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Survival of Honey Bee (Hymenoptera: Apidae) Spermatozoa After Two Years in Liquid Nitrogen (−196°C)[1]

JOHN R. HARBO

Bee Breeding and Stock Center Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Baton Rouge, Louisiana[2]

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ABSTRACT Genetic markers showed that spermatozoa of the honey bee, *Apis mellifera* L., can produce progeny after storage for 2 years at −196°C. Progeny counts indicated that a loss of viability may have occurred between 4 days and 2 years of storage. Nine queens inseminated with spermatozoa stored 4 days produced 22% worker brood (range = 8–55%); eight queens inseminated with spermatozoa stored 2 years produced 8% worker brood (range = 1–25%) ($P < 0.05$).

Spermatozoa of honey bees, *Apis mellifera* L., have survived storage in liquid nitrogen (Melnichenko and Vavilov 1976, Harbo 1977, 1979). If this method is to be practical, one must know the effect of time in storage on the survival of the spermatozoa.

Materials and Methods

On 7 May 1980, semen was collected into capillary tubes (ID = 1.1 to 1.2 mm), in a 3:2 ratio (semen-diluent). The diluent consisted of 25% dimethyl sulfoxide (Me_2SO), 25% egg yolk, and 50% buffer solution at pH 7.2 (1.1 g of $NaH_2PO_4 \cdot H_2O$ and 0.845 g of $NaHPO_4 \cdot 7H_2O$ and 0.25 g of dihydrostreptomycin sulfate in 100 ml total water solution). When 22 μl of diluted semen was in each tube, it was sealed with petrolatum (Harbo 1979) and stored in a refrigerator (5°C).

The following day all tubes were removed from the refrigerator to liquid nitrogen. Cooling rate (3°C/min) was monitored by a thermocouple in a capillary tube of diluent sealed in the same manner as the other tubes (Harbo 1979).

After each storage interval (4 days; 5, 11, and 24 months) at least 2 tubes of semen were removed from storage, thawed, and used to inseminate 10 to 12 queens (5 or 6 per tube). I rapidly thawed each tube by rolling it between my fingers after removal from liquid nitrogen. With this method, the semen reached room temperature in about 12 sec. Each queen received 3.3 μl of diluted semen.

The matings were evaluated by measuring the percent female progeny from each queen and estimating the number of spermatozoa in their spermathecae. After insemination, each queen laid eggs in a small colony for about 2 weeks. Spermatozoa in their spermathecae were then counted with a hemacytometer. When the brood pupated, the worker and drone pupae in 100 worker-sized cells were counted. These cells normally contain workers, so the presence of drone pupae indicates that the eggs had not been successfully fertilized (normal drones develop from unfertilized eggs; workers and queens develop from fertilized eggs).

Table 1. Evaluation of inseminations made with semen stored in liquid nitrogen for varying periods of time[a]

Period in storage (−196°C)	No. of queens	Thousands of spermatozoa in spermatheca ($\bar{x} \pm SD$)	% Worker brood produced	
			$\bar{x}$	Range
4 days	9	134 ± 59[b]	22	8–55a[c]
5 mo	8	182 ± 209	12	0–40a,b
11 mo	8	212 ± 135	14	1–44a,b
24 mo	8	101 ± 62	8	1–25b

[a]Each insemination included 2 μl of semen (ca. 15 × 10^6 cells). Semen was diluted 3:2 (semen-diluent) and stored in May 1980.

[b]Analysis of variance indicated no significant differences among the four groups ($P < 0.05$).

[c]Percent worker brood was evaluated with the Kolmogorov-Smirnov two-sample test. Ranges followed by different letters are significantly different at the 0.05 level.

Mutant eye markers were used to prove parentage. The single gene recessive markers snow (*s*) in the queen and tan (*s'*) in the stored spermatozoa are allelic. When homozygous they are white and tan, respectively; when heterozygous (*s*/*s'*), they are red eyed (Laidlaw et al. 1964). Thus, the appearance of red-eyed workers or queens proves that the progeny were from eggs fertilized with the stored spermatozoa and not parthenogenetic females.

Results and Discussion

After every test interval, red-eyed workers were found in the progeny of queens inseminated with the stored spermatozoa. This proved that frozen spermatozoa produced adult progeny. At the 2-year interval, red-eyed queens were also reared to show that germplasm can be perpetuated after 2 years in storage.

Two years of storage caused a significant drop in the percent female progeny. This difference was found only between spermatozoa stored 4 days and those stored for 2 years ($P < 0.05$). No other significant difference were found (Table 1). These results are similar to results with bovine spermatozoa, which began to show a decline in viability after 12 months in liquid nitrogen (Salisbury and Hart 1970).

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[2]Postal address: Bee Breeding and Stock Center Laboratory, Route 3, Box 82-B, Ben Hur Road, Baton Rouge, LA 70808.

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