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ENHANCED FERTILITY OF HONEY BEE SEMEN STORED *IN VITRO* AND POSSIBLY A REVERSAL OF SENESCENCE

by

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SUMMARY

Semen from the honey bee, *Apis mellifera*, was stored for 113 days in three different treatments : *a)* rapid cool, *b)* slow cool, and *c)* over N_2 gas. The aged semen was added to equal parts of fresh semen and used for insemination of virgin queen bees. Progeny from eggs fertilized by stored semen could be distinguished from that of fresh semen by a genetic marker.

The storage of bee semen under N_2, at 13 °C and in tubes dusted with streptomycin sulfate, yielded greater fertility after insemination than has previously been reported.

Four progeny samples collected at least 2-4 weeks apart showed increasing proportions of progeny from the stored, aged, and senescent semen. We conclude that the mixture of fresh semen with aged semen probably reversed the senescent effect of the aged semen.

INTRODUCTION

Stale, aged or senescent sperm cells are generally associated with abnormal growth and development of the progeny of the animal involved. Storage and aging of sperm *in vitro* resulted in decreased fertility and/or embryonic death for the invertebrate sea urchin (MEDES, 1917; DUNGAY, 1913), the frog (HART and SALISBURY, 1967) and for mammals such as rabbits (MILLER and BLACKSHAW, 1968), cattle (SALISBURY, 1968), and pigs (DZUIK and HENSHAW, 1958). When aged rabbit semen (ROCHE *et al.*, 1968) was mixed with fresh semen, no progeny were produced

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from the aged semen. The implication made is that the aged and therefore senescent rabbit semen, though shown capable of fertilizing the ova, was not as competitive when placed with fresh semen.

A review and discussion of gamete aging of both the egg and sperm cell is presented by SALISBURY and HART (1970). They point out that regardless of *in vitro* storage conditions of the gamete, all authors they reviewed showed either a chemical or biological change in the gamete during storage that led to eventual non-fertility. Much less is known about invertebrate or particularly *Apis* spermatozoa than mammalian spermatozoa. But in fact, invertebrate and vertebrate spermatozoa show (TABER, 1977) characteristics that are comparable in many respects.

The proportions of progeny produced by mixtures of genetically distinguishable bee *(Apis mellifera)* semen might change (TABER, 1955) with passage of time (subsequent to) artificial insemination. Experiments also showed that spermatozoa do not mix randomly in the spermatheca.

The purpose of these experiments was to determine a better method of *in vitro* semen preservation and to design the experiment in such a way that a change in the progeny ratio might be associated with the use of semen stored *in vitro*. Such a change might be explained by at least 3 testable hypotheses :

1) the fresh semen could be affected by toxic products accompanying aged and senescent semen, or,

2) the fertility capacity of aged semen could be improved in the spermatheca of the queen in the presence of fresh semen and or semenal plasma, or,

3) the proportion of progeny from stored vs. fresh semen changes because of non-mixing (TABER, 1955).

Evidence for the first hypothesis would be favored by a reduction in the proportion of fertilized eggs laid by the queen and in a rapid exhaustion of sperm from the spermatheca all evidenced by drone laying. Supporting evidence for the second hypothesis would be provided by an increase in the proportion of progeny that come from eggs fertilized from stored semen. Evidence for the third hypothesis would be a random change in proportion of offspring.

Loss of fertility after *in vitro* storage of honey bee semen has been reported (TABER, 1960; POOLE and TABER, 1970). In contrast, the present experiments demonstrate that after storage at above freezing temperatures, sperm fertility is reduced but then increases to an approximately normal level when the aged sperm are accompanied by fresh sperm cells.

We show that overlaying with N_2 during storage of the semen increases its fertility after a second period of storage within the laying queen bee's spermatheca.

METHODS AND MATERIALS

In these experiments the recessive mutant red-eye was used in conjunction with its wild-type alleles. [Stock was supplied by H. H. LAIDLAW, CA.] The insemination of red-eye virgin queens with wild-type semen yield wild-type offspring, whereas the insemination with red-eye semen yields only red-eye offspring. If both types of sperm are inseminated into a red-eye virgin, then progeny will be of red-eye and wild-types in equal proportions.

Sperm was collected for storage from free flying wild-type drones. The semen (ca 35 μl) was placed in heat sealed capillary tubes previously dusted with streptomycin sulfate and stored at 13°-15 °C for 113 days (POOLE and TABER, 1970). Three treatments were given the semen that was stored as follows (Fig. 1) :

a) The tube containing the semen was placed (from the room temperature, 25°-28 °C, at which it had been collected) directly into a Dewar flask at 13 °C.

b) Semen was cooled 5°/hour from room temperature until reaching 13° where it was then placed in the Dewar flask with treatment (a).

c) The gas space remaining in the unsealed capillary tube containing the semen was flushed 5 times with N_2 in a vacuum desicator to remove O_2 and CO_2, and then heat-sealed and immediately placed within the same Dewar flask with all other tubes.

The control (d) or fourth-treatment consisted of red-eye virgins being inseminated with fresh semen only, 2 μl from red-eye drones and 2 μl from wild-type drones.

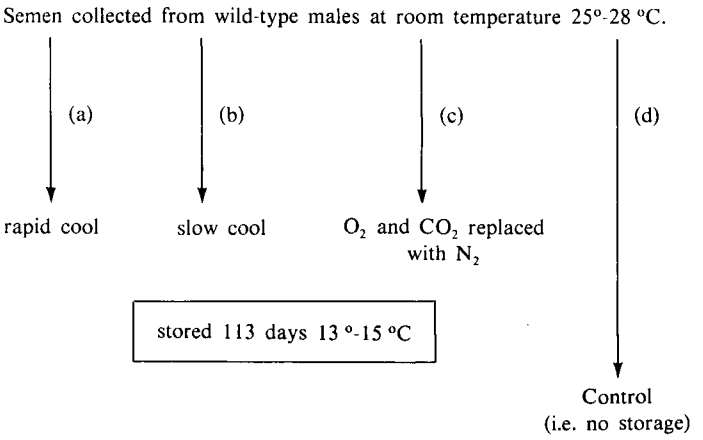

FIG. 1. — *Flow diagram of the 4 semen treatments.*

Inseminations : Red-eye virgin queens were kept in small cages within the nucleus colony. Each was inseminated with 2 μl of stored wild-type semen and 2 μl of fresh semen from red-eye drones. The order of semen type in the insemination syringe was reversed for alternate queens inseminated. The queens in each of the three treatments were divided into two groups with each group receiving stored semen from one tube (Tabl. 1).

After artificial insemination, all queens received two treatment of CO_2 to induce oviposition (MACKENSEN, 1947). They were then distinctively marked and the wings were clipped to prevent loss by flight or additional matings. Each queen was released in a nucleus colony for initial oviposition. One to two weeks after the queen's egg laying commenced, they were transferred to larger bee colonies where

TABL. 1. — *Flow diagram of red-eye virgin queen inseminations.*

Queen No.	Insemination syringe loaded and injected in the following order.
	Semen Rapid Cooled
1, 3, 5	2 μl semen treatment (a) followed by 2 μl fresh semen from red-eye drone.
2, 4	2 μl fresh semen from red-eye drones followed by 2 μl semen treatment (a).
	Semen Slow Cooled
6, 8, 10, 12	2 μl semen treatment (b) followed by 2 μl fresh semen from red-eye drones.
7, 9, 11	2 μl fresh semen from red-eye drones followed by 2 μl semen treatment (b).
	N_2 Treatment of Semen
13, 15, 17, 19, 21, 23, 25, 27	2 μl semen treatment (c) followed by 2 μl fresh semen from red-eye drones.
14, 16, 18, 20, 22, 24, 26	2 μl fresh semen from red-eye drones followed by 2 μl semen treatment (c).
	Control Treatment
28, 30, 32, 34	2 μl fresh semen from wild-type drones followed by 2 μl fresh semen from red-eye drones.
29, 31, 33	2 μl fresh semen from red-eye drones followed by 2 μl of fresh semen from wild-type drones.

progeny samples would be obtained. Samples from each queen had to meet certain minimum criteria, which included no less than 4 samples that had to total at least 1,000 progeny. Each of the 4 samples was taken at least at two week intervals. Samples were obtained by placing combs of emerging bees into an incubator-isolator. All bees that emerged in a 24 hour period were frozen and stored until the eye color for each could be determined.

RESULTS

The data in Table 2 are samples of the progeny produced by 2 queens since each queen produced from 200 to 1,000 progeny daily. Large progeny sample sizes exceeding 250 bees are of no statistical advantage, but the longer interval between samples is an advantage in determining sperm content proportions of the spermatheca and fertilization capacity of the spermatozoa. The periods between sample collections were sometimes 6 weeks apart and probably averaged 3 weeks.

The data in Table 3 are a sum off all samples in all treatments and queens. Variations in the numbers of bees per sample is determined by several factors, primarily adult bees to care for and feed the queen and growing larvae and to the food supply. We intentionally limited the bee colony population units so that sample sizes would not exeed 1,000.

The analysis of variance in Table 4 contains three highly significant F values which are interpreted to mean that the control treatments with untreated semen was the

TABL. 2. — *Progeny samples from honey bee queens inseminated with both fresh and stored semen. The 2 queens were selected to illustrate methods and data.*

Queen 14 May 7, 1974 — artificially inseminated with 2 µl fresh : 2 µl old semen.
June 6, 1974 — first progeny emerge.

Sample Date	Progeny				
	Days from 6-6-74	Fresh semen	Stored semen	Total	% From Stored Semen
7-18	42	318	75	393	19.1
8-30	85	193	63	256	24.6
9-13	99	70	254	324	78.4
10-2	118	109	94	203	46.3
Totals		690	486	1 176	42.1

Queen 13 May 7, 1974 — artificially inseminated with 2 µl old : 2 µl fresh.
June 6, 1974 — first progeny emerge.

Sample Date	Progeny				
	Days from 6-6-74	Fresh semen	Stored semen	Total	% From Stored Semen
7-12	36	142	38	180	21.1
8-23	78	1 130	58	1 188	4.9
9-20	106	400	61	461	13.2
10-4	120	248	164	412	39.8
Totals		1 920	321	2 241	19.8

TABL. 3. — *Sum of progeny samples from all queens and treatments collected over 4 periods of time from inseminations of mixtures of stored and fresh semen.* Numerator is the number of progeny in samples from stored or wild-type semen. The denomenator is the sum of all samples taken during that time period. Each time period is from 2 to 4 weeks.

Treatment	TIME PERIODS					
	Number of Queens	1	2	3	4	Total
(a)	5	201 / 2 249	221 / 2 057	340 / 2 513	960 / 3 496	1 722 / 10,315
(b) slow cool	7	135 / 1 867	110 / 2 740	454 / 4 061	361 / 2 143	1 060 / 10,811
(c) N_2	14	743 / 4 875	1 176 / 7 592	2 235 / 5 627	1 600 / 3 983	5 754 / 22,077
(d) control	7	1 616 / 2 364	1 484 / 2 960	1 883 / 3 628	1 141 / 2 083	6 124 / 11,035

TABL. 4 — *Results of analysis of variance of the four semen treatments. See text for explanation.*

		F	//
Treatment	3 df	16.1	**
Time	3 df	6.3	**
Treatment × time interaction	9 df	2.7	**

(//) The 3 F values show a significant difference at the 1 % level of probability.

best and that the storage with N_2 gas was the best of 3 storage treatments. The third highly significant F value shows the time periods different but a regression fits none of the usual curves in that the increase in progeny occurred after the second time period.

DISCUSSION AND CONCLUSION

This investigation demonstrated that the viability of honey bee semen stored at above freezing temperatures was greater than previously observed (POOLE and TABER, 1970) after additional treatments that included : *a)* replacing the air in the capillary storage tube with N_2, *b)* using a favorable storage temperature (13 °C), and *c)* dusting the storage tubes with streptimycin sulfate.

During the 3 months that these observations were taken, it was noted that none of the queens developed as « drone layers ». In honey bees, queens lay unfertilized eggs when an insufficient supply of viable spermatozoa is present in the spermatheca (MACKENSEN and ROBERTS, 1948). Use or exhaustion of semen by drone-laying queens in a three-month period while these queens were producing from 300-1,000 bees per day would have indicated that the 2 μl of fresh semen had been detrimentally affected by the inclusion of stored semen.

Our series of experiments were similar to those of ROCHES's *et al.* (1968); however, the results were markedly different. We do not know if the discrepancy between the two experiments could have been caused by the fact that (a) vastly different species are involved in the experiments, and there are no compelling reasons to believe that rabbit ova and spermatozoa should be anywhere near similar to those of the honey bee, or (b) perhaps ROCHE *et al.* are correct in that when fresh and aged spermatozoa were mixed the differences in ratio might be due to the faster penetration of the eggs by the fresh spermatozoa in the honey bee.

In the honey bee it has been estimated that between 4-100 spermatozoa are released by the sperm pump (SNODGRASS, 1925) and placed near the micropyle of the egg for its fertilization. Spermatozoa from multiple matings do not mix appreciably in the oviduct or spermatheca of the queen (TABER, 1955) so that most of the

spermatozoa deposited on any egg or successively laid eggs would consist of either all stored or all fresh semen. However, for a period of 24 hours, during multiple oviposition occur, it has usually been found that both types of spermatozoa will be present and fertilize eggs. Further, because of the non-mixing of semen in the queen it is difficult to estimate the proportion of each type of spermatozoa that reaches the spermatheca.

Of particular interest in a study such as this is the possibility of increased mutagenic damage to the sperm cells caused by their aging, as was shown to occur in *Drosophila* (BYERS and MULLER, 1952). Male bees are haploid, and mutations would be readly discernible. No adverse mutation rate was detected in our bees (LAIDLAW *et al.*, 1977).

Our experiments suggest a reversal of senescence of aged semen in the presence of fresh semen impling a chemical transfer of energy. At this time we do not have evidence to support that idea. However, the postulation was to our mind so interesting we felt it should be presented so that experiments with other animal species could be designed to show a chemical transfer of energy from new to old cells.

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RÉSUMÉ

FERTILITÉ ACCRUE DU SPERME D'ABEILLE CONSERVÉ *IN VITRO* ET POSSIBILITÉ D'UNE ANNULATION DE LA SÉNESCENCE

Le sperme de l'abeille, *Apis mellifica* L., a été conservé pendant 113 jours selon 3 modalités différentes : *a)* refroidissement rapide, *b)* refroidissement lent, *c)* sur N_2. On a ajouté le sperme âgé en quantités égales au sperme frais et l'ensemble a été utilisé pour inséminer des reines vierges. On a pu distinguer par un marqueur génétique la descendance des œufs fertilisés par le sperme stocké de celle des œufs fertilisés par le sperme frais.

La conservation du sperme de l'abeille sous N_2, à 13 °C et dans des tubes saupoudrés de sulfate de streptomycine a fourni une plus grande fertilité après insémination, qu'il n'avait été reporté jusqu'à présent.

Quatre échantillons prélevés dans la descendance à au moins 2 à 4 semaines d'intervalle ont montré des proportions croissantes de descendance provenant de sperme stocké, âgé et sénescent. On en conclut que le mélange de sperme frais à du sperme âgé a probablement annulé l'effet sénescent du sperme âgé.

ZUSAMMENFASSUNG

ERHÖHTE FERTILITÄT VON BIENENSPERMA NACH LAGERUNG *IN VITRO* MÖGLICHERWEISE EINE REVERSION VON ALTERUNGSVORGÄNGEN

Samen der Honigbiene, *Apis mellifera,* wurde für 113 Tage unter drei verschiedenen Versuchsbedingungen gehalten : *a)* Rasche Abkühlung (auf 13 °C), *b)* langsame Abkühlung, *c)* unter N_2-Gas. Der alte Samen wurde zu gleichen Teilen mit frischem Samen gemischt und zur Besamung von virginellen

Königinnen benutzt. Die Tiere, die aus mit gelagerten Spermien besamten Eiern hervorgingen, konnten durch genetische Marken von Tieren aus frischem Sperma unterschieden werden.

Die Lagerung von Samen bei 13 °C unter N_2-Gas, in Kapillaren eingestäubt mit Streptomycinsulfat, ergab nach der Besamung eine höhere Fertilität als bei den bisherigen Versuchsanordnungen.

Vier Proben von Nachkommen, entnommen in Abständen von mindestens 2-4 Wochen, zeigten einen steigenden Anteil der Nachkommenschaft aus gelagerten, alten und gealterten Spermien. Wir schliessen daraus, dass die Beimischung von frischem Samen zu altem Samen wahrscheinlich den Alterungseffekt von altem Samen rückgängig macht.

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