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NUMBERS OF SPERMATOZOA IN THE SPERMATHECA OF THE QUEEN HONEYBEE AFTER MULTIPLE INSEMINATIONS WITH SMALL VOLUMES OF SEMEN[1]

ALAN B. BOLTEN[2] AND JOHN R. HARBO
Bee Breeding and Stock Center Laboratory, ARS, USDA, Baton Rouge, LA 70808, USA

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Summary

Multiple inseminations of the queen honeybee with small volumes of diluted semen resulted in significantly more spermatozoa entering the spermatheca than did single inseminations of the same total volumes ($P < 0{\cdot}01$). In three experiments with small, equal insemination volumes, the second insemination contributed more spermatozoa to the spermatheca than did the first. For inseminations of semen that had been stored in liquid nitrogen (−196°C), the second insemination contributed 77% and 68% in two different experiments. With fresh diluted semen the second insemination contributed 61%.

Introduction

Mackensen and Roberts (1948), Mackensen (1964), and Woyke (1960, 1962), found that the number of spermatozoa reaching the spermatheca of the queen honeybee (*Apis mellifera*) increased as the volume of semen inseminated increased. However, with larger volumes, a lower percentage of the spermatozoa entered the spermatheca. Mackensen (1964) showed that there were more spermatozoa in the spermatheca after multiple inseminations than after a single insemination, when the same total volume of semen was inseminated. For example, more spermatozoa entered the spermatheca after two inseminations with 2 µl than after one insemination with 4 µl. Also, the queens receiving two inseminations showed a lower variance in the number of spermatozoa in the spermatheca. Woyke's (1960, 1962) results were similar. Both Mackensen and Woyke showed that the second insemination contributed fewer of the spermatozoa in the spermatheca than the first and that, as the total insemination volume increased, the relative contribution of the second decreased.

Our experiments were designed to test whether Mackensen's and Woyke's results with large volumes of semen were obtained with small volumes of both stored and fresh semen. The insemination apparatus designed by Harbo (1979) enabled us to make precise inseminations with small volumes. Earlier studies had shown that queens inseminated with spermatozoa stored in liquid nitrogen (−196°C) had relatively few spermatozoa in the spermatheca (Harbo, 1977, 1979). Our first experiment here was designed to determine if multiple inseminations with stored spermatozoa significantly increased the numbers of spermatozoa entering the spermatheca. Results from this experiment indicated that second and third inseminations with stored spermatozoa each contributed more spermatozoa to the spermatheca than the first. Therefore, a second experiment was done to test the effect of varying the volumes of the first and second inseminations, keeping the total volume constant. The third experiment analysed the relative contributions of the first and second inseminations when small volumes of fresh semen were used.

Methods and Materials

The genetically uniform queens used in each experiment were sisters, hybrids from a cross of two inbred lines. Each group of queens was grafted into a single cell-building colony, and emerged in an incubator (35°). Except when they were brought into the laboratory to be inseminated, the queens were always stored in colonies, caged in holes on boards holding 18 queens each. The storage colonies had no laying queen, and the stored queens were screened from the workers with wire mesh (⅛ inch, 3·2 mm).

Queens were inseminated at 7-10 days of age, using the apparatus designed by Harbo (1979). We collected semen from wild-type drones which had been stored in cages in queenless

[1] In co-operation with Louisiana Agricultural Experiment Station.
[2] Present address: Department of Zoology, University of Florida, Gainesville, FL 32611, USA.

colonies. Multiple inseminations were given at 24-h intervals. To equalize possible differences between storage tubes, the insemination sequences were randomized. Treatment groups that were inseminated only once were divided, so that some of the queens were inseminated on each of the days that multiple inseminations were given.

Spermatozoa in the spermatheca were counted 2-4 days after inseminations were completed. Counts were made using a haemacytometer except in experiment B, where a spectrophotometer was used (Harbo, 1975).

Differences between treatments were analysed using the *t*-test; $\alpha = 0{\cdot}05$. Coefficient of variation (CV) was used to compare variance between treatments.

Experiment A: Single versus multiple inseminations with stored semen

Semen was collected and mixed with a diluent using a fine glass stirring rod and small glass cone. Each 100 ml of the diluent consisted of 25 ml dimethylsulphoxide (DMSO), 25 ml fresh egg yolk, and 50 ml PO_4 buffer (1·1 g $NaH_2PO_4\,H_2O$, 0·845 g $NaHPO_4\,7H_2O$, 0·1 g dihydrostreptomycin sulphate, and water to make 100 ml of solution). The ratio of semen to diluent was 3 : 2 (v : v). This semen-diluent mixture was collected into capillary tubes, sealed with petroleum jelly, and then stored in liquid nitrogen (−196°) for 13-15 days (Harbo, 1979). Tubes of semen were thawed just before use. The test queens were given one of four treatments, as follows:

1. one insemination with 1·3 µl
2. two inseminations with 1·3 µl each
3. three inseminations with 1·3 µl each
4. one insemination with 3·9 µl.

Experiment B: Variations in the volumes of successive inseminations

Semen was collected and mixed with a diluent in a 1 : 1 (v : v) ratio, by collecting into capillary tubes alternately 1·0 µl of fresh semen and 1·0 µl of diluent. Each 100 ml of the diluent consisted of 20 ml DMSO, 20 ml fresh egg yolk and 60 ml PO_4 buffer. After the tubes were 75% filled in this manner, the contents were mixed by drawing the liquid up and down the tube. The tubes were then sealed with petroleum jelly and stored in liquid nitrogen as described above. The storage period was 1-2 days. The test queens were given one of four treatments, as follows:

5. one insemination with 2·2 µl
6. two inseminations with 2·2 µl each
7. two inseminations, 1·1 µl then 3·3 µl
8. two inseminations, 3·3 µl then 1·1 µl.

Experiment C: Single and multiple inseminations with fresh, diluted semen

Semen was collected and mixed with a diluent, using a fine glass stirring rod and small glass cone. Each 100 ml of the diluent contained 0·9 g NaCl and 0·25 g dihydrostreptomycin sulphate in water. The ratio of semen to diluent was 1 : 1 (v : v).

Relatively few spermatozoa were used in this experiment (equivalent to 0·35 µl of undiluted semen for the smaller insemination), so dilution was necessary in order to increase the precision of the insemination. The concentration of spermatozoa was checked with a haemacytometer on the day of each insemination. Thus, equal numbers of spermatozoa were injected, by slightly adjusting the insemination volume on the second day. The concentration of spermatozoa was approximately 3·2 million per µl. The test queens were divided into three treatments, as follows:

9. one insemination with 2·2 million spermatozoa
10. two inseminations with 2·2 million spermatozoa each
11. one insemination with 4·4 million spermatozoa.

Results and Discussion

The results are summarized in Table 1. Multiple inseminations with small volumes resulted in more spermatozoa in the spermatheca than single inseminations with the same total volume (compare treatments 3 and 4, 10 and 11; $P < 0{\cdot}01$). However, there was not a consistent reduction in variance as has been reported when larger volumes of semen were used (Mackensen & Roberts, 1948; Mackensen, 1964).

TABLE 1. Number of spermatozoa in the spermatheca of queens following single and multiple inseminations. The semen was diluted 3 : 2 in experiment A and 1 : 1 in B and C. Results followed by different letters are significantly different ($P < 0.01$), but comparisons can be made only between treatments within an experiment, not between experiments. *CV* = coefficient of variance.

Amount inseminated	*No. queens*	*Spermatozoa in spermatheca: mean no. ± SD (millions)*	*CV*
Experiment A: Stored semen			
1: 1·3 µl	8	0·049 ± 0·046a	93·9
2: 1·3 µl + 1·3 µl	9	0·212 ± 0·075b, c	35·4
3: 1·3 µl + 1·3 µl + 1·3 µl	9	0·307 ± 0·132c	43·0
4: 3·9 µl	10	0·153 ± 0·077b	50·3
Experiment B: Stored semen			
5: 2·2 µl	7	0·261 ± 0·163a*	62·4
6: 2·2 µl + 2·2 µl	7	0·823 ± 0·580b	70·5
7: 1·1 µl + 3·3 µl	8	0·946 ± 0·330b	34·9
8: 3·3 µl + 1·1 µl	8	0·925 ± 0·451b	48·8
Experiment C: Fresh semen			
9: 2·2 million spermatozoa	10	0·683 ± 0·332a	48·6
10: 2·2 million + 2·2 million spermatozoa	9	1·74 ± 0·53b	30·7
11: 4·4 million spermatozoa	12	1·19 ± 0·33c	28·1

*Result 5 different from 6 at the 0·05 level.

With small insemination volumes, the second insemination contributed more spermatozoa to the spermatheca of the queen than the first. Extrapolating from Mackensen's data (1964), we expected that contributions of both the first and second inseminations would approach 50% as insemination volumes decreased. They did not (cf. treatments 1 and 2). Assuming that the first insemination of treatment 2 was equal to the insemination in treatment 1, the contribution of the second insemination was calculated. Thus, in treatment 2, the first and second inseminations contributed 23% and 77%, respectively. The contribution of the second insemination in treatment 2 was greater than that of the first ($P < 0.01$). This same analysis was used for treatment 6 (32% and 68% contribution of the first and second inseminations, $P < 0.01$), and for treatment 10 (39 and 61% contribution of the first and second inseminations, $P < 0.05$). The third insemination in treatment 3 contributed numerically more than the first insemination, 31% and 16%, respectively ($0.05 < P < 0.10$). Although the three experiments (A, B, C) are not directly comparable because of different experimental designs, the second insemination consistently contributed more spermatozoa to the spermatheca than the first when small insemination volumes were given.

Our results concerning the relative importance of the second insemination may seem inconsistent with those reported by Mackensen and Roberts (1948), Mackensen (1964) and Woyke (1960, 1962). However, the second insemination contributed more than the first only when very small volumes were inseminated or when frozen spermatozoa were used. (The first and second inseminations of frozen spermatozoa gave results similar to inseminations with smaller volumes of fresh semen, containing fewer spermatozoa.) For inseminations with 2µl or more of undiluted, fresh semen, the first insemination significantly filled the spermatheca and thus preventing the contribution of the second insemination from reaching its potential.

Only diluted semen was used in these experiments, and our conclusions apply only to semen that has been diluted about 1 : 1. However, Mackensen (1969) found no difference in the number of spermatozoa in the spermatheca after inseminations with 9 million spermatozoa that were diluted (1 : 1) or undiluted.

Spermatozoa stored in liquid nitrogen had a low migration efficiency, but the number of spermatozoa entering the spermatheca increased significantly with multiple inseminations. Therefore, until better techniques are developed for storing spermatozoa in liquid nitrogen, multiple inseminations with small volumes are strongly recommended.

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