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Bioassay for Diluents of Honey Bee Semen[1]

JON L. WILLIAMS AND JOHN R. HARBO

Bee Breeding and Stock Center Laboratory, ARS, USDA, Baton Rouge, Louisiana 70808

ABSTRACT

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A bioassay was developed to evaluate diluents of honey bee (*Apis mellifera* L.) semen. Using a simple diluent (1.1% NaCl, 0.1% glucose, 0.01% each L-arginine HCl, L-glutamic acid, L-lysine, 0.02% dihydrostreptomycin sulfate, and 0.012% penicillin G sodium in 0.05 M Tris buffer at pH 8.7), 100-fold dilution, incubation at 35 ± 1°C for 10 min, and reconcentration did not reduce the number of spermatozoa that reached the spermathecae of instrumentally inseminated queens. Undiluted control semen had a 10.9% transfer efficiency when 15×10^6 spermatozoa were injected into the oviducts of each queen. Treated groups of semen incubated for 0.17, 1.5, 3.0, 4.5, 6.0, 7.5, and 19.5 h had transfer efficiencies of 10.7, 8.8, 9.8, 5.1, 2.1, 4.3, and 2.4%, respectively.

Several diluents are used routinely in minute quantities for instrumental insemination of honey bees to lubricate the queen's reproductive tract and to prevent the viscous semen from obstructing the insemination tip during collection and insemination (Laidlaw 1976, Mackensen and Tucker 1970, Ruttner 1976). Spermatozoa thus exposed to diluents exhibit no apparent damage. Conversely, holding diluted semen for more than brief periods alters the quality of the spermatozoa or seminal plasma. For example, Verma (1978) reported that spermatozoa from semen mixed with various diluents and stored for 60 to 90 days had a markedly reduced ability to migrate to the spermathecae of instrumentally inseminated queens. Harbo (1979a,b, 1980) observed that low-temperature (–196°C) storage of honey bee semen diminished the reproductive capacity of instrumentally inseminated queens and resulted in (1) decreased sperm transfer, (2) reduced egg hatch rate, (3) decreased percentage of worker brood, and (4) male mosaicism. These anomalies may be partially related to suboptimal diluents. Also, the washing of honey bee semen described by Kaftanoglu and Peng (1980) delayed the onset of oviposition in instrumentally inseminated queens.

Preliminary observations by one of us (Williams, unpublished data) indicated that spermatozoa sometimes maintained near-normal patterns of mortality for ca. 20 h when honey bee semen was diluted 100-fold in a 1.11% NaCl-based medium and incubated at 35°C. In these tests, various diluent formulations were assessed by microscopically ranking the sperm motility of hanging drop preparations of incubated semen. However, use of the technique was discontinued because of the great variation in sperm motility among replicates of incubated semen.

To design a bioassay, we selected only the percentage of inseminated spermatozoa reaching the queens' spermathecae (transfer efficiency) as the measure of success. Although desirable, progeny tests were excluded from the bioassay because of their logistical requirements (i.e., a colony of worker bees for each test queen and ca. 30 days for results). We chose to reserve progeny tests for diluents which the bioassay indicates as having special merit.

Materials and Methods

Semen donors throughout the study consisted of wild-type, free flying drones taken at the entrances of colonies in several apiaries and stored 3 to 7 days in a populous nursery colony supplied continuously with pollen supplement and sugar syrup. The entire lot of semen needed for an experiment was collected in a sterile glass capillary tube (Corning 7099-S, total capacity ca. 185 μl), expelled into a small glass cone, mixed with a fine glass rod, and then recollected in a duplicate tube. Semen was held overnight at 14 to 15°C (Poole and Taber 1970) in capillary tubes sealed with petrolatum. The apparatuses designed by Harbo (1979b) were used for collecting semen and instrumentally inseminating queens. The diluent used in this study was modified from Verma (1978) as follows: 1.11% NaCl, 0.1% glucose, 0.01% each L-arginine HCl, L-glumatic acid, and L-lysine, 0.02% dihydrostreptomycin sulfate, 0.012% penicillin G sodium; all in 0.05 M Tris buffer (hydroxymethyl)-aminomethane at ca. pH 8.7. Inorganic chemicals and glucose, reagent grade; other organic chemicals, Sigma Chemical Co., St. Louis. Solutions were prepared on the day of use with glass-distilled water.

After reserving one fraction of semen for the control matings, equal fractions of semen were diluted 100-fold (generally 40 μl of semen and 3,960 μl of diluent) in sterile 15-ml plastic centrifuge tubes (Falcon 2095), gently shaken 25 times, and incubated, on a staggered schedule, in a water bath maintained at 35 ± 1°C. Suspensions of incubated semen were transferred to sterile 3-ml conical glass tubes (Corning 8060) sealed with Parafilm, and centrifuged at 12,100 × *g* for 10 min at ca. 12 to 14°C in a Sorvall RC-2B centrifuge. After discarding the supernatant, the pelletized material (spermatozoa plus insoluble fraction of seminal plasma) was stirred and collected in the insemination apparatus, and the numbers of spermatozoa per unit of volume were determined

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Table 1.—Number of spermatozoa in the spermathecae of queens after instrumental insemination with ca. 15 × 10^6 cells incubated from 0 to 19.5 h at 35 ± 1°C and 100-fold dilution[a]

Time (h) of semen incubation at 35 ± 1°C	No. of queens[b]	Spermatozoa (×10^6) in queens' spermathecae ($\bar{x}$ ± SD[c])	Vol of insemination (μl/queen)	Transfer efficiency (%)
0.0 (Control)	8*	1.28 ± 0.37	2.1	
	10**	1.77 ± 0.74	2.2	
	9***	1.92 ± 0.37	1.7	
Total (Control)	27	1.67 ± 0.58a		10.9
0.17	8*	1.61 ± 0.37a,b	2.0	10.7
1.5	7*	1.51 ± 0.28	3.4	
	10**	1.11 ± 0.54	3.4	
Total (1.5 h)	17	1.28 ± 0.49b		8.8
3.0	11**	1.47 ± 0.69a,b	4.0	9.8
4.5	10***	0.77 ± 0.40c	3.3	5.1
6.0	12**	0.32 ± 0.13c	3.4	2.1
7.5	11***	0.65 ± 0.22c	3.0	4.3
19.5	10*	0.36 ± 0.16c	3.3	2.4

[a]Pooled semen was held overnight at 14 to 15°C after mixing. One portion of each collection was used for insemination without further alteration (control); all other portions were diluted, incubated at the times indicated, and reconcentrated before insemination.

[b]*, Experiment 1; **, experiment 2; ***, experiment 3.

[c]Means followed by different letters are significantly different at $P < 0.01$, F = 19.76, df = 7 and 98.

spectrophotometrically (Harbo 1975).

Three tests were performed varying the incubation period as indicated in Table 1. The number of queens per treatment (7 to 12) varied by the mortality of virgin and mated queens in nursery colonies and amounts of incubated semen recovered. Sister queens were instrumentally inseminated at 14 to 15 days of age with ca. 15 × 10^6 spermatozoa per queen; all inseminations were performed by one of us (J.R.H.). Inseminated queens were returned to the nursery colony for a minimum of 48 h before the number of spermatozoa in each queen's spermatheca was counted with a hemocytometer. The data were analyzed by analysis of variance and a least significant difference test. Data from the three experiments were combined for analysis based on nonsignificant differences of the mean counts of spermatozoa from queens inseminated with nonincubated semen ($P < 0.05$).

Results and Discussion

An average sperm transfer efficiency of 10.9% was obtained for queens inseminated with nonincubated semen (Table 1). This is similar to the transfer efficiency of 12.4% reported by Mackensen (1964) for inseminations with 2 μl of freshly collected semen, assuming 7.5 × 10^6 spermatozoa per μl. Consequently, our mixing and overnight storage of undiluted semen had little or no effect on transfer efficiency.

In this study, transfer efficiency was not significantly reduced when semen was diluted 100-fold, incubated at 35 ± 1°C for 3 h or less, and reconcentrated (centrifuged). Incubation for periods of 4.5 h or longer significantly reduced the number of spermatozoa capable of migration to the spermathecae of instrumentally inseminated queens, but the mean values of spermathecal spermatozoa incubated for 4.5, 6.0, 7.5, and 19.5 h were not significantly different from one another. The data indicate that the decreased cell density of reconcentrated semen (1.6 to 1.8 times the volume of whole semen within experiments) had little or no effect on sperm transfer efficiency. Similarly, Mackensen (1964) found that diluting fresh semen with equal volumes of various diluents did not alter the transfer efficiency of honey bee spermatozoa.

The present diluent can be used as a base line to develop improved diluent formulations. Incubations of about 19.5 h should be used initially for screening purposes, whereas improved diluents likely will require longer semen-diluent exposures.

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