worker brood. However, most queens produced more drone than worker brood. Thus progress has been made, but a perfect system for storing honeybee spermatozoa in liquid nitrogen has not yet been developed.

Three variables that need special consideration when freezing spermatozoa are the freezing rate, the chemicals that are mixed with the semen, and the ratio between amounts of semen and diluent. For routine storage and especially during experimentation, these variables must be measured accurately, so I developed a system for metering semen and monitoring temperature changes.

This paper describes the basic techniques used to measure the freezing rate, the dilution ratio, and the insemination dosage, and reports on the dilution, freezing rate and insemination of bee spermatozoa.

Materials and Methods

Equipment

Because of the need for precision, I redesigned an earlier syringe (Harbo, 1974); the glass insemination tip, however, was merely shortened to make room for a storage tube. The new syringes meter the semen and diluent as they are collected and expelled, thus eliminating the need to calibrate tips or storage tubes. Two syringe meters are shown in Fig. 1. The meter in Fig. 1*a* is simpler to use and costs a third as much as the meter in 1*b* which is more accurate, and therefore recommended for research work. The design of the syringe barrel, storage tube, and tip is shown in Fig. 2.

The length and diameter of the storage tube, between the tip and the syringe, can vary. Its length is limited by the distance from the end of the syringe holder to the queen holder on the Mackensen insemination stand. Therefore during (but only during) insemination, the combined length of the storage tube and tip must be less than 60 mm.

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[1]In co-operation with Louisiana Agricultural Experiment Station.
[2]Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

Allowing about 20 mm for the tip, 40 mm is the limit for a storage tube. It is possible to use storage tubes as long as 60 mm by moving the column of semen into the tip just before insemination, and then breaking off the basal end of the storage tube.

When the meter in Fig. 1*b* is used, the micrometer must be calibrated to measure the absolute volumes that are collected. With a 250-μl Hamilton syringe mounted in the microburette, 1 μl corresponded to about 9·4 units. During use, however, it is easier to work directly with units than to convert constantly to μl.

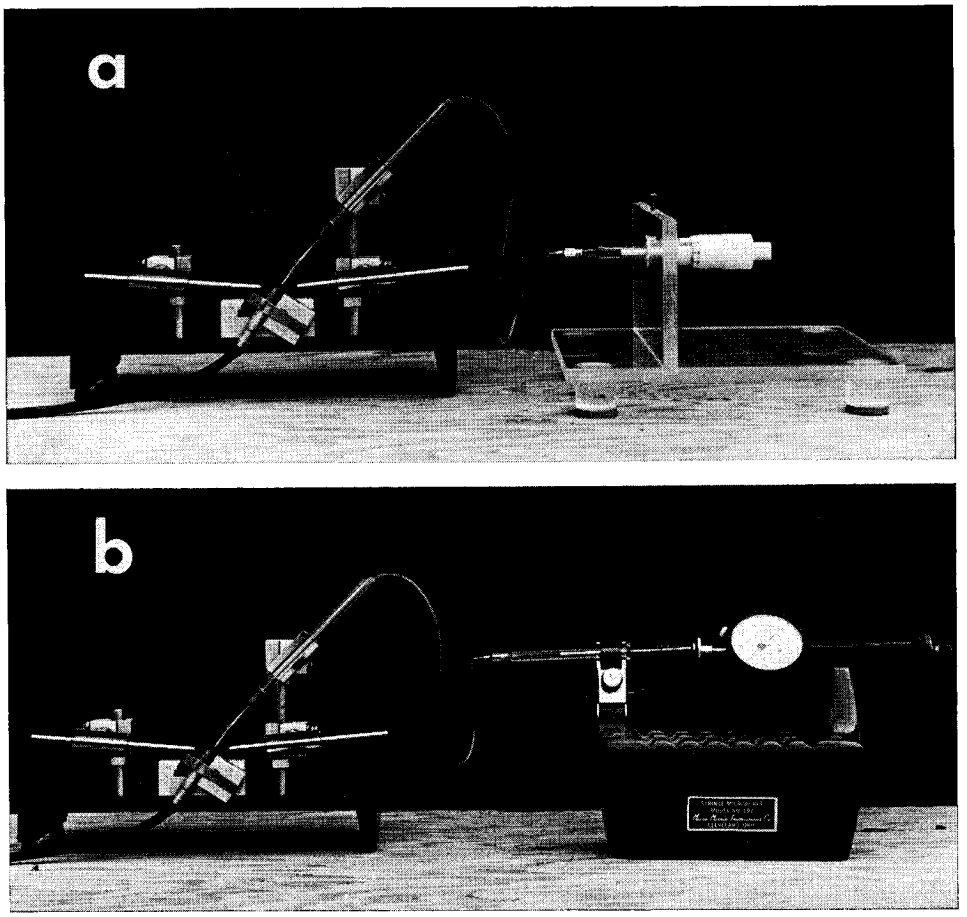

FIG. 1. Two metered systems for semen collection and insemination.

FIG. 1*a*. Gilmont syringe mounted on a plexiglass stand and connected to the syringe barrel in Fig. 2.

FIG. 1*b*. Microburette fitted with a 250-μl laboratory syringe and connected to the syringe barrel in Fig. 2. A spring pulls the plunger out, keeping it in contact with the micrometer. A small amount of fine oil around the plunger prevented air bubbles entering the syringe during suction.

Collecting the semen

The first step was to fill completely the syringe storage tube and tip with boiled saline. No air bubbles should remain in the tubing or syringe, for this would reduce the responsive suction. Boiled saline was used because it does not generate air bubbles in the

system. A 3-μl air space was established between the saline and the incoming semen, and prior planning ensured that the meter reading was precisely 800 or 900 units (syringe, Fig. 1*b*) or 200 μl (Fig. 1*a*) when collection began.

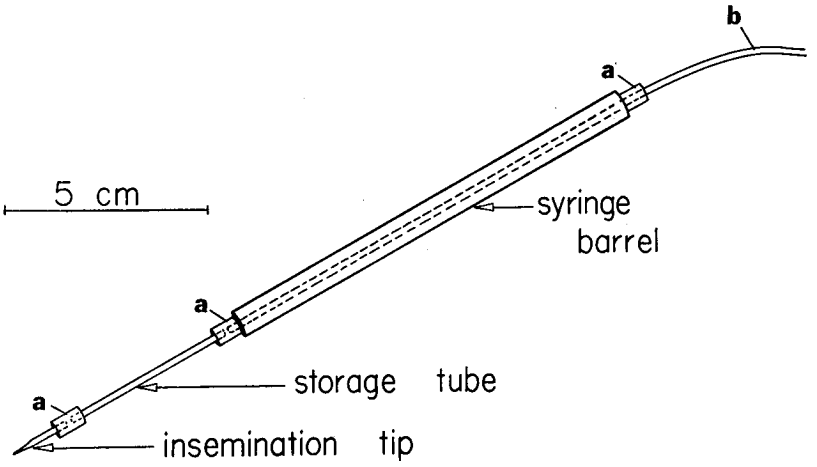

FIG. 2. Syringe barrel, storage tube and tip.

The tip is a glass capillary tube drawn to a point, cut at an angle, ground, and fire-polished (Harbo, 1974). Internal and external diameters (ID, OD) at the point are about 0·18 and 0· 3mm.

a. Sections of latex tubing (ID 1·2, OD 4·5 mm) that hold the capillary tubes together and keep tube *b* in position.

Syringe barrel. Section of glass tubing with OD 7 mm to fit the Mackensen insemination stand and ID 2 mm, slightly larger than that of tube *b*.

b. Tube of polyvinyl tubing (Tygon) (ID 0·5, OD 1·5 mm), its end fitted onto a syringe needle that has been shortened and bent. Connection between needle and syringe tightly sealed with stopcock grease.

Assuming the use of syringe 1*b*, the collection procedure involved drawing in 12 units of semen, then 8 units of dimethyl sulphoxide (DMSO) saline, 12 units of semen, and so on, 8, 12, 8 . . . until the desired amount of semen was collected. The DMSO saline contained 25% DMSO and 75% saline (0·85% NcCl), so the resulting mixture of semen and saline contained 60% semen, 10% DMSO and 30% saline. When the required amount of material had been collected (up to 50 μl), it was further mixed either by moving it back and forth in the storage tube or by depositing it in a small container, stirring with the tip, and recollecting.

The storage tube containing semen was then disconnected from the tip and the syringe, and sealed with a petrolatum (petroleum jelly) plug at each end. To establish the plug, the semen was moved to one end of the tube by pressure from the syringe, or by mouth pressure with a micropipette tube and mouthpiece. This end was then pushed through a mound of petrolatum so that a plug of it collected inside. Then, the column of semen, followed by a plug, was pushed to the other end, and a similar plug was established there. No air remained between the plug and the semen (Harbo, 1973, 1974).

Freezing the semen mixture

A thermocouple was inserted through one of the petrolatum plugs and into the semen mixture. I used thermocouples of no. 36 copper-constantan or no. 30 chromel-alumel

wires; the thinner wire (no. 36) was better. The thermocouple and tube were then put into a 28-cm plastic tube that was sealed at the bottom and labeled at the top (Fig. 3). The outside tube, in turn, was put into an open canister and lowered into the tank of liquid nitrogen.

A recorder with a 10-mV span recorded temperatures from the thermocouple throughout the range from ambient to −196°C. By setting the chart to move at a convenient rate such as 1 cm/min, I monitored and controlled the freezing curve by controlling the rate at which the canister was lowered into liquid nitrogen.

To thaw the semen mixture, the plastic tube was quickly pulled from the nitrogen tank, inverted, and tapped on the table to expel the capillary tube of semen. The tube was immediately picked up and warmed between the fingers, giving a thawing time of about 12 sec or a rate of 1100°C/min.

The tube was then reattached to the syringe for insemination. After connecting it with the syringe barrel, I moved the column of material in the storage tube forward so that the plug at the distal end could be easily removed. The tip was then attached, and the column of semen, followed by the basal plug, was moved into the tip ready for insemination (Harbo, 1973). If the storage tube was too long, it was cut where the petrolatum plug met the saline from the syringe.

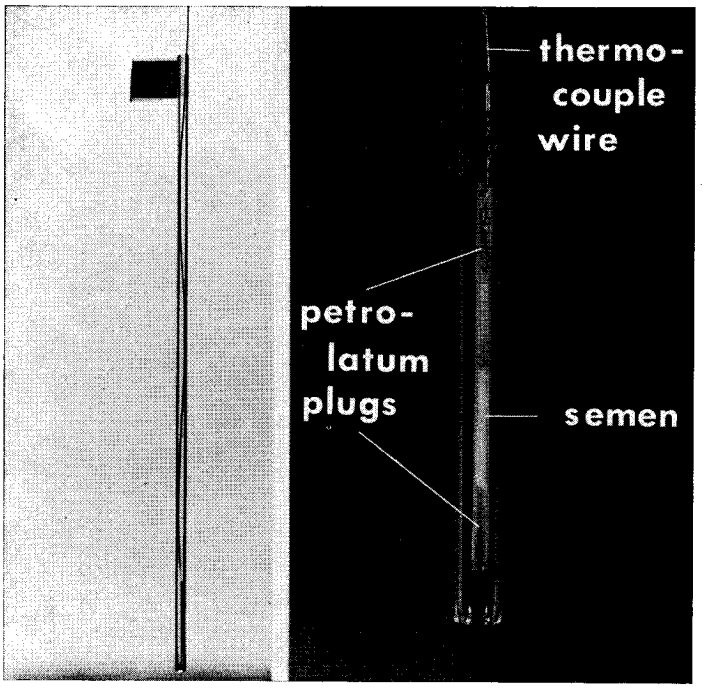

FIG. 3. Tube of semen within a larger tube, as inserted into the tank of liquid nitrogen; it was large enough to handle and find in the tank, and it also served to label the semen.

The outside tube was sealed at the bottom to keep the tube of semen from dropping out; this insulated the tube of semen from direct contact with the liquid nitrogen, which may or may not have been significant.

Experimental design

When testing freezing rates, cryoprotective chemicals, or dilution ratios, I used one basic experimental design. A group of sister queens were numbered and stored (caged) in a queenless colony. If there were three tubes of semen to test, I randomly established the following : three groups of 6 or 7 queens, an insemination sequence within each group, and an insemination sequence for the three tubes. All queens were then inseminated with equal amounts of semen.

Results for the inseminated queens were analysed by measuring the worker-drone brood ratio, or by counting the number of spermatozoa that reached the spermatheca, or both. Sperm counts were made with a haemacytometer. The worker-drone brood ratio was measured after 19 days or more, by uncapping the cells on a frame and counting the worker and drone pupae. This gives only a rough estimate, because drone mortality in worker cells can be high (Tucker, 1978), and because the age and duration of worker and drone pupation are not the same.

Results and Discussion

Efficiency

With this method of storing spermatozoa, the average loss of volume was 6·6% (SD 2·4%). This was calculated by comparing the amount of semen collected with the amount that a storage tube yielded during insemination. Such a high efficiency was caused largely by the petrolatum plug that separates the semen from the saline in the syringe; in addition to preventing the semen from mixing with the saline, it reduced the number of spermatozoa that were left on the side of the capillary tube.

Cryoprotective agents and diluents

Dimethyl sulphoxide (DMSO) was used almost exclusively as the cryoprotective additive. All concentrations from 8% to 15% gave some success, but 10% DMSO was used most frequently. Without the freezing step, semen containing 10% DMSO and 30% saline did not differ from controls in the number of spermatozoa that reached the spermatheca (Harbo, 1977).

TABLE 1. Effect of diluting semen before storage in liquid nitrogen (cooling rate 18°C/min). The insemination volume was adjusted to give each queen the equivalent of 2½ μl semen.

Storage dilution	*No. queens inseminated*	*Mean no. spermatozoa per spermatheca*
10% DMSO, 10% water	7	0
10% DMSO, 20% water	8	7 500
10% DMSO, 30% water	10	35 500

Dilution of the semen seemed beneficial. In an experiment where the semen was diluted with 10% DMSO and 10, 20 or 30% water before storage, the queens inseminated with the most dilute semen retained the most spermatozoa in the spermatheca (Table 1).

When 10% glycerol was used in place of DMSO as a cryoprotectant, some spermatozoa did survive the extreme cold. However, in a test comparing DMSO with glycerol, DMSO was markedly superior (Table 2). The 0·5% of worker brood that was protected with glycerol represents one worker produced by one queen. The worker was normal-eyed, and the queen was homozygous snow-eyed, mated to wild-type sperm that had been stored in liquid nitrogen, so it is very likely that the worker came from this latter sperm.

Use of DMSO and glycerol together did not produce any further benefits. A final dilution, using 8% DMSO, 8% glycerol and 30% saline with 50% semen, was inferior to the usual 10% DMSO, 30% saline and 60% semen, when frozen at 13°/min.

TABLE 2. Comparison of dimethyl sulphoxide and glycerol as cryoprotective agents. The final storage mixtures were 60% semen, 30% saline (0·85% NaCl), and 10% DMSO or glycerol. The cooling rate was 10°/min to 0°C, 40°/min between 0 and −60°, and 15°/min to −196°.

Dilution	*No. queens*	*Mean no. spermatozoa in spermatheca ± SD*	*% Worker brood*
10% DMSO	5	193 000 ± 51 000	31%
10% glycerol	3	12 000 ± 8 500	<0·5%

Freezing rate

Sperm survived freezing at many rates, and successful inseminations were produced from average rates of 40° to 4°/min. However, a freezing rate was seldom constant. A typical freezing required 9 min, an average rate of 25°/min. Spermatozoa did not survive a freezing rate as rapid as 300°/min.

Thawing rate

Rapid thawing has been reported generally as advantageous for animal and plant cells (Mazur, 1977). If thawing is slow, intracellular ice crystals can grow or agglomerate, and cause cell damage (Mazur, 1966). Hence I used a standard rapid thawing rate, about 1100°/min.

Insemination results

Very diverse results were often obtained from sister queens inseminated with semen from one tube. For example, semen in a tube containing 60% semen, 10% DMSO and 30% saline, and frozen at 13°/min, was used to inseminate the 6 sister queens in Table 3. The variation here is typical, although the percentage of workers produced was higher than usual.

TABLE 3. Inseminations from a single tube containing 60% semen, 30% saline (0·85% NaCl), 10% DMSO (cooling rate 13°/min).
Spermatozoa were counted after the queen had laid 2 or 3 frames of eggs.

Queen no.	*Insemination volume (μl semen)*	*% worker brood*	*No. spermatozoa in spermatheca*
1	1·1	55%	602 000
2	2·2	98%	552 000
3	2·2	11%	69 000
4	2·2	8%	73 000
5	2·2	24%	89 000
6	1·6	0	0

Conclusions

Stored spermatozoa could be used in two ways. The first and more limited way is solely to maintain stock or store germplasm, survival of spermatozoa being the only concern. The queens inseminated with spermatozoa stored in liquid nitrogen would perform well enough to produce queen progeny and thus to propagate a line, but perhaps not well

enough to head a productive colony. The second way, of much wider application, is to inseminate queens with frozen spermatozoa so that they will perform as well as queens inseminated with fresh spermatozoa. If this could be achieved, the use of frozen spermatozoa could be expanded vastly, into commercial queen-rearing operations that offer a multitude of hybrid combinations at nearly any time of year.

As yet the queens inseminated with spermatozoa stored in liquid nitrogen have not performed well enough to head large honey-producing colonies : the capability at the second level has not been achieved. It is possible, however, to produce female progeny consistently from a tube of semen stored in liquid nitrogen. It usually requires the insemination of more than one queen to produce larvae for grafting (e.g. Table 3), but mutant markers as well as other germplasm can be stored in liquid nitrogen. I do not recommend the system for germplasm storage because spermatozoa have not been stored in liquid nitrogen for more than 3 months, nor has the F_1 progeny been tested adequately.

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