Viability of Honey Bee[1] Eggs From Progeny of Frozen Spermatozoa[2]

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ABSTRACT

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Honey bee, *Apis mellifera* L., queens were inseminated with semen that had been stored in liquid nitrogen (−196°C). Daughters (F_1 queens) were produced, and eggs from these F_1 queens were measured for viability. All eggs were kept in an incubator (35°C, 50 to 70% RH) for their entire developmental period. The percent mortality of fertilized eggs (F_2) in one test group differed significantly ($P < 0.05$) from controls (mean [$\bar{x}$] ± SD was 5.6 ± 5.0% for eggs in the test group and 2.6 ± 1.5% for controls). No significant differences were found between test and control eggs in another unrelated group of fertilized eggs or between test and control eggs that were not fertilized. In a survey of fertilized eggs in 46 normal colonies of bees near Baton Rouge, La., the mean mortality was 7% and the median was 5%. Unfertilized (haploid) eggs had a consistently higher mortality rate than fertilized (diploid) eggs. A typical group of queens, 12 producing fertilized eggs and 12 others producing unfertilized eggs, averaged 5.0 ± 5.0% ($\bar{x}$ ± SD) mortality for fertilized eggs and 18.4 ± 9.8% for unfertilized eggs.

Nonhatching of eggs (Harbo 1979a) of the honey bee, *Apis mellifera* L., and the apparent inability of spermatozoa to unite with egg pronuclei (Harbo 1980) are two phenomena that have been linked to frozen sperm. If these and other adverse effects are limited to the F_1 generation, then storage of spermatozoa in liquid nitrogen, even in its present state of technology (Harbo 1979b), may be a practical way to maintain germplasm. However, if these phenomena persist in subsequent generations, the present techniques may be detrimental and must be discarded.

The major objective of this work was to evaluate the mortality of the eggs produced by the F_1 generation which were progeny of spermatozoa stored in liquid nitrogen. Both fertilized and unfertilized eggs were examined. In this paper these fertilized eggs are called the F_2 progeny of frozen sperm, and the unfertilized eggs are called unfertilized eggs from F_1 progeny of frozen sperm (Fig. 1). In honey bees, unfertilized eggs are normally viable and develop into males, so unfertilized eggs provide an opportunity to examine single gametes of the F_1 queens. As background for the whole study, I sampled egg mortality from a normal population in the Baton Rouge area.

Materials and Methods

To standardize the environment for egg development and hatching, the eggs for all the experiments were laid and kept in an incubator (35°C, 50 to 70% RH). Two days before eggs were collected, the colonies were fed a pollen supplement (Harbo 1979a). Each queen to be tested was removed from the colony in which she was laying eggs and placed in a cage with about 1,000 workers from that colony. In the cage with the bees were a feeder containing about 15 ml of sugar syrup and an empty comb containing 3 ml of pollen tamped into an upper corner. The cage was put into an incubator. After 24 h in the cage, the bees and queen were returned to their colonies, and the frames of eggs were left in the incubator. Because eggs require about 72 h from oviposition to hatch, the number of hatched and unhatched eggs were counted at 80 to 92 h, 8 to 20 h after the hatching period of the youngest eggs was completed.

Each data point consisted of 30 or more eggs from a single queen. When a queen produced hundreds of eggs, the sample was usually limited to 200. Mated queens lay more eggs per day in worker-sized cells than do unmated queens (Harbo 1976), so the samples of fertilized eggs usually consisted of more eggs than the samples of unfertilized eggs.

To ascertain parentage, mutant markers were used in the parent generation of all the experiments. The three experiments (Fig. 2B–D) were conducted at different times and used unrelated stocks of bees. Semen was collected and stored in the manner described by Harbo (1979b).

Survey of Egg Mortality

Forty-six colonies were randomly chosen from a group of 281 miscellaneous colonies in the Baton Rouge, La., area. The objective was to measure the percent mortality of the fertilized eggs. Most of the colonies were headed by free mated queens, and none had frozen spermatozoa in their ancestry. Neither the size of the colonies nor the age of the queens was controlled, and only fertilized eggs were evaluated. After the survey, the queens in the six colonies with the highest egg mortalities traded places with the queens in the six colonies that had the lowest egg mortalities. Two weeks after the switch, when the 12 queens were safely reestablished and before new queens produced any adult progeny, egg mortalities were measured again.

Mortality of Fertilized Eggs

Fertilized eggs produced by F_1 queens (F_2 eggs) were evaluated for mortality. The experiment, therefore, involved a two-generation breeding program. Data were collected from two unrelated groups of queens, one in 1977 and the other in 1978. The freezing rates for the test semen, the semen storage procedures, and the percent mortality of eggs produced by the parent generations (F_1 eggs) were published (Harbo 1979a). The percent mortality of the F_1 eggs produced by the test groups

[1] Hymenoptera: Apidae.
[2] In cooperation with the La. Agric. Exp. Stn.

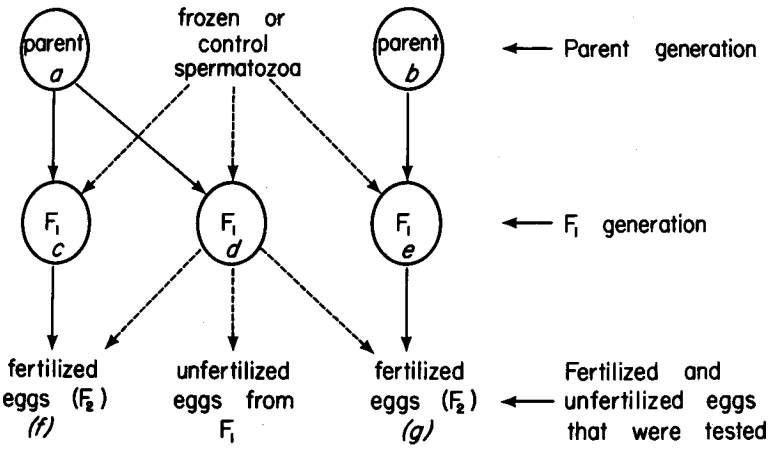

FIG. 1.—Matings above are examples of all the matings mentioned in the text. The pedigree format follows Polhemus et al. (1950) and Harbo and Rinderer (1980). Each oval represents a female and each line represents a gamete: male gametes (----), female gametes (——). Males develop from unfertilized (haploid) eggs, and the spermatozoa that they produce are simply replicates of the gamete that was originally produced by their mother. Thus, in a pedigree, males (drones) are only vehicles for delivering their mother's gamete; they do not belong to any generation. The following is a verbal description of the figure, using the same terminology as in the text. Test or control spermatozoa were mated to sister queens in the parent generation (queens *a* and *b*) to produce the F_1 generation (females represented by *c*, *d*, and *e*). Males were produced from unfertilized eggs of F_1 queens and mated to other F_1 queens to produce the F_2 generation (*f* and *g*). Mating queen *c* with drones from queen *d* is called a sister mating; mating queen *e* with drones from queen *d* is called a cousin mating.

were significantly higher than that of controls ($P < 0.01$).

To produce F_2 eggs, males (drones) from F_1 queens were mated to other F_1 queens. Semen from three drones were used for each mating. These F_1 crosses were either sister or cousin matings (terminology by Polhemus et al. 1950; see Fig. 1). Many of the F_1 queens in the 1977 experiment were mated to wild-type drones (6 of 10 in the test group, 3 of 6 in the DMSO control, and 5 of 10 in the undiluted and unstored control).

Mortality of Unfertilized Eggs

Unfertilized eggs from the F_1 progeny of frozen sperm were evaluated twice and from two different stocks. The semen diluent for both groups consisted of 25% dimethylsulfoxide (DMSO), 25% egg yolk, and 50% phosphate buffer (1.1 g of $NaH_2PO_4 \cdot H_2O$, 0.845 g of $NaHPO_4 \cdot 7H_2O$, and 0.25 g of dihydrostreptomycin sulfate in 100 ml total water solution). The final mixture contained 60% semen and 40% diluent. The freezing rates were 16°C/min for the group in Fig. 2C and 5°C/min for the group in Fig. 2D. The test semen remained in liquid nitrogen (−196°C) for 2 to 3 days before insemination of the parent generations. The controls were of two types: (1) DMSO control, in which the semen was diluted and stored just as the frozen semen was, but at 12°C rather than at −196°C, and (2) undiluted, unstored semen.

The F_1 queens that produced the eggs for Fig. 2C were progeny of 7 queens in the parent generation, and those that produced eggs for Fig. 2D were progeny of 11 queens in the parent generation. To obtain unfertilized eggs, these F_1 test and control queens were not inseminated and laid unfertilized eggs in worker-sized cells.

Comparing Mortality of Fertilized and Unfertilized Eggs

The mortalities of fertilized and unfertilized eggs were compared in two ways. First, the percent egg mortalities of sister queens, some mated and some unmated, were compared at the same time. Second, the egg mortalities of unmated queens were measured, the queens were then inseminated, and the mortalities of their eggs (now fertilized) were measured again. Most of these queens were from the group in Fig. 2D whose unfertilized eggs had a high mortality rate. Only queens B and J (Fig. 2E) were not from that group. Some of these queens continued to produce mostly unfertilized eggs after being inseminated; these queens were discarded. Data were used only from queens that produced over 85% worker progeny after the insemination. In all cases, the eggs were laid in worker-sized cells.

Results

Survey of Egg Mortality

Half of the queens in the population had egg mortalities of 5% or less; 80% had mortalities less than 10%. The mean mortality of 7% was misleading because of the skewed distribution (Fig. 2A).

The egg mortalities measured after switching the six queens having the very high egg mortalities (12 to 45%) with the six having very low mortalities (1 to 3%) showed that the groups were no longer different (Table 1).

Mortality of Fertilized Eggs (F_2)

In the 1977 experiment, eggs from 10 queens in the test group averaged 9.5 ± 7.6% ($\bar{x}$ ± SD) mortality, eggs from 6 queens in the DMSO control group averaged 7.7 ± 6.5% mortality, and eggs from 10 queens in the undiluted, unstored control group averaged 7.2 ± 5.0%. Analysis with the *t* test showed that there was only a 0.6 probability that the test group differed from the latter control group, so the difference was not considered significant.

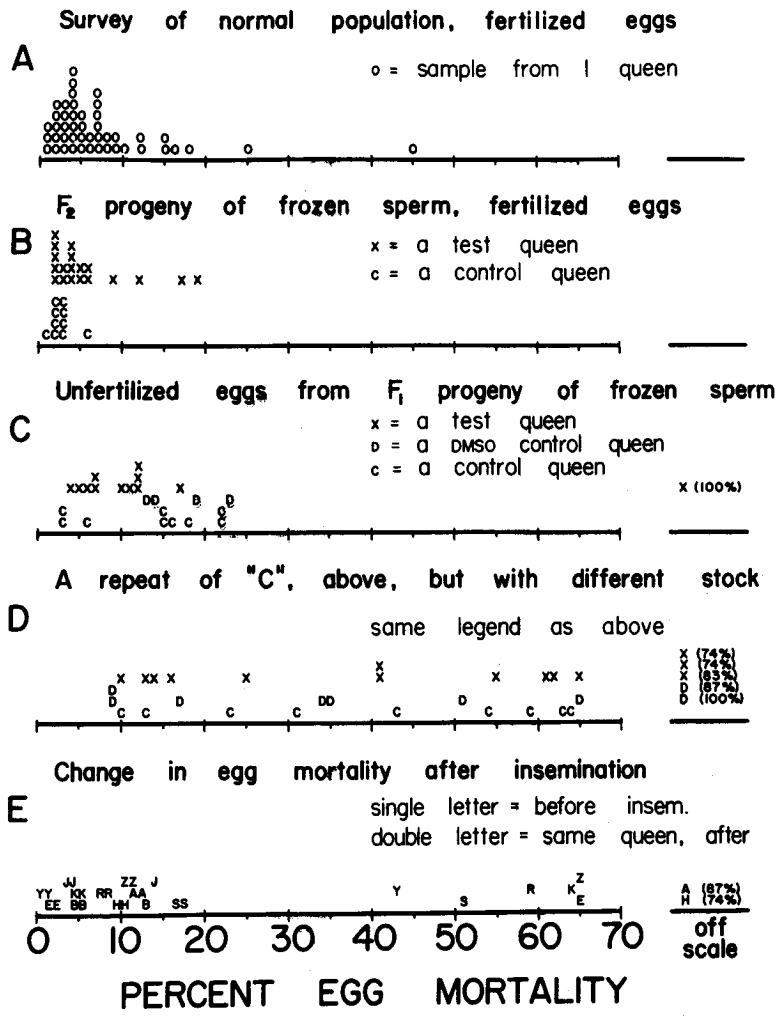

FIG. 2.—Percent eggs that did not hatch when kept in an incubator (35°C, 50 to 70% RH) without adult bees. Each data point represents the percent mortality of eggs of a different queen ($n > 30$ eggs per queen). (A) Fertilized egg samples from 46 randomly chosen colonies near Baton Rouge, La. (B) Fertilized eggs produced by $F_1 \times F_1$ matings. The test queens were progeny of frozen spermatozoa. (C and D) Unfertilized eggs from F_1 queens. DMSO controls were progeny of spermatozoa that had been diluted and stored just as the test semen but at 12°C rather than at −196°C. Controls were progeny of undiluted, unstored spermatozoa. (E) Mortalities of unfertilized eggs of 10 queens before insemination, and the mortalities of fertilized eggs from the same queens after insemination. Most of these queens were not progeny of frozen spermatozoa.

In the 1978 experiment, eggs from 19 test queens averaged 5.6 ± 5.0% ($\bar{x}$ ± SD) mortality and eggs from 10 controls averaged 2.6 ± 1.5% (Table 2, Fig. 2B). Analysis with the *t* test indicated that this difference was significant ($P < 0.05$).

Mortality of Unfertilized Eggs

The mortality of unfertilized eggs produced by the F_1 progeny of frozen spermatozoa was not significantly different from controls. Egg mortalities of the group in Fig. 2C averaged 17.8 ± 26.6% ($\bar{x}$ ± SD) for the test group ($n = 12$), 17.3 ± 4.6% for the DMSO controls ($n = 4$), and 13.3 ± 7.5% for the undiluted, unstored controls ($n = 9$). The group in Fig. 2D had even greater variability within treatments; eggs from test queens averaged 45.3 ± 25.8% ($\bar{x}$ ± SD) mortality ($n = 14$), eggs from DMSO controls averaged 45.2 ± 33.2% ($n = 9$), and eggs from the undiluted, unstored controls averaged 40.0 ± 21.4% ($n = 9$).

Two queens produce eggs that were all nonviable (Fig. 2C and D). These total nonhatches were not caused by the incubator technique because no larvae were found in their outdoor colonies either.

Comparing Mortalities of Fertilized and Unfertilized Eggs

Mortalities of eggs from 10 queens before insemination were much higher than after insemination (Fig. 2E). Unfertilized eggs from the 10 queens averaged 53.5 ± 24.2% ($\bar{x}$ ± SD) mortality and fertilized eggs from the same queens averaged 6.9 ± 4.9%.

Table 1.—Queens that produced fertilized eggs having the highest and lowest egg mortalities during a survey[a]

Queen no.	1st egg collection[b]		2nd egg collection[c]	
	Colony	Egg mortality (%)	Colony	Egg mortality (%)
High 1	A	45	B	18
Low 2	B	2	A	2
High 3	C	25	D	2
Low 4	D	2	C	24
High 5	E	18	F	5
Low 6	F	3	E	6
High 7	G	16	H	2
Low 8	H	2	G	2
High 9	I	12	J	7
Low 10	J	2	I	9
High 11	K	12	L	7
Low 12	L	3	K	6

[a] Queens in colonies that measured a high egg mortality were exchanged with queens in colonies having low egg mortalities.
[b] From the first collection, eggs from the "high" queens averaged 21.3 ± 12.5% ($\bar{x}$ ± SD) mortality and eggs from "low" queens averaged 2.3 ± 0.5%.
[c] From the second collection, eggs from the "high" queens averaged 6.8 ± 5.9% ($\bar{x}$ ± SD) mortality and eggs from "low" queens averaged 8.2 ± 8.2%.

The mortality rates of unfertilized eggs were also higher than those of fertilized eggs when eggs of mated and unmated sister queens were compared. Group 1 had eggs mortalities of 5.0 ± 5.0% (x ± SD) for fertilized eggs (eggs produced by 12 queens, n = 12) and 18.4 ± 9.8% (n = 12) for unfertilized eggs. Group 2 had egg mortalities of 6.7 ± 4.9% (n = 7) and 51.0 ± 24.7% (n = 7), respectively. Group 3 had 6.7 ± 3.9% (n = 4) and 61.3 ± 28.2% (n = 4), respectively. Group 4 had 4.6 ± 3.3% (n = 4) and 15.9 ± 3.1% (n = 4), respectively. Group 1 included some of the mated queens in Fig. 2B and group 2 included some of the unmated queens in Fig. 2D.

Table 2.—Parentage of fertilized (F_2) eggs and their percent mortality

Group	F_1 females that produced female gametes (eggs)	F_1 females that produced male gametes[a] (drones and sperms)	% Mortality of F_2 eggs[b]
Test	9 (P1)[c]	2 (P2)	18.5%
	22 (P1)	3 (P3)	17.0
	8 (P2)	2 (P2)	11.5
	16 (P1)	2 (P2)	9.0
	14 (P1)	2 (P2)	6.0
	19 (P1)	1 (P2)	5.2
	11 (P1)	2 (P2)	5.0
	7 (P2)	2 (P2)	4.5
	4 (P3)	3 (P3)	3.5
	6 (P2)	1 (P2)	3.5
	18 (P1)	1 (P2)	3.5
	21 (P1)	3 (P3)	3.5
	5 (P2)	1 (P2)	3.0
	17 (P1)	1 (P2)	3.0
	13 (P1)	2 (P2)	2.3
	10 (P1)	1 (P2)	2.0
	20 (P1)	1 (P2)	2.0
	12 (P1)	2 (P2)	1.8
	15 (P1)	2 (P2)	1.5
Control	41 (P5)	32 (P7)	6.0
	33 (P6)	31 (P6)	3.3
	35 (P4)	31 (P6)	3.0
	39 (P5)	32 (P7)	3.0
	42 (P5)	32 (P7)	3.0
	34 (P4)	31 (P6)	1.5
	36 (P4)	31 (P6)	1.5
	38 (P4)	31 (P6)	1.5
	40 (P5)	32 (P7)	1.5
	37 (P4)	31 (P6)	1.3

[a] See Fig. 1 for explanation of why queens are a source of male gametes.
[b] There were 200 or more eggs in each sample.
[c] The female parent of each F_1 is in parentheses to show relationships between F_1 queens. There was only one male parent for all the F_1 queens. All drones were produced by this male parent (a queen), and spermatozoa from these drones were subdivided into test (frozen) and control (nonfrozen) groups.

Discussion

General Egg Mortality

Data indicate that there are many factors that cause egg mortality in honey bees. Genotype of the egg is not the only factor and perhaps not the major one. Nevertheless, percent egg hatch is still a valid technique for measuring detrimental effects in bees as long as test groups are only compared with their controls. Unfortunately, the natural factors may increase the variance and thus make true differences between test and control groups more difficult to detect.

Egg Mortality in the F_2 Generation

There is evidence that genetic damage may exist in the F_2 progeny of frozen sperm. The results were not overwhelming (a 95% probability that the treated group was different from the control group), but the results have detered this laboratory from beginning a program of storing bee semen in liquid nitrogen on a routine basis. This study did not prove that detrimental effects persist to the F_2 generation, but if detrimental effects do persist to the F_2, the effects are greatly diminished and therefore much more difficult to detect than those in the F_1 generation.

Mortality of Unfertilized Eggs

Measuring the mortality of unfertilized (haploid) eggs was not a good technique for evaluating possible genetic damage. The high variance in the mortalities of haploid eggs would tend to mask slight differences that may exist between test and control groups. I expected haploid eggs to magnify the slight differences measured in the diploid (F_2) eggs above. They did not. If one disregards the variance and compares only the means, the test and control groups were similar.

Total nonhatching of all the eggs produced by one queen in Fig. 2C and one in Fig. 2D may be important. Nonhatching of all of a queen's eggs is extremely rare—so rare that Hitchcock (1956) published on this oddity. If such a queen had not been sent to this laboratory a few years ago, I would never have seen it before. I have seen it four times since I began working with frozen sperm, the two mentioned above and two F_1 queens that were mated to wild-type drones (Harbo, unpublished data). All were progeny of frozen sperm except the queen in Fig. 2D, which was a DMSO control. This seems to implicate DMSO as a possible cause, but because of high mortality in that entire group, the observation in Fig. 2D was not as exceptional as the other three. Those three represent about 3% of the F_1 queens that I have observed from frozen sperm. Although low, 3% is not rare and cannot be ignored as a possible indicator of serious genetic damage.

Comparing Mortalities of Fertilized and Unfertilized Eggs

It is not surprising that haploid eggs normally have a higher mortality rate than diploids. Deleterious genes would likely have a greater effect on a haploid than on a diploid genome.

The mean mortality rates of unfertilized eggs (the means between groups) were much more variable than the means for fertilized eggs. For five samples of unfertilized eggs, the means ranged from 13 to 63%. In contrast, the mean mortality rates of fertilized eggs (nontreated groups) were always between 2.6 and 7.9%.

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