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Fertility Differences among Male Rabbits Determined by Heterospermic Insemination of Fluorochrome-labeled Spermatozoa[1]

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ABSTRACT

Spermatozoa from different bucks were stained with different fluorochromes, mixed, and inseminated heterospermically. By altering the interval between insemination and luteinizing hormone injection, spermatozoa were allowed to reside in the female tract approximately 5, 10, or 15 h prior to ovulation. The number of functional spermatozoa, from each male of a pair used, that was transported to the site of fertilization was estimated by counting total number of differently stained spermatozoa that surrounded or fertilized each oocyte. Spermatozoa from split ejaculates within a male competed against each other equally, indicating that the staining procedure did not affect fertilization or functional spermatozoal transport rates. Three pairs of males with high initial semen quality (>80% motility) differed in fertility primarily due to functional spermatozoal transport. Spermatozoal survival in the female tract and capacitation time played a role in differences in male fertility when heterospermic insemination occurred at variable times relative to ovulation. Differences in fertilization not accounted for by spermatozoal transport ratio raised the possibility that rate of egg penetration due to acrosomal enzyme differences may be important in determining male fertility. Therefore, total acrosin, hyaluronidase, and arylsulfatase activity in spermatozoa from specific bucks used in fertilization experiments were determined. Although there were trends favoring high fertility when enzyme content was higher, the difference was significant only for arylsulfatase in one buck.

INTRODUCTION

Males with apparently normal semen quality often differ in fertility. It is important to identify factors that are responsible for some of these differences in fertility in order to focus investigations on processes underlying them. Factors of interest in the current study included spermatozoal transport and survival in the female tract, capacitation, and egg penetration rate.

Sensitive measurements of fertility are important in reducing costs of fertility assessment. Heterospermic insemination is much more sensitive in detecting differences in fertility (Beatty et al., 1969) than homospermic insemination (Dunn et al., 1956). A major drawback to heterospermic insemination has been the need to establish paternal parentage of conceptuses and offspring, resulting in expensive procedures and long waiting periods. Labeling spermatozoa with contrasting fluorescent dyes provides an opportunity, as suggested by Blazak et al. (1981), to identify both the accessory and fertilizing spermatozoa.

Heterospermic insemination with fluorochrome-labeled spermatozoa was used to estimate functional spermatozoal transport to the site of fertilization by measuring accessory spermatozoa and determining the fertilizing spermatozoon in the same female. The rabbit was chosen as the animal model because spermatozoa can be collected easily, the female is an induced ovulator, and multiple oocytes are obtained easily by superovulation. Manipulation of the interval from insemination to induced ovulation (by exogenous luteinizing hormone) allows one to design studies simulating insemination at different times in spontaneously ovulating domestic farm animals and humans. Specifically, experiments were designed to use two fluorescent labels with heterospermic insemination to investigate the role of spermatozoal transport and number of spermatozoa present within the female tract, egg penetration rate, and capacitation time on differences in male fertility.

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MATERIALS AND METHODS

Fluorescent Labeling of Spermatozoa

Stock solutions of the fluorescent stains fluorescein isothiocyanate (FITC; Sigma Chemical Co., St. Louis, MO) or tetramethylrhodamine B isothiocyanate (TRITC; Sigma) were prepared by mixing 10 mg of stain with 1 ml of 0.1 M KOH for not more than 15 s and then diluting to 100 ml with sterile phosphate-buffered saline containing 55.5 mM glucose (PBSG). The stock solution (0.1 mg stain/ml) was stored at 5°C in serum bottles wrapped in aluminum foil for up to 3 mo.

Batches of stain vary in toxicity and efficacy of labeling sperm. For each batch of stain prepared, the amount used to label spermatozoa was determined as the lowest level that allowed detectable fluorescence and did not affect spermatozoan motility. Semen was collected with an artificial vagina from male Dutch rabbits. Ejaculates were diluted by adding three volumes of PBSG. All subsequent work with semen and fluorescent stains was performed under incandescent light. Stock stain preparations were diluted with PBSG to yield 5 or 10 μg/ml of FITC and 3 or 7 μg/ml of TRITC. To 0.5 ml of diluted stain was added 0.125 ml of semen. The mixture of semen plus stain was incubated in the dark for 10 min at 37°C, then added to 5 ml of a modified Tyrode's medium (TALP; Bavister and Yanagimachi, 1977) supplemented with 6 mg/ml bovine serum albumin (BSA, Fraction V; Sigma), at 37°C. The mixture was centrifuged at 210 × *g* for 6 min. The spermatozoal pellet was resuspended in TALP.

Superovulation and Insemination

Female Dutch Belted rabbits utilized were at least 5 mo of age. Does not previously superovulated received subcutaneously 0.3 mg of follicle-stimulating hormone (FSH; Burns-Biotech) Lab., Inc., Omaha, NE) every 12 h for a total of six injections. Does previously superovulated received 0.5 mg FSH at each injection. Each doe received 2.5 mg of luteinizing hormone (pLH; Burns Biotech) intravenously 12 h after the last FSH injection.

Does were inseminated with 0.2 ml of treated semen drawn into polyethylene tubing to avoid possible selective sticking to glass of spermatozoa stained with the fluorescent labels. The tubing was threaded through a regular glass inseminating catheter already placed at the anterior vagina and the semen was expelled.

Does were inseminated 5 h after (+5), at the time of (0), or 5 h before (−5) receiving LH, as shown in Fig. 1. The LH injections were staggered so that all does were inseminated with the same ejaculate of semen. Sperm thus resided in the female approximately 5, 10, or 15 h prior to ovulation (10–11 h after LH). The does were laparotomized and oviducts were flushed with PBSG containing 0.3% BSA, to collect zygotes from all treated does 18 h after LH. This interval allowed time for egg penetration by the fer-

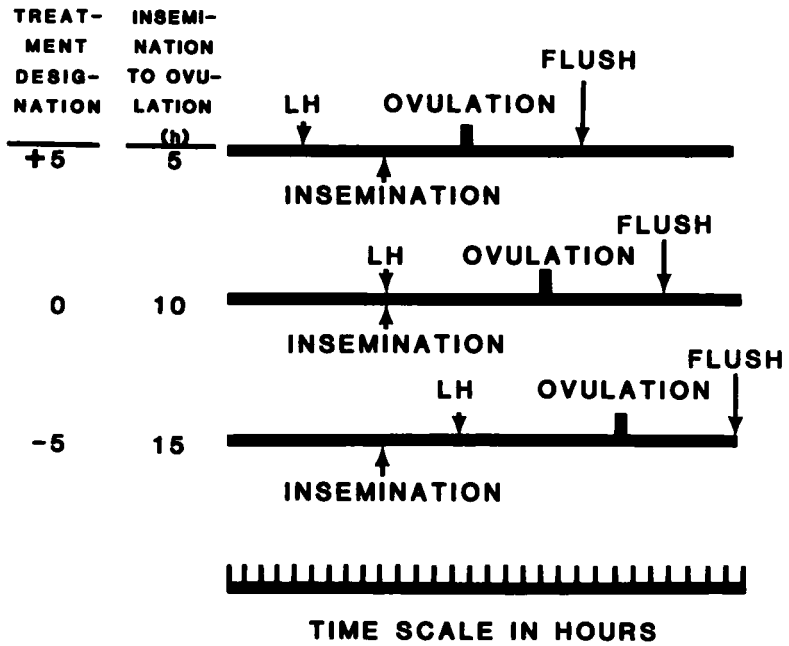

FIG. 1. Treatment regimen used to permit sperm to reside in the female for approximately 5, 10, or 15 h before ovulation. All three groups of animals were inseminated with aliquots of the same semen at the same time for each replicate performed. Each tick mark represents 1 h.

tilizing spermatozoon and it was the earliest time at which oocytes or zygotes had sufficient numbers of cumulus cells removed to be able to identify individual spermatozoa in or on the ova.

Spermatozoal Transport and Fertilization Determination

Each zygote or unfertilized egg was mounted on a slide covered with a paraffin-Vaseline-supported coverslip and examined at magnifications of 200X and 500X with a Zeiss Universal microscope equipped with epifluorescence. Fluorescent filter combinations utilized were: 1) excitation at 450–490 nm and barrier filter at 520 nm (Zeiss filter set 487709) for FITC; and 2) excitation at 546 nm and barrier filter at 590 nm for TRITC (Zeiss filter set 487715). With these filter combinations, FITC-labeled spermatozoa fluoresced green and TRITC-labeled spermatozoa were red.

To measure fertilization rate, the color of any fertilizing spermatozoon as visualized by the fluorescent midpiece was recorded. The fertilizing spermatozoon was often present as a male pronucleus but decondensing spermatozoon heads with an associated tail also were seen. The fertilizing spermatozoon was easily distinguishable from accessory spermatozoa. The midpiece of the fertilizing spermatozoon often was split and lay within the egg cytoplasm. Accessory spermatozoa were only in the perivitelline space, in the zona pellucida, or on the vitelline membrane. The midpieces of accessory spermatozoa were clearly intact, with distinct mitochondria. Of the 818 embryos examined in this study, 3 were found to be polyspermic.

The total number of spermatozoa stained red or green surrounding each oocyte (in the cumulus, zona pellucida, and perivitelline space) was recorded as a proposed measure of transport to the site of fertilization of spermatozoa capable of fertilization. While this determined the number of spermatozoa that remained associated with the egg or its investments approximately 7–8 h after ovulation, we believe it may estimate the number of spermatozoa present at the time of fertilization; however, this was not directly verified. If a spermatozoon was to fertilize an oocyte, then it would have belonged to this population of spermatozoa. These data were compared with data on the color of the fertilizing spermatozoon.

Experiment 1

Experiment 1 was a procedural test to determine whether labeling sperm with the fluorescent stains FITC and TRITC would affect functional spermatozoal transport or fertilization rate. Ejaculates from each of two bucks were divided equally and stained with FITC (10 μg/ml) or TRITC (3 μg/ml). Then 1.25 × 10^6 spermatozoa from each stained suspension (FITC and TRITC), from one buck, were combined and diluted to 1 ml with TALP. The equal numbers of spermatozoa were verified by direct counting with phase-contrast epifluorescence. Does previously treated with FSH were inseminated with 0.2 ml of mixed semen (0.5 × 10^6 spermatozoa) at 1 of 3 times (Fig. 1). The total number of spermatozoa inseminated was determined in preliminary experiments as the number that never gave more than 50 spermatozoa, FITC or TRITC labeled, attached to each oocyte. Thus, spermatozoa from the same male stained differently were competing against each other.

Experiment 2

The objective of Experiment 2 was to determine if functional spermatozoal transport, capacitation time, or egg penetration rate could account for differences in fertility between males. To test this hypothesis, a competitive heterospermic insemination experiment was designed. Three pairs of bucks were selected whose spermatozoa exhibited greater than 80% motility. Spermatozoa from one buck of each pair were stained with 7 μg/ml FITC and spermatozoa from the other member of a pair were stained with 5 μg/ml TRITC. The labels for spermatozoa were reversed each time a particular pair of bucks was used to average out any possible fluorochrome effect. Does were inseminated with 0.2 ml of a 50–50 mixture of FITC- and TRITC-stained spermatozoa (0.5 × 10^6 spermatozoa) at the times previously described and shown in Fig. 1. The equal number of spermatozoa was verified with phase-contrast epifluorescent microscopy.

Experiment 3

Experiment 3 was conducted to determine if differences found between functional spermatozoal transport and fertilization in Experiment 2 were due to acrosomal enzyme content of the spermatozoa. The amounts of acrosin, hyaluronidase, and arylsulfatase were measured on four different ejaculates from each of two pairs of bucks used in Experiment 2. Spermatozoa were prepared prior to extraction as described for fluorescent labeling with the exception that stain was not present in the PBSG.

Acrosin. Acrosin extraction was modified from procedures of Polakoski et al. (1972), Huang-Yang and Meizel (1975), and Goodpasture et al. (1980) for use with rabbit spermatozoa (Parrish, 1984). The extraction was performed by layering the semen sample on 3 ml of 1 M sucrose solution and centrifuging at 3000 × *g* for 20 min (5°C). The supernatant was removed and the pellet was resuspended in 0.75 ml of 10% (v/v) glycerol in water (pH 2.8, adjusted with 1 N HCl). This mixture was incubated for 3 h at 5°C. A subsample was removed and concentration of spermatozoa was determined with a hemacytometer. After 3 h, 0.5 ml of activation solution (40 mM HEPES/100 mM $CaCl_2$; pH 7.0) was added to 0.5 ml of the spermatozoal suspension at 5°C for 15 min to activate proacrosin conversion to acrosin. Twenty microliters of 1 N HCl was added to adjust the pH to 2.8 and the solution was incubated for 15 min at 25°C. The spermatozoa were removed by centrifugation at 27,000 × *g* for 30 min at 5°C. The supernatant was poured off and stored in capped plastic tubes at 5°C until assayed.

The total acrosin content was determined within 12 h of extraction by measuring its esterolytic activity, using a modification of the procedure described by Goodpasture et al. (1980). One-tenth milliliter of extract was added to 0.9 ml of 0.05 M Tris buffer (pH 8.0) containing 50 mM $CaCl_2$ and 0.5 mM α-*N*-benzoyl-L-arginine ethyl ester (BAEE) as substrate at 25°C. Samples were placed in silicone-coated quartz cuvettes. The change in absorbance over 4 min was determined with a Bausch and Lomb Spectronic 21 (253 nm) con-

nected to a strip chart recorder. A molar absorbance difference of 1150 $M^{-1} \cdot cm^{-1}$ was used to convert the change in absorbance to micromoles of BAEE hydrolyzed (Whitaker and Bender, 1965). One milli-IU activity was the amount of enzyme that caused the hydrolysis of 1 nmol BAEE in 1 min at 25°C.

Hyaluronidase. The semen sample was layered on 3 ml of 0.438 M sucrose and centrifuged at 375 × *g* for 20 min at 5°C to remove cytoplasmic droplets (Srivastava et al., 1979) but not to disrupt membranes as did 1 M sucrose in the acrosin extraction. The pellet was resuspended with 0.5 ml of a 0.9 g/100 ml NaCl + 0.6 g/100 ml BSA mixture (saline-BSA). A subsample was removed for determination of concentration with a hemacytometer. Equal volumes of the diluted spermatozoa and 2% Triton X-100 in saline-BSA (Rodgers and Yanagimachi, 1975) were mixed and incubated for 15 min at 22°C. The spermatozoa were removed by centrifugation at 27,000 × *g* for 30 min and the supernatant was stored at 5°C until assayed.

Hyaluronidase was measured by the procedure of Triana et al. (1980) with the following modifications. Standards of pure *N*-acetyl-glucosamine (NAG) (0–100 nmol/0.1 ml) were prepared in the extract solution (1% Triton X-100, 0.3% BSA, 0.9% NaCl). Hyaluronidase-positive controls were prepared in the extract solution at 0 to 50 μg/0.1 ml (bovine testicular hyaluronidase; Sigma). The action of hyaluronidase on NAG reaction was halted by addition of 50 μl freshly prepared stopping reagent (8.9 ml of 0.76 M boric acid, pH 9.0, and 1.1 ml of 5 M potassium hydroxide). After color development, all tubes were centrifuged at 200 × *g* for 15 min at 5°C to remove precipitates. The optical density was determined immediately at 544 nm for each sample. The nmol/min of NAG formed were calculated from a standard curve of 20–100 nmol NAG. A unit of activity was defined as the amount of enzyme liberating 1 μmol NAG/min. Each assay was checked for linearity with hyaluronidase controls.

Arylsulfatase. The amounts of arylsulfatase were determined in rabbit spermatozoa because, along with acrosin and hyaluronidase, they have been implicated in zona pellucida penetration (Akruk et al., 1979). Extraction of arylsulfatase with 1% Triton X-100, as described previously for hyaluronidase, or the sequential method using $MgCl_2$ treatment followed by 0.5% Hyamine 2389 and 0.5% Triton X-100 (Yang and Srivastava, 1974), yielded equal values (Parrish, 1984). The 1% Triton X-100 method was therefore chosen because of its simplicity and simultaneous extraction of both hyaluronidase and arylsulfatase. The measurement of arylsulfatases was performed as described by Akruk et al. (1979). All standards of 4-nitrocatechol were prepared in saline-BSA containing 1% Triton X-100.

Statistical Analysis

All competitive insemination data were first analyzed with the generalized least-squares procedure for categorical data, Funcat, in the Statistical Analysis System. Treatment differences were then compared with orthogonal contrasts or a Scheffe-type multiple comparison procedure as suggested by Grizzle et al. (1969). The sperm transport and fertilization data were compared with chi-square goodness-of-fit tests. Differences in enzyme levels were determined with the aid of analysis of variance for each pair of bucks.

RESULTS

Experiment 1

There was no interaction between buck and time of insemination ($P>0.05$) for spermatozoal transport (spermatozoal attachment to the egg or its investments) or fertilization. The data, therefore, are presented by buck in Table 1 and by insemination time in Table 2. The data indicate that neither buck ($P>0.05$) nor time of insemination ($P>0.05$) influenced the proportion of functional spermatozoa transported or ova fertilized by fluorochrome-labeled spermatozoa. However, there was a marked decrease in number of spermatozoa attached to the zygotes and cumulus cells as residence time in the female increased. Never were more than five spermatozoa present in flushes that were not attached to the egg or its investments. Within a time of insemination and buck, the total number of spermatozoa counted between does varied from 0 to 1463. The range obtained from individual does of percent spermatozoa transported that were labeled with TRITC was 40–59%, while the range of fertilization was 8–67%. The larger range in fertilization results was probably due to fewer fertilizing spermatozoa than accessory spermatozoa present per doe, increasing the binomial variation.

Experiment 2

Bucks 329 and 381 (Table 3). When using these bucks insemination time affected both functional spermatozoal transport ($P=0.0001$) and fertilization rate ($P=0.034$). The significant difference ($P<0.05$) in spermatozoal transport rates between +5 h and 0 h insemination times was small, but in the −5 h treatment (i.e., 15 h before ovulation), spermatozoa from Buck 381 were present at the site of fertilization in considerably lower numbers than spermatozoa from Buck 329. A similar striking trend existed for fertilization data. When results from all does within an insemination time are pooled and fertilization rates are predicted from the functional spermatozoal transport ratio, spermatozoa from Buck 329 resulted overall in more embryos (86%, $P<0.05$) than would be expected based on the percentage of spermatozoa estimated to reach the site of fertilization (74%).

Bucks 329 and 381 were used previously in a heterospermic insemination study with equal

TABLE 1. Effect of FITC or TRITC fluorochrome labeling of spermatozoa and of different bucks on spermatozoal transport and fertilization (Experiment 1).

Bucks	Number does bred	Sperm transport (%)		Total sperm counted	Fertilization (%)		Total embryos
		TRITC labeled	FITC labeled		TRITC labeled	FITC labeled	
329	6	50[b]	50[b]	1360	56[b]	44[b]	97
380	6	50[b]	50[b]	1772	45[b]	55[b]	83
Overall	12	50	50	3132	51	49	180

[a]Based on the total fertilizing sperm and sperm attached to the ova or zygote for all does per group.

[b]Values with the same superscript within a column are not different ($P>0.05$).

TABLE 2. Effect of insemination time on spermatozoal transport and fertilization by FITC or TRITC fluorochrome-labeled spermatozoa (Experiment 1).

Insemination time relative to		Number of different does bred	Sperm transport (%)		Total sperm counted	Fertilization (%)		Total embryos
LH (h)	Expected ovul. (h)		TRITC labeled	FITC labeled		TRITC labeled	FITC labeled	
+5	5	4	49[b]	51[b]	1867	50[b]	50[b]	66
0	10	4	52[b]	48[b]	971	55[b]	45[b]	64
−5	15	4	50[b]	50[b]	294	46[b]	54[b]	50
Overall			50	50	3132	51	49	180

[a]Based on the total fertilizing sperm and sperm attached to the ova or zygote for all does per group.

[b]Values with the same superscript within a column are not different ($P>.05$).

numbers of spermatozoa (J. Vallet, unpublished where does were allowed to kindle following LH injection at the time of insemination. Paternal parentage of offspring was determined by coat color, as one buck was a homozygous recessive for albino coat color (of Dutch parentage) and one was a homozygous dominant for Dutch coat color. Of the 34 young born, 75% were sired by Buck 329. This is similar to the 81% of the 177 embryos recovered (Table 3) that were fertilized by spermatozoa from Buck 329. The kindling and fertilization percentages were not different ($\chi^2 = 0.93$, $P=0.33$).

Bucks 051 and 080. The time of insemination affected both functional spermatozoal transport ($P=0.0001$) and fertilization ($P=0.008$) for Bucks 051 and 080. Spermatozoal transport and fertilization at the 0 h time period was lower for Buck 051 as compared to the other two time periods. The same trends always appeared in both spermatozoal transport and fertilization for these two bucks. Buck 051 fertilized more eggs (72%) than was predicted by estimated spermatozoal transport (60%, $P=0.016$).

Bucks 080 and 271. Fewer does than planned were bred with spermatozoa from Bucks 080 and 271 due to the death of Buck 271 before replication was completed. There was no effect of the time of insemination of functional spermatozoal transport ($P=0.64$). However, the fertilization frequency was different ($P=0.04$) at the +5 h time of insemination than at the 0 h and −5 h time periods. Also at this +5 h time the 50% fertilization rate for Buck 271 was different ($P=0.04$) from what would have been predicted by 24% spermatozoal transport.

Experiment 3

Results are presented in Table 4 by pairs

TABLE 3. Effect of insemination time on spermatozoal transport and fertilization with competitive insemination of labeled spermatozoa from three pairs of bucks (Experiment 2).

Insemination time relative to LH (h)	Insemination time relative to Expected ovul. (h)	No. of does[a]	Sperm transport[b] (%)		Total sperm counted	Fertilization (%)		Total embryos
			Buck 381	Buck 329		Buck 381	Buck 329	
+5	5	7	27[c]	73[c]	1496	14[c]	86[c]	74
0	10	7	30[d]	70[d]	2555	19[d]	81[d]	177
−5	15	4	11[e]	89[e]	843	7[c]	93[c]	92
Overall		18	26	74	4894	14	86	343
			Buck 051	Buck 080		Buck 051	Buck 080	
+5	5	3	62[c]	38[c]	2587	75[c]	25[c]	113
0	10	3	54[d]	46[d]	2215	62[d]	38[d]	120
−5	15	3	65[c]	35[c]	1666	79[c]	21[c]	124
Overall		9	60	40	6468	72	28	357
			Buck 080	Buck 271		Buck 080	Buck 271	
+5	5	2	76[c]	24[c]	699	50[c]	50[c]	46
0	10	2	77[c]	23[c]	874	74[d]	26[d]	39
−5	15	2	78[c]	22[c]	177	73[d]	27[d]	33
Overall		6	77	23	1750	64	36	118

[a]Does with more than two fertilized ova.

[b]Based on the total fertilizing sperm and sperm attached to the ova, zygote, or its investments for all does per group.

[c-e]Values with different superscripts within a column and buck pairs are different ($P<0.05$).

TABLE 4. Acrosin, hyaluronidase, and arylsulfatase activity in rabbit spermatozoa (Experiment 3).

Buck	Acrosin (mU/10^7 sperm)[a]	Hyaluronidase (mU/10^8 sperm)[a]	Arylsulfatase (mU/10^9 sperm)[a]
381	127.6 ± 25.0[b]	101.9 ± 4.4	241.5 ± 25.3
329	153.5 ± 22.6	116.4 ± 1.4	259.7 ± 18.6
051	88.6 ± 13.5	115.7 ± 1.6	273.6 ± 6.9[a]
080	132.3 ± 22.5	118.6 ± 2.3	171.7 ± 14.7[d]

[a]1 mU = 1 nmol of substrate cleaved/min.

[b]Mean ± SEM ($n = 4$).

[c-d]Values with different superscripts are different ($P<0.05$).

previously tested (Table 3). Due to the death of Buck 271 only four ejaculates each from Bucks 329, 381, 080, and 051 were analyzed for acrosomal enzyme content. No significant differences between Bucks 329 and 381 were found in the levels of acrosin (P=0.74), hyaluronidase (P=0.16), or arylsulfatase (P=0.72) in spermatozoa, although there was a trend for the actual values of all three enzymes to be higher in spermatozoa from Buck 381.

No difference in acrosin (P=0.28) or hyaluronidase (P=0.51) activity was found between spermatozoa from Bucks 051 and 080. There was significantly less arylsulfatase activity (P=0.040) in spermatozoa from Buck 080 than from Buck 051.

DISCUSSION

Competitive fertilization is a highly sensitive technique for comparing fertility of males (Beatty et al., 1969). Coat colors (Beatty et al., 1969; O'Reilly et al., 1972), blood groups (Stewart et al., 1974), and chemical or radiation mutagens (Overstreet and Adams, 1971; Fischer and Adams, 1981) have been used to establish paternal parentage of conceptuses and offspring.

In the present study fluorochrome labeling of spermatozoa was used. Concentrations of TRITC and FITC were established that fulfilled the desired conditions of not altering sperm transport to the site of fertilization or ability to fertilize ova relative to each other. Both could be studied in the same doe. When Bucks 381 and 329 were compared at 0 h (Table 3), 70% of the spermatozoa were transported and 81% of the eggs were fertilized by sperm from Buck 329. A competitive breeding test, without the use of dyes, between this pair consisting of a true-breeding Dutch and an albino buck established a birth ratio of 75% young sired by Buck 329 and 25% by Buck 381. These results are not different. Therefore, FITC and TRITC labeling of spermatozoa does not appear to alter the ability of a male's spermatozoa to compete successfully in heterospermic inseminations, under the conditions of dye concentrations used here.

Advantage was taken of the rabbit as an induced ovulator to control the interval from insemination to ovulation of about 5, 10, and 15 h. However, animals were flushed 18 h after LH injection, or about 8 h after ovulation. Spermatozoa were thus in the female tract a total of 13, 18, and 23 h, respectively. By using a low number of spermatozoa inseminated per female (0.5×10^6), when oviducts were flushed only a few spermatozoa (<5), motile or not, were not attached to the oocyte or its investments. The count of spermatozoa attached to the oocyte or its investments therefore represents the number of spermatozoa recoverable from the oviduct at that time. Spermatozoa did not appear to accumulate in the oviduct with increasing time in the female since the number of sperm counted decreased. This may be due to a gradual removal of spermatozoa from the uterus, uterotubal junction, and lower isthmus of the oviduct, with spermatozoa then moving up to the site of fertilization at the time of ovulation (Overstreet and Cooper, 1979). These results support the contention that counting accessory and fertilizing spermatozoa is a useful means of determining functional spermatozoal transport. Furthermore, conventional methods of counting spermatozoa transport by flushing techniques have been found to yield highly variable results with substantial errors (Waugh, 1984).

From the results presented in Table 3 it appears that males differ in fertility primarily because of the number of functional spermatozoa transported. When the female is inseminated with the same number of spermatozoa from each male, spermatozoa from one male reached the site of fertilization and bound to ova in higher numbers than spermatozoa from another male. Heterospermic insemination tests have been shown to be more sensitive and highly correlated to homospermic insemination results (Beatty et al., 1969; Martin and Dziuk, 1977). Therefore, it is likely that differences among males in the selective transport of functional spermatozoa by the female may play a role in determining fertility of males following homospermic insemination.

In the present experiments it was observed that different females inseminated with the same number of spermatozoa from the same ejaculate had vastly different numbers of spermatozoa attached to oocytes (Experiment 1). Females with the ability to transport large numbers of functional spermatozoa to the oviduct, even when small numbers of spermatozoa are inseminated, would likely not show differences in fertility with homospermic inseminations from different males because all ovulated ova would be fertilized. However, the females with inherent ability to transport only a few functional spermatozoa when homo-

spermically inseminated with the same number of spermatozoa from different males may reflect large differences in fertility between the males, as was found here (Table 3).

The fertilization rate was also affected by the time of insemination relative to ovulation. It appears that spermatozoa from Buck 381 did not survive as long as spermatozoa from Buck 329 (Table 3) or they were selectively eliminated. This normally would not affect results when insemination was followed immediately by LH injection or endogenous LH release, as would occur in natural mating in rabbits. This finding can have implications for cattle artificial insemination. With variable estrus detection procedures in cattle, and variable ovulation, insemination takes place at varying times relative to ovulation. Therefore, fertility of semen from different males could be differentially affected by time of insemination if survival and transport rates within the tract of different females differs.

The reasons for differential transport and survival in females were not investigated in these studies. However, a few speculative possibilities include: 1) spermatozoal viability when in female tract secretions for variable periods of time; 2) preferential removal of spermatozoa from the tract by phagocytosis; or 3) inability of spermatozoa to pass a barrier that is a function of the period of time they are exposed to female tract secretions.

Another example of the effect of interval of time from insemination to ovulation is the result for Bucks 271 and 080 (Table 3). With 5 h elapsing between insemination and ovulation, 50% of the eggs were fertilized by Buck 271 with only 24% of the spermatozoa present surrounding the zygotes coming from Buck 271. Spermatozoa of Buck 271 may capacitate faster relative to spermatozoa of Buck 080. Capacitation occurs over a period of time and a population of capacitated spermatozoa is expected to accumulate in the lower isthmus of the oviduct with the passage of time. Heterospermic insemination with semen from males producing spermatozoa with different rates of capacitation could result in spermatozoa from the faster capacitating male being able to fertilize ova sooner after arriving at the site of fertilization. This has been observed in double mating experiments (Dziuk, 1965) where the first male sired the majority of the litter. The advantage of rapid capacitation would be particularly apparent when insemination occurs near the time of ovulation. In contrast, total spermatozoa transported to the oviduct would be the major determinant of fertilization rate when there was adequate time for a large number of spermatozoa to be capacitated before ovulation. These factors may play a role in homospermic insemination of cattle when the female is inseminated close to or even after ovulation, and the ovum may age sufficiently prior to fertilization that a viable zygote does not result.

Another competitive advantage of spermatozoa from different males could be associated with potency of acrosomal enzymes and speed of egg penetration, and this was investigated. In Experiment 2 (Table 3), Bucks 329 and 051 fertilized more eggs at all insemination times than might be expected from the transport studies. Since this occurred at all times studied, it appears to be an egg penetration advantage rather than a capacitation advantage, as appears to be the case with Buck 271. This suggests that acrosomal enzymes may regulate differences in fertility between males. While numerous enzymes are located in the acrosome, with limited number of spermatozoa in an ejaculate, only a few enzymes can be quantified. Acrosin, hyaluronidase, and arylsulfatase have all been proposed to be involved in fertilization. Any one or a combination of these enzymes may be responsible for differences seen.

To test the hypothesis that acrosomal enzyme content of spermatozoa can affect competitive fertilization results, the content of acrosin, hyaluronidase, and arylsulfatase was evaluated in spermatozoa from two pairs of males. There tended to be small differences between pairs, but only arylsulfatase was found to be different in one pair of the males. Arylsulfatases have been suggested to desulfate mucopolysaccharide in the zona pellucida (Akruk et al., 1979). While arylsulfatases cannot in themselves dissolve the zona pellucida, they may intensify the action of other enzymes by providing better accessibility to a substrate. The more fertile males tended to have more of the acrosomal enzymes. A multiple index based upon the three enzymes was considered, but it was not applied to the limited data because exact roles and importance of particular enzymes are not known. Acrosin, hyaluronidase, and arylsulfatase may well interact or be involved with other acrosomal enzymes in mediating ability of spermatozoa to fertilize oocytes.

These experiments demonstrated directly that the fluorochromes used to stain spermatozoa with dye concentrations sufficient to identify paternal parentage up to the pronuclear stage of development do not reduce fertility. Such stained spermatozoa used for heterospermic insemination can isolate possible causes of fertility differences. These include spermatozoal transport and survival, spermatozoal capacitation time, and egg penetration asssociated with acrosomal enzyme content. While all these effects contribute to fertility at a single time of insemination relative to ovulation, by varying this period, insight into the importance of the individual effects was obtained. The number of spermatozoa transported that have fertilizing potential appears to be the most important factor in these experiments.

Although the test was performed with the rabbit, an induced ovulator, the design with three intervals of time from insemination to ovulation permits extrapolation of results to species that are spontaneous ovulators. These may be species that either exhibit behavioral estrus (cow, sow, mare, ewe) or do not exhibit estrus (humans). The implications of the results are particularly important in considering potential male effects on fertility in humans, where semen deposition can occur at any time during the menstrual cycle, rather than a defined period of estrus and associated ovulation.

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