Effect of Osmotic Pressure on Insemination Success Using Incubated Honey Bee Semen[1]

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ABSTRACT

The effect of osmotic pressure of semen diluents on honey bee (*Apis mellifera* L.) semen was studied using instrumental insemination. Rates of sperm transfer to the queen's spermatheca were determined from unaltered semen and semen diluted 100-fold before incubation at 35 ± 1°C for 18 h. Diluents contained 16.7 mM glucose, 300 IU penicillin G sodium and 300 ug dihydrostreptomycin sulfate/ml, 50 mM Tris-HCl buffer in glass distilled water at ca. pH 8.7, and amounts of NaCl to give 9 osmotic concentrations. Unaltered semen had a transfer efficiency of 13.7% when queens were inseminated with ca. 15 x 10^6 spermatozoa, whereas, semen exposed to diluents of 280, 380, 480, 580, 680, 780, 830, 880, and 980 mOsm/L had transfer efficiencies of 0.1, 3.3, 5.6, 8.1, 7.7, 9.1, 8.1, 3.3, and 0.1%, respectively; the mean number of spermathecal spermatozoa from queens instrumentally inseminated with unaltered semen was significantly higher than all those from queens inseminated with semen reconcentrated after incubation in diluents of 280 to 980 mOsm/L. Excluding comparisons of the values for semen incubated at 380, 480 and 780 mOsm/L, mean numbers of spermathecal spermatozoa obtained from semen incubated at 480, 580, 680, 780, and 830 mOsm/L were significantly higher than those from semen incubated at higher or lower osmotic pressures. Maintenance of relatively uniform transfer efficiencies over such a wide range of osmotic pressures may be related to interaction of diluent hypertonicity and concentrations of ions and other substances in diluted semen. Further reduction of sperm transfer efficiency was not observed when queens were inseminated with incubated semen still slightly diluted (ca. 1.8 to 4X original volume) after centrifugation.

INTRODUCTION

THE technology of instrumental insemination is well developed commercially for dairy cattle, horses, chickens and turkeys and considerable progress is being made in its application to additional domestic animals. Recently artificial breeding has been employed with endangered species and other wild animals (Lake, 1978).

Breeding programs for animal production are dependent upon precise information and techniques concerning the behavior, nutrition, and reproductive physiology of the species of interest. Requisite procedures for breeding programs include techniques of maintaining the breeding stock in optimum health, collecting, handling and storing semen, and properly timing the breeding operation. Of these, success in the collection and processing of semen greatly affects the economics of artificial breeding programs; hence a major portion of such endeavors involves research on the physical, chemical, and biological properties of semen (Hafez, 1974).

Semen diluents are universally employed for processing and storing semen to be used in the instrumental insemination of birds and mammals. The formulation of semen diluents generally varies according to the species involved and the period of time between semen collection and the insemination process. For avian species, simple diluents composed of several inorganic salts, including a buffer and sometimes an energy source, suffice to hold semen ca. 40 min. at nonfreezing temperatures (5 to 20° C), but specialized formulations are required for longer holding periods. In addition to an energy bearing compound and inorganic salts, the more complex diluents for avian and mammalian semen include a source of protein (e.g. egg yolk or serum albumin), a buffer, one or more antibiotics, and frequently other chemicals (Harrison, 1976; Hughes and Varley, 1980; Lake 1978). The concentrations of the various diluent components are adjusted to approximate the osmotic pressure (tonicity) and pH of the semen or seminal plasma of the species involved to minimize damage to membranes, enzymes and other subcellular components of spermatozoa.

Diluents currently in use for semen collection and instrumental insemination of queen honey bees (*Apis mellifera* L.) are considerably hypotonic to drone semen and the queen's spermathecal fluid. The 0.875% to 0.9% sodium chloride and the 2.43% sodium citrate-based solution (Laidlaw, 1976; Mackensen and Tucker, 1970; Ruttner, 1976) are ca. 165 to 185 mOsm/L less than the average osmotic pressure of honey bee semen (467 ± 13 mOsm/L, Verma, 1973a) or spermathecal fluid (477 ± 47 mOsm/L, Gessner and Gessner, 1976). Insemination diluents have no significant effect on bee semen because the minute amounts

Table 1: Effect of osmotic pressure on sperm transfer in honey bee queens instrumentally inseminated with ca. 15 million cells incubated 0-18 h in a sodium chloride diluent at 100-fold dilution, ca. pH 8.7, and 35 ± 1°C.[1]

Osmolarity of diluent (mOsm/L)	Number of queens	ul semen/queen /treatment	Spermatozoa (x 10^6) in queens' spermathecae ± SD[2]	Transfer efficiency (%)
Unaltered semen (control)	42	1.8, 1.9, 1.9, 2.0	2.05 ± .079 e	13.7
280	15	4.2, 7.6	0.01 ± 0.14 a	0.1
380	24	3.8, 5.0, 5.3, 5.9	0.49 ± 0.35 b	3.3
480	26	4.2, 5.3, 8.1	0.84 ± 0.47 b,c	5.6
580	14	3.2, 4.2, 4.5, 5.1	1.22 ± 0.53 c,d	8.1
680	20	3.4, 5.9	1.16 ± 0.61 c,d	7.7
780	10	4.0	1.36 ± 0.64 d	9.1
830	7	4.4	1.22 ± 0.42 c,d	8.1
880	8	4.7	0.49 ± 0.25 a,b,c	3.3
980	6	8.2	0.02 ± 0.25 a,b	0.1

[1] Pooled data from four experiments comprised of four to six treatments each and five to twelve queens per treatment.

[2] Means followed by different letters are significantly different at $P < 0.01$, $F = 43.67$, d.f. = 9 and 162.

employed act as a lubricant for the inner surfaces of the insemination apparatus and the queen's reproductive tract, as well as a carrier for antibiotics to control the growth of microbial contaminants. On the other hand, recent observations on the effect of diluent composition (Williams, unpublished) indicate that hypotonic solutions may adversely affect honey bee semen when used at moderate rates of dilution as a washing medium.

The objective of this study was to determine the effect of the osmotic concentration of diluent solutions on the ability of incubated spermatozoa to migrate to the queen's spermatheca after insemination.

MATERIALS AND METHODS

The bioassay developed by Williams and Harbo (1982) was followed in the present study unless otherwise indicated. All diluents contained 16.7 mM glucose, 300 IU penicillin G sodium and 300 μg dihydrostreptomycin sulfate/ml, 50 mM Tris [tris (hydroxymethyl) aminomethane] buffer adjusted with 0.1 N HCl to ca. pH 8.7 at 35°C, glass distilled water, and requisite amounts of sodium chloride to produce desired total osmotic concentrations. Artificial diluents were prepared in 9 different osmotic concentrations from 280 to 980 mOsm/L (Table 1). Osmotic pressures of diluent solutions, measured with a Wescor 3150-B vapor pressure osmometer, varied ± 7.0 mOsm/L from expected values. After storage overnight for ca. 16 to 18 h at 15°C. semen was diluted 100-fold in 15 ml centrifuge tubes, and then incubated for 18 h in a water bath at 35 ± 1°C. Ordinarily the possibility of temperature effect would be eliminated in this type of study by uniformly incubating all semen subsamples, including control or unaltered semen. Preliminary trials included two control treatments of one subsample each of unaltered semen held at 15° and 35°C for 18 h. However, traces of mucus formed unusually firm clumps and blocked the insemination tip despite removal efforts, frequently causing the loss of subsamples incubated at 35°C. Further work showed no statistical difference ($P < 0.01$) in the number of spermathecal spermatozoa of queens instrumentally inseminated with whole semen (ca. 15 million cells/queen) incubated 18 h at either 15° or 35°C: 15°, $\bar{x} \pm SD = 1.41 \pm 0.27$ million spermatozoa, $N = 8$ queens; 35°, $\bar{x} \pm SD = 1.50 \pm 0.26$ million spermatozoa, $N = 6$ queens; $F = 0.39$, $df = 1$ and 12. Consequently, in the test reported here, 15° instead of 35°C was used to maintain control semen until queens were inseminated. Supersister hybrid queens (771 x Ka) 13 to 14 days old were inseminated with a volume of reconcentrated or unaltered (control) semen containing ca. 15 x 10^6 spermatozoa.

Four experiments were performed consisting of 4 to 6 treatments each according to the amount of semen and the number of queens available on a given date; treatments were randomized within experiments to include a control (undiluted semen), diluents of 380 and 480 mOsm/L, and 1 to 3 additional diluents of concentrations from 280 to 980 mOsm/L. Duplicate counts of spermathecal spermatozoa were made with a hemocytometer. Data from the four experiments were pooled on the basis of nonsignificant differences among mean counts of spermatozoa from queens inseminated with nonincubated semen ($P < 0.01$). Statistical treatment of combined data was done with an analysis of variance and a least significant difference test.

RESULTS AND DISCUSSION

The number of spermatozoa which migrate into a queen's spermatheca relates inversely to the volume of unaltered semen injected into her oviducts by instrumental insemination. However, this volume effect apparently is eliminated whenever diluted semen is used to inseminate queens.

Mackensen (1964) noted that transfer efficiency decreased as the volume of semen used to instrumentally inseminate queens was increased; transfer efficiencies of 12.4, 11.6, 9.5, 6.9 to 7.6%, and 3.2% were obtained from inseminations of 2, 3, 4, 6 and 28 μl semen per queen, respectively. On the other hand, the effect of semen volume on transfer efficiency is eliminated with a mixture of small amounts of semen and diluent. For example, mean counts of spermathecal spermatozoa from queens instrumentally inseminated with either 1.25 μl of fresh semen or 2.5 μl of diluted semen (1:1) were not significantly different (Mackensen, 1969). In the present study, there were no significant differences between mean counts of spermathecal spermatozoa obtained from queens inseminated with 4.5 (A) or 6.4 (B) μl of semen (ca. 15 million spermatozoa) reconcentrated as much as possible after incubation at 1:100 dilution (18 h at 35 ± 1° C) in solutions of 580 and 600 mOsm/L, respectively, and corresponding queens that received ca. 15 million spermatozoa each in 1.9 (C) and 1.8 (D) μl of unaltered semen (mean counts ± SD: 0.88 (B), 1.81 ± 0.49 (C),1.70 ± 0.36 (A), 1.33 ± and 1.74 ± 0.68 (D) million spermatozoa per queen ($P < 0.01$)). Further, mean numbers of spermatozoa from queens inseminated with ca. 15 million spermatozoa each, either as 4.3 μl of semen reconcentrated after incubation in modified Kiev solution at 1:100 dilution (18 h at 35 ± 1° C) or 2 μl of unaltered semen, were not significantly different ($P < 0.01$) (Williams, unpublished). Therefore, in these experiments, the increased volumes of reconcentrated semen required to deliver ca. 15 million spermatozoa (range: 3.2 to 8.2 μl), as compared to unaltered semen (range: 1.8 to 2.0 μl), apparently had no influence on transfer efficiency of incubated spermatozoa.

Overnight incubation of honey bee semen diluted in saline diluents of 9 different osmotic concentrations, including that of fresh bee semen, significantly reduced insemination success (rate of sperm transfer to the spermatheca) with instrumentally inseminated queens, as compared to the results obtained with unaltered semen (Table 1). Insemination success was significantly higher with semen incubated in diluents ranging from 580 to 830 mOsm/L than that from semen incubated in diluents of 280, 380, 480, 880, or 980 mOsm/L. In addition to decreased sperm transfer, nearly all queens inseminated with semen incubated in 280 or 980 mOsm/L diluents contained dead spermatozoa with tails broken into several pieces and severely damaged membranes. Diluents hypotonic or hypertonic to bee semen may also damage spermatozoa without preventing their migration to the queen's spermatheca.

Verma (1973b) found that *in vitro* motility was reversibly inactivated when fresh semen was incubated, presumably for 2 h, as a 1:1 mixture with a simple diluent of Tris buffer, sodium, and potassium at ca. 890 mOsm/L (total OP). The relationship of diluent composition to honey bee spermatozoan survival *in vitro* and their subsequent insemination quality has been studied by few others. Mackensen (1969) found in attempts to improve insemination efficiency that mixing fresh bee semen at a 1:1 ratio with a saline diluent alone or as various combinations with phosphate buffer, fructose, glucose, and trehalose, did not increase the transfer capacity of spermatozoa in instrumentally inseminated queens.

Only one diluent parameter, osmotic pressure, was varied in this study because larger experiments were unmanageable. Because such characteristics of semen diluents as the ionic composition, pH and osmotic pressure interact physiologically, conclusions about the present results of the effect of diluent osmotic pressure on honey bee spermatozoa apply only to very restricted conditions, specifically pH 8.7 and the simple diluent consisting of sodium chloride, Tris-HCl and glucose. Under these conditions, sperm transfer capacity of honey bee spermatozoa was reduced similarly over a wide

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This situation is not comparable to the storage of semen of higher animals, where, out of billions of sperm injected, only a few thousands reach the site of the fertilization, and ultimately only a single sperm cell is responsible for the fertilization of the ovum and the production of a new offspring (Mann, 1964).

SUMMARY

Different diluents used in the present investigation for deep freezing of honey bee semen in liquid nitrogen at -196°C gave good results in terms of sperm motility. Drone haemolymph was as good as or better than other diluents used, but haemolymph tended to coagulate and thus made insemination difficult.

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range of hypertonic osmotic pressures, ca. 580 to 830 mOsm/L, by a compositionally simple diluent.

The determination of an optimal diluent tonicity for handling and storage of honey bee semen will require further studies, including progeny tests, of the combined effect on spermatozoa of holding temperature, rate of dilution, and diluent composition, osmotic pressure, and pH. More complex diluents with osmotic pressures hypertonic to bee semen may be helpful in this regard.

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FOOTNOTES

[1] In cooperation with the Louisiana Agricultural Experiment Station. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

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