Egg Hatch of Honey Bees Fertilized with Frozen Spermatozoa[1,2]

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ABSTRACT

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Honey bee (*Apis mellifera* L.) semen was diluted with water (or 0.85% NaCl) and dimethyl sulfoxide in a 6:3:1 ratio and stored in liquid nitrogen (−196° C). Queens inseminated with this semen produced a higher proportion of males (unfertilized eggs) and a lower egg hatching rate than control queens. Low hatching rates were associated with the fertilized eggs rather than with the unfertilized eggs within the test group. Egg hatching rates averaged 82±11% (x̄±SD) in test queens and 95±2% in controls.

A normal honey bee (*Apis mellifera* L.) egg will hatch even though it is not fertilized. Unfertilized eggs develop into normal males (drones); fertilized eggs develop into females (workers or queens) if heterozygous at the sex locus or into nonviable males (killed as larvae by the workers) if homozygous at that locus (Mackensen 1951, Woyke 1962).

Hatching rates < 1% have been reported both as natural phenomena and as a result of exposing honey bee spermatozoa to mutagenic agents. Spermatozoa exposed to gamma irradiation (Lee 1958) and the chemosterilant *tris* (l-aziridinyl) phosphine oxide (tepa) (Taber and Borkovec 1969) caused otherwise viable eggs not to hatch. Hitchcock (1956) found natural cases of nonhatching to be caused by the egg rather than the spermatozoa.

Many kinds of environmental stresses on spermatozoa have caused death to eggs. As early as 1913, Dungay observed death after 1st cleavage in some eggs of the marine worm *Nereis limbata* when fertilized with refrigerated or heated spermatozoa. A significant number of sea urchin eggs (*Arbacia punctulata*) died after early cleavage and frog eggs (*Rana pipiens*) died in the gastrula stage after being fertilized with spermatozoa that had been aged ca. 1 day (Hart 1967). Chicken eggs (*Gallus gallus*) fertilized by aged spermatozoa had a higher death rate than eggs fertilized by fresh spermatozoa (Nalbandov and Card 1943).

My purpose was to determine whether storage of spermatozoa in liquid nitrogen (−196° C) can affect egg hatch. The experiment was run in 1977 and repeated, with modifications, in 1978.

Methods

1977 Experiment

Treatment Groups.—Sister queens were divided into 3 treatment groups (A, B, and C). Group A, consisting of 6 queens, was inseminated with semen mixed with dimethyl sulfoxide (DMSO) and water and stored in liquid nitrogen for 2 days. The final mixture consisted of 60% semen, 30% water, and 10% DMSO. Group B, 6 queens, was inseminated with semen prepared identically to group A but stored for 2 days at 13° C rather than in liquid nitrogen. Group C, 4 queens, was inseminated with fresh, undiluted, unstored semen.

Semen Storage and Inseminations.—All semen was collected from mutant drones (tan eyes), and queens were all heterozygous for snow eyes. Snow and tan are allelic, and when both are present in a diploid, the eyes are red (Laidlaw et al. 1964). I used these markers to be certain that the resulting progeny came from the frozen sperm. Semen for group A ~~and B~~ was stored in capillary tubes (Harbo 1979) and frozen at a rate described in Fig. 1. Queens from all 3 groups were each inseminated with 1½ μl of semen and released into small colonies to lay eggs.

Egg Collection.—After the queens had been laying for ca. 2 wk, a clean, empty comb was introduced to the broodnest. These combs were left in the colonies for 24 h; then combs, containing eggs but without bees, were put into an incubator (35° C and 50–60% RH) until the 3-day embryonic period was over. Three and one half days after eggs were removed from the colony, hatched larvae and unhatched eggs were counted. A 2nd comb of eggs was taken ca. one mo later.

1978 Experiment

This was a repeat of the 1977 experiment with the following modifications: 1. Subgroup B was omitted because it was nearly identical to group C controls in 1977. There were 6 test queens and 6 controls. 2. Saline (0.85% NaCl) was substituted for water in the semen diluent. 3. Queens were homozygous for body color markers cordovan (c) and black (bl), wing marker diminutive (di), and eye marker chartreuse Benson (ch^B). They were heterozygous for the eye marker brick (bk). All are single gene recessive markers. Semen was from nonmutant brother drones. 4. The cooling rate was slightly different (Fig. 1). 5. Fewer eggs were counted (Table 1).

The greatest difference was in the egg collecting technique. The 1978 procedure began by feeding pollen supplement [13% pollen, 17% torula yeast, 9% lactalbumin, 48% sugar, 0.5% Terremycin® (TM 50), and 12.5% water (by weight)] to each colony. Two days later, the queen and ca. 1000 of the workers in her colony were shaken into a screen cage that contained an empty comb with 5 grams of pollen tamped into cells in an upper corner and 15 ml of 60% (w/w) sugar syrup in a feeder. These caged bees were left in an incubator (35° C and 50–60% RH) for 24 h, then everything was removed except the combs that now contained eggs. Hereafter, eggs were handled as in 1977.

[1] Mention of commercial products in this paper does not constitute an endorsement of these products by the USDA.

[2] In cooperation with the Louisiana Agricultural Experiment Station. Received for publication Jan. 8, 1979.

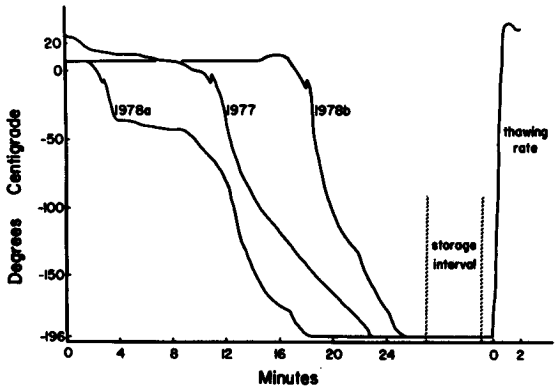

FIG. 1.—Freezing and thawing rates used in 1977 and 1978 experiments as recorded by a thermocouple in the semen. In 1978 the semen was stored 1 day at 7° C before freezing. Two freezing rates were used in 1978; queens inseminated with semen frozen at the 1978b rate are footnoted in Table 2. Thawing rates were the same for all samples. Storage intervals were 2 days in 1977 and 1 day in 1978.

Table 1.—Sample size and percent egg hatch of all queens.

Test queens[a]		Control queens	
No. of eggs counted	% hatch	No. of eggs counted	% hatch
1977 experiment			
Group A		Group B	
287	91	1389	98
319	90	960	97
313	84	535	96
367	83	1614	96
175	75	1067	94
507	55	1298	93
		Group C	
		1118	98
		1808	98
		1289	96
		1103	93
1978 experiment[b]			
251	92	400	96
232	90	475	95
270	87	400	95
288	86	494	93
183	85	400	93
409	65	400	92

[a] The test queens laid fewer eggs, probably because they were laying many unfertilized eggs in worker sized cells (Harbo 1976).
[b] In 1978 I counted only 100 eggs on each side of a frame; if there were fewer than 200 eggs on a frame, I counted them all.

Analysis

Egg hatching rates of test queens were compared with hatching rates of controls by analysis of variance.

I explored the possibility that another factor caused the reduced egg hatch in test queens. For example, test queens produced many unfertilized eggs, usually over 50%, and controls produced none. Perhaps these unfertilized eggs had a lower hatching rate than fertilized eggs. To test this, I evaluated the test queens' ability to produce queen progeny. Those producing the highest percentage of queens from unsexed larvae were judged to be those producing the highest proportion of female larvae (fertilized eggs). My objective was to determine whether the nonhatching phenomenon was associated more with the fertilized eggs or with the unfertilized eggs within the test groups. Therefore, I correlated egg hatch with percent queen progeny using linear regression analysis.

Results and Discussion

Queens inseminated with spermatozoa stored in liquid nitrogen had a lower egg hatching rate than control queens (Table 1). Egg hatching rates averaged 82±11% (x̄±SD) in test queens and 95±2% in controls. This difference was statistically significant ($P < 0.01$). In addition, test queens laid fewer eggs, probably because they were laying many unfertilized eggs in worker sized cells (Harbo 1976).

The nonhatching phenomenon in the test group was strongly linked to test queens that produced the highest proportion of female progeny. Correlation between egg hatch and ability to produce queen progeny was $r = -0.89$ in 1977 and $r = -0.98$ in 1978 (Table 2). Since queens develop from fertilized eggs, I infer that most nonhatching was among fertilized eggs of test queens. Conversely, this means that the nonhatching was not linked to the many unfertilized eggs in the test group.

Percent egg hatch of group B controls (x̄±SD = 96.1±2.0%) was nearly identical to egg hatch for group C controls (96.3±2.7%) in 1977. Since group B differed from the test group only in the storage temperature (13° vs. −196° C), I conclude that storage in liquid nitrogen and not aging, dilution, or chemical treatment of the

Table 2.—Relationship between egg hatch and production of female progeny among 12 queens inseminated with semen stored in liquid nitrogen.[a]

Queens produced from grafted larvae[b]		
No.	%	% eggs hatching
1977 group		
0	0[c]	91
0/20	0	90
1/40	3	75
5/80	6	84
7/80	9	83
7/36	19	55
1978 group		
0/80	0	92
1/80	1[d]	90
2/80	3	85
2/80	3	87
7/80	9	86
28/80	35[d]	65

[a] With simple linear regression analysis, the correlations (r) were −0.89 in 1977 and −0.98 in 1978.
[b] Grafting is the transfer of young larvae from the parent colony to wax cups for the production of queens. Since only females can develop into queens, the percent queens produced reflects the percent female larvae. Conversely, fewer queens produced indicate a higher proportion of male larvae. Controls from 1977 and 1978 produced 100% female progeny, and 76% (91/120) of their grafted larvae developed into queens.
[c] This queen produced no worker brood, so I assumed that she could produce no queens.
[d] Semen used to inseminate these queens was frozen at the rate identified as 1978b in Fig. 1.

spermatozoa caused many of the F_1 eggs not to hatch.

Although environmental stresses have long been known to damage spermatozoa, nonhatching eggs or early abortions have not heretofore been reported from spermatozoa stored in liquid nitrogen. The reason may be because most sperm storage work has been with cattle which usually produce 1 or 2 offspring/insemination. If a spontaneous abortion occurred within the 1st 21 days it would likely go undetected and regarded as an unsuccessful insemination. Alternatively, liquid nitrogen storage may not damage bovine spermatozoa in this way or the damaged spermatozoa may not be competitive with nondamaged spermatozoa.

How does this affect plans to bank honey bee and other insect spermatozoa in liquid nitrogen? It has very little effect if the nonhatching phenomenon is limited to the F_1 generation, for germplasm can be propagated through the viable sisters which accompany the nonhatching eggs. A problem occurs only if nonhatching or other deleterious effects are observed in subsequent generations. The F_2 generation is presently being evaluated.

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William Puissegur and Douglas Barbarousse, biological technicians at the Bee Breeding and Stock Center Laboratory, grafted larvae and helped with egg counting.

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