

**Separation of Bovine Spermatozoa by Density  
on Water Insoluble Newtonian Gels and  
Their Use for Insemination**

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**ABSTRACT**

Bovine spermatozoa were separated into different density subpopulations utilizing water insoluble hydrocarbon and silicone gels of defined specific gravity. Sperm density profiles were generated for 13 bulls. The separations were found to be repeatable and characteristic of the bull examined. Considerable density variation among animals was demonstrated. Analysis of the separated spermatozoa, before and after freezing, demonstrated that good motility and acrosomal integrity of spermatozoa were maintained. When the least dense fraction of spermatozoa was used for insemination, conception rates were similar to those obtained routinely by artificial insemination with unfractionated spermatozoa. Therefore, this system may be useful in separating spermatozoa of various densities and for removing extraneous matter from semen. However, the sex ratio, among 51 60-day-old fetuses recovered from heifers inseminated with the lowest density fraction of spermatozoa, was 26 males:25 females.

**INTRODUCTION**

Control of sex ratio in livestock, poultry and other animals to increase efficiency of food production is potentially worth millions of dollars annually (Foote and Miller, 1971). There have been many reports of attempted "sexing of semen" (Beatty, 1974; Anonymous, 1978). However, whenever extensive testing is done the sex ratio of progeny resulting from sexed semen is either unaffected or only slightly altered. Furthermore, it is likely that there is a bias which favors publishing positive results but not negative ones.

The techniques primarily used to separate the 2 types of spermatozoa are based upon assumptions that they differ in 1) density, 2) electrical charge or 3) surface immunochemical properties. Techniques based upon the latter 2

assumptions, while potentially exciting, have yielded overall nonreproducible and negative results. This is also true of studies based upon density. However, following the publication by Ericsson et al. (1973) of the use of bovine serum albumin (BSA) gradients, there was renewed interest in density gradient separation, but the large scale trial of this technique (Ericsson, 1980) gave mainly negative results.

The difference in density between X- and Y-chromosome containing spermatozoa is expected to be only about 3% (Bahr, 1971), with much overlap in density expected due to the variation in several components of the sperm cell. Thus, complete separation of the 2 types of spermatozoa is not expected, but with highly sophisticated techniques of density separation it should be possible to isolate fractions differing greatly in density and in the proportion of the 2 types. Recently, it has been demonstrated that lymphocytes can be separated from other formed elements in blood of slightly different specific gravity using thixotropic water insoluble gels of precisely defined density (Luderer et al., 1979). Only those cells of lower density than

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the gel remain at the gel-aqueous interface following centrifugation. Cellular elements of higher density pass through the nonaqueous gel, pelleting at the tube bottom.

The objectives of the experiments reported were to examine bovine spermatozoa in this gel separation system with respect to: 1) effectiveness of this system for separating spermatozoa by density for any purpose, 2) the motility and morphology of separated spermatozoa, and 3) fertility and sex ratio of fetuses using the least dense fraction of spermatozoa.

## MATERIALS AND METHODS

### *Preparation of Newtonian Gels*

The hydrocarbon and silicone fluid based gels were prepared using identical procedures and equipment. The difference between the 2 gels was the amount of silica filler added to each of the base fluids to arrive at the same specific gravity. Water insoluble, nonthixotropic hydrocarbon based gels (Zine, 1974; Zine, 1977; Hertl, 1976) were formulated from polybutadiene oil ARCO-R-45-HT (Arco Chemicals, Philadelphia, PA) and OX-50 (Degussa Corporation, Teterboro, NJ), a fumed nonmethylated silica, according to Luderer et al. (1980). The water insoluble, nonthixotropic, silicone based gels were formulated from Dow Corning 200 fluid (30000 centistokes, Dow Corning Corp., Midland, MI) and OX-50 according to Zine (1974, 1977) and Hertl (1976). Both the hydrocarbon and silicone based gels were Newtonian, possessing thixotropic indices  $<1.0$ , and thus behaved like fluids with high viscosity.

High density base gels (1.1093 g/cc hydrocarbon base, 1.1143 g/cc silicone base) were prepared by the addition of 45 g and 29.01 g OX-50 to 100 g of the hydrocarbon and silicone oil respectively. The components were mixed using a metal spatula and then passed 3 times through a 3 roll mill to insure that the mixture was homogenous. Gels were then placed in a vacuum oven for 5 h at 180°C, and remained in the vacuum oven at 120°C for an additional 16 h. Gels were removed from the oven, cooled to room temperature and passed 3 times through a 3 roll mill. The milled gels were re-treated in the vacuum oven at 180°C for an additional 3 h, allowed to cool to room temperature and then passed through a 3 roll mill 3 more times. Gels were then degassed under vacuum to remove any air entrained by the milling process.

Gels of varying specific gravities were prepared by diluting the stock gel with varying amounts of either polybutadiene oil ARCO-R-45-HT for the hydrocarbon base gel or Dow Corning 200 fluid for the silicone base gel. After the fluid addition, gels were mixed, milled and degassed prior to specific gravity measurement. Gels were stored dry under vacuum until used.

### *Semen*

Bull semen was obtained from Eastern Artificial Insemination Cooperative, Inc., Ithaca, NY, following collection with an artificial vagina. The sperm cell concentration was estimated spectroscopically. Their

progressive motility was estimated conventionally by use of a bright field microscope, and a 37°C heat stage. Individual samples were extended in sterile buffer consisting of Dulbecco's phosphate-buffered saline, pH 7.2, 0.15 M (Grand Island Biologicals, Grand Island, NY), supplemented with 1% BSA (Miles Labs, Elkhart, IN) and 100 µg/ml gentamycin. Semen was transported to Sullivan Park, Corning, NY, in a closed styrofoam container which contained water initially at 37°C. During transport, the water and semen cooled very slowly, preventing thermal shock. Upon arrival, samples were removed from the container and allowed to equilibrate to 22°C.

### *Gel-Spermatozoa Separation*

One ml of desired specific gravity gel was carefully deposited on the bottom of 10 X 75 mm plastic test tubes followed by brief 2000 X g centrifugation to uniformly seat the gel in the tube bottom. One ml of extended bull semen (typically 8 to 10 X 10<sup>8</sup> cells) was pipetted directly on top of the gel followed by centrifugation in a Sorvall RC-2B refrigerated centrifuge (Dupont, Wilmington, DL), at 22°C, for 1 h at 5000 X g. The spermatozoa placed on the gel are referred to as input, and recoveries are expressed as a percent of this input. Alternatively, separations were performed in a Beckman #L5-75 ultracentrifuge, utilizing a swinging bucket rotor (SW-65), at 22°C for 1 h at 62000 X g (25000 rpm). At completion of centrifugation, the spermatozoa deposited on top of the gel barrier were gently removed. When all spermatozoa on top were removed, the tube was rotated to the horizontal position and the gel barrier penetrated with a fresh Pasteur pipet. Buffer was then forced between the water insoluble gel and the semen pellet until all sperm on the tube bottom were recovered.

### *Counting Spermatozoa and Volume Estimation.*

Sperm cell counts were performed on a Coulter Model ZBI Counter (Coulter Electronics, Hialeah, FL) utilizing a 50/60 µ diameter aperture tube. Spermatozoa were sized on a Coulter H-4 channelyzer calibrated with spherical particles 2.02 µm in a diameter (Coulter Electronics).

### *Freezing and Evaluating Spermatozoa for Insemination*

This phase of the study was conducted with great care to insure validity of the data, but is described only briefly because of the lack of effect on sex ratio. Separated spermatozoa were centrifuged at 1500 X g for 10 min at 22°C, the sperm pellet resuspended in standard heated bovine milk extender (Eastern Artificial Insemination Coop., Inc.) and cooled slowly to 4°C. Semen was frozen the next day by Eastern after packaging 40 X 10<sup>6</sup> spermatozoa per 0.5 ml French straw.

A random set of straws was thawed and evaluated for the percentage of motile spermatozoa and the proportion of intact acrosomes by a person who did not know the coding. The inseminator also did not know the coding.

One breeding experiment was performed to test fertility of isolated light and heavy fractions of sper-

matzoza. A second experiment was designed to test the sex ratio of separated spermatozoa from a bull for which the sex ratio of 1909 recent progeny was known. Thus, the sex ratio obtained from the unseparated semen produced by this bull was accurately established.

Cattle inseminated were purchased beef heifers transported to Willow Lane Farm, West Berne, NY. Any animals found pregnant or with infantile reproductive tracts were sold. Those retained were inseminated with coded semen approximately 12 to 24 h after estrus was first observed. For the fertility trial, inseminated animals which did not conceive, and which recycled, were reinseminated. For the sexing trial, only 1 insemination per heifer was performed. Animals were pregnancy checked by a veterinarian 45 days after the last insemination. In the sexing trial confirmed pregnant heifers were slaughtered 15 days later (pregnant 60 days), the individual uteri collected and the fetal sex determined by gross examination.

Statistical analysis of sex ratio data was performed utilizing the chi-square test and the null hypothesis, with  $P < 0.05$  being required to reject the hypothesis. Because there are only 2 types of events, male and female, there is  $(n-1)=1$  degree of freedom.

## RESULTS

### *Separation of Sperm on the Basis of Density*

When semen is centrifuged in the presence of water insoluble hydrocarbon gels of known specific gravity, separation of the sperm into

various density populations is achieved. Figure 1 illustrates 1 series of separations of the extended semen from donor bull H ( $5000 \times g$ , 1 h at  $22^\circ\text{C}$ ) in Newtonian gels of 6 increasing densities. The percentage of total sperm cells recovered on the gel surface and in the pellet are plotted as a function of gel specific gravity. Cell recoveries were always less than 100% and typically were 80 to 85%. From microscopic examination of the gel after centrifugation, it appeared that the unrecovered sperm cells were trapped in the gel. Some of these cells were believed to be at buoyant equilibrium. For purposes of discussion, all unrecovered cells are assumed to be present in the gel phase and are included in the figure as such. At specific gravities of 1.0802 g/cc and below, the majority of spermatozoa are found beneath the gel in the pellet. Five to 6% remain on the gel surface, and 15 to 16% appear trapped within the gel. At specific gravities of 1.0822 g/cc and above, approximately 85% of all cells were found at the gel surface, 15% with the gel and no cells beneath the gel barrier in a pellet. The most striking feature of this particular sperm separation profile is the extremely narrow specific gravity range within which a shift of cells from bottom to top of gel occurs. The point of intersection of the curves should approximate

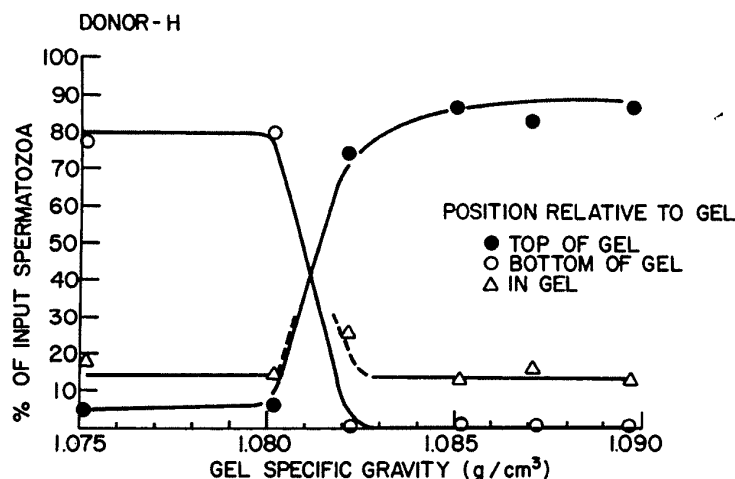


FIG. 1. Newtonian hydrocarbon gel separation of donor bull H ( $5000 \times g \times 1 \text{ h}$ ,  $22^\circ\text{C}$ ). The percent of input spermatozoa recovered on the gel surface or in the pellet beneath the gel are plotted as a function of gel specific gravity. Unrecovered spermatozoa are plotted as percent within the gel. Recoveries generally range between 80 to 85%. ●— spermatozoa on top of gel; ○— spermatozoa in the pellet beneath the gel; △— spermatozoa within the gel.

the average specific gravity of sperm cells under the conditions of the experiment. The width of the inflection should approximate the specific gravity distribution of cells.

The slopes of the cell density profiles were not always steep. The density profile of sperm from bull *I* is shown in Fig. 2. With this sperm sample, the crossover was broad, covering 0.008 density units, 4-fold broader than bull *H*. This suggests that the sperm cell density distribution was broader than that of bull *H*. It should be noted that on the 1.0851 g/cc gel, 50% of the cells were recovered above and 50% recovered below the gel. The average specific gravity of spermatozoa in this sample would be 1.0851 g/cc under these conditions.

Within another bull, *J* (not shown), little or no sperm were found on the gel surface or in the pellet near the point of crossover. The cells were all within the gel as verified by microscopy of a gel smear. If gels are homogeneous with respect to specific gravity (i.e. a gel gradient is not set up) and if the cell density distribution is narrow, one might expect all cells within the gel to be at their buoyant position at the crossover specific gravity. This would appear to be the case for this ejaculate of semen.

#### Among and Within Animal Variation

Three ejaculates from Bull *H* collected at 2-week intervals were separated in the gel system at 5 gel-specific gravities (see Fig. 1 for 1 of these ejaculates). One can draw a bar so that its length approximates the specific gravity

range which defines the curve's steepness (95% response), and a line within the bar represents the crossover point. The bar length also approximates the cell density distribution under these conditions. In Fig. 3 the 3 ejaculates for bull *H* are shown. The width of the crossover remained constant, but the crossover point shifted slightly during the 3 collections over a period of 1 month. Also shown in Fig. 3 are results with other bulls separated under identical conditions. Not only does one observe a wide variation in crossover points between different bulls (vertical lines on bars) but also a large variation in width of the crossover. This is particularly apparent in bulls *M*, *P* and *Q*, suggesting that these animals have wide variation in sperm cell specific gravity.

#### Comparison of Sperm Density Profiles with Coulter Volume Analysis

Sperm cells, as well as other cell types, mammalian or bacterial, display a wide variation of volumes. This is not due primarily to differences in nucleic acid content, but to variations within the cytoplasm caused by age and other factors (Bredderman and Foote, 1969; Foote and Bredderman, 1969). An example of a typical sperm cell volume distribution is given in Fig. 4. A 2-fold variation in volume from  $16 \mu^3$  to  $32 \mu^3$  is typically observed. One would expect that this should result in a large density variation. This has been a problem complicating the density-based method of separation. The

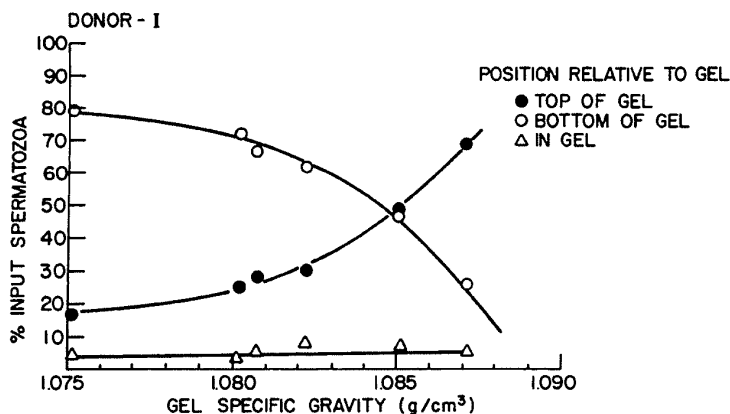


FIG. 2. Newtonian gel separation of donor bull *I*. See legend of Fig. 1.

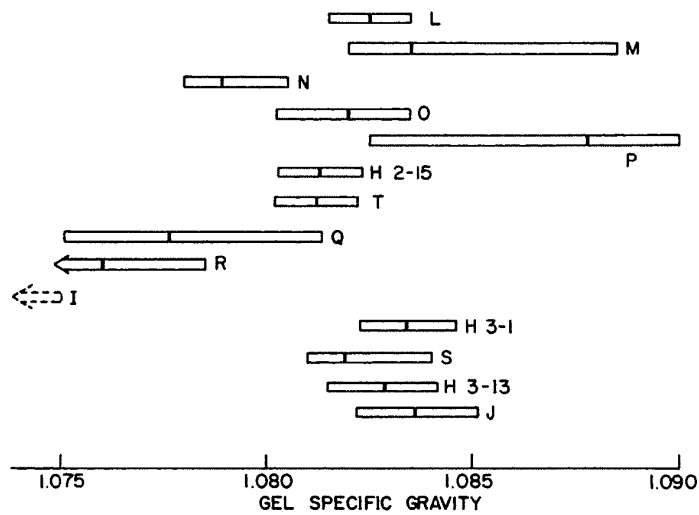


FIG. 3. Among and within animal variation. Summary of individual bulls separated on Newtonian hydrocarbon gels of defined specific gravity at 5000 X g for 1 h at 22°C. Crossover point is represented by the line within the bar and the length of the bar approximates the specific gravity range which defines the curve's steepness (95% response).

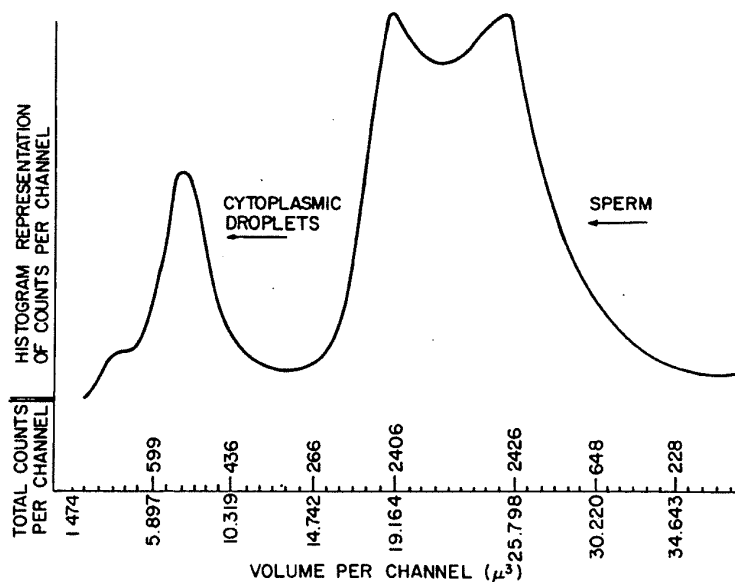


FIG. 4. Coulter H-4 channelyzer analysis of bull sperm. The number of counts per channel is plotted against channel number which was previously calibrated to 0.73  $\mu^3$ /channel.

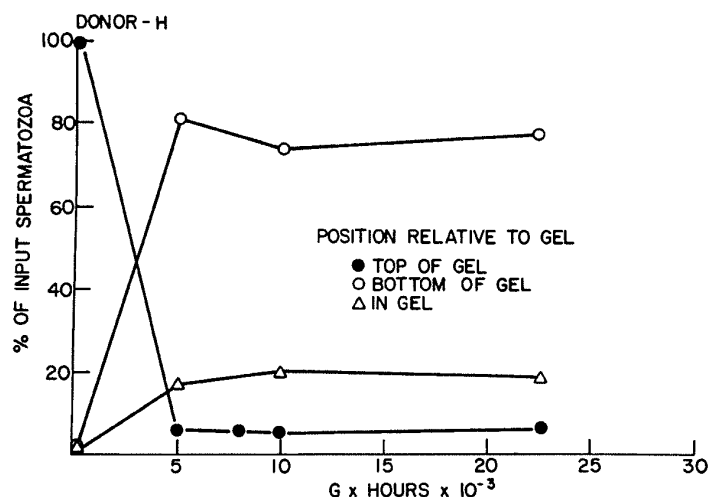


FIG. 5. Equilibrium studies on donor bull *H* spermatozoa, utilizing Newtonian hydrocarbon gel specific gravity=1.0802. Separations were performed at 5000  $\times g$  for 1 and 2 h and at 22000  $\times g$  for 1 h, 22°C. The percent of total input spermatozoa are plotted as a function of  $g \times h$ . ●— spermatozoa on top of gel; ○— spermatozoa in pellet beneath gel; △— spermatozoa within gel.

same cells which display wide volume variation in the Coulter counter have characteristically narrow density distributions when evaluated in the gel systems. The reason for this is unknown. The fact that volume analysis of spermatozoa separated on gels (either top or bottom fraction) reveals no significant alteration in volume distributions from the initial distribution may reflect fluid influx in the counting fluid.

#### Equilibrium Conditions

The question of whether the separation of sperm cells was based upon specific gravity differences, or whether we were simply monitoring a kinetic phenomena rather than reflecting specific gravity equilibrium was examined. To ascertain whether the Newtonian gel separations were achieving equilibrium conditions, separations were performed at 5000  $\times g$  for 1 and 2 h and at 22000  $\times g$  for 1 h on a gel of specific gravity 1.0802 g/cc. The results are shown in Fig. 5. The percent of total input cells recovered on the gel surface and in the pellet are plotted as a function of  $g \times h$ . Unrecovered spermatozoa are plotted, for purposes of discussion, as percent sperm cells within the gel. Equilibrium appears to be achieved at 5000  $\times g \times 1$  h, as evidenced by no further change in sperm distribution as either time or  $g$  increased.

The percent of spermatozoa in the gel (total input-total top and bottom recovered) is a constant 20% and appears to represent entrapment of cells in the gel, most likely cells with a density similar to the gel.

In a related series of experiments, semen from 3 bulls was separated for 1 h on hydrocarbon and silicone based gels at various specific gravities under 2 g loads, 5000  $\times g$  and 62200  $\times g$ . Top and bottom fractions were isolated, counted and the data are given in Table 1. Although large errors can be introduced during recovery, dilution and Coulter counting, data for both gel systems adequately redemonstrate that the system is at equilibrium under standard (5000  $\times g$ , 1 h) separation conditions.

#### Reseparation Studies

If the gels are truly fractionating cell populations on the basis of specific gravity, without mixing of cells due to nonequilibrium or meniscus entrapment, then cells initially purified as low and high density fractions should reseparate on the same specific gravity gels as pure fractions. Refractionation experiments were performed for both top and bottom fractions and the results are presented in Table 2. In most instances, low density cell populations (initially 2 to 6% of total cells), when

TABLE 1. Comparative separation data when utilizing identical gels at low and high G forces.

Gel type	Specific gravity of gel	Bull	Cells on top, %	
			5500 × g	62200 × g
Silicone	1.0683	A	4.86	4.02
Silicone	1.0683	B	2.42	3.88
Silicone	1.0762	B	5.33	6.21
Silicone	1.1143	C	100	100
Hydrocarbon	1.0699	A	3.46	1.93
Hydrocarbon	1.0699	B	4.29	1.65
Hydrocarbon	1.0751	B	3.88	5.73
Hydrocarbon	2.0751	C	5.60	2.04
Hydrocarbon	1.0807	C	4.07	5.80
Hydrocarbon	1.1172	C	100	100 <sup>a</sup>

<sup>a</sup>31000 × g.

resedimented, were recovered on the gel surface for both of the gel systems (range 63 to 102% recovered). In no instance were any cells observed beneath the gel. Low density fractions appear, therefore, not to be system artifacts but truly are of low specific gravity.

High density cells, when resedimented, were recovered with 73 to 126% efficiency. The quantitative recovery of bottom fraction spermatozoa is extremely difficult, due primarily to the necessity of manipulating the gel away from the pellet, followed by gradual dilution of the pellet and bottom exposure to remove all spermatozoa. In all instances 10 to 15% of the

input cells were found above the meniscus. Apparently some cells, when passed through a nonsoluble gel and resuspended, have altered densities. These data generally support our previous data indicating that spermatozoa are separating according to specific gravity.

#### *Physiological Properties of the Separated Spermatozoa*

Preliminary physiological analysis of gel-separated spermatozoa was performed at Cornell University on coded samples. These data (not presented) indicated that *both* top and

TABLE 2. Sperm isolated from top or bottom of the gel separation systems and recentrifuged to their original position.

Gel type	Specific gravity	Donor	1st separation <sup>a</sup>	2nd separation <sup>b</sup>	% Recovery 2nd separation
<i>Top fraction</i>					
Silicone	1.0683	B	$8.22 \times 10^6$	$5.18 \times 10^6$	63
Hydrocarbon	1.0699	B	$5.32 \times 10^6$	$4.45 \times 10^6$	84
Hydrocarbon	1.0699	A	$1.99 \times 10^7$	$1.85 \times 10^7$	93
Hydrocarbon	1.0751	B	$9.61 \times 10^6$	$9.77 \times 10^6$	102
<i>Bottom fraction</i>					
Silicone	1.0683	D	$2.85 \times 10^8$	$2.08 \times 10^8$	73
Silicone	1.0762	D	$1.96 \times 10^8$	$1.85 \times 10^8$	94
Hydrocarbon	1.0751	D	$1.29 \times 10^8$	$1.63 \times 10^8$	126

<sup>a</sup>Spermatozoa recovered from their respective top or bottom gel fraction 5500 × g × 1 h and reapplied to a new gel of identical specific gravity.

<sup>b</sup>Number of spermatozoa recovered from the top or bottom gel fraction after the second 5500 × g × 1 h centrifugation.

TABLE 3. Motility and acrosomal characteristics of cryopreserved gel separated spermatozoa.

Bull	Gel specific gravity	Gel fraction	% of input	Straw code	Post-thaw evaluation	
					% motile	% intact acrosomes <sup>a</sup>
E	1.0751 <sup>b</sup>	Top	22	Yellow	35	54
E	1.0802 <sup>b</sup>	Top	33	Red	35	71
F	1.0751 <sup>b</sup>	Top	13	Purple	35	33
G	1.0751 <sup>b</sup>	Top	22	Gray	30	55
F	1.0851 <sup>c</sup>	Bottom	44	Blue	15	ND <sup>d</sup>

<sup>a</sup>Expressed as the percent of spermatozoa demonstrating intact, undamaged acrosomes; 200 sperm examined.

<sup>b</sup> $1.6 \times 10^8$ /ml.

<sup>c</sup> $1.42 \times 10^7$ /ml.

<sup>d</sup>ND=not done.

bottom fractions of spermatozoa retained a significant portion of their progressive and total motility when compared to sham-treated samples. To address whether separated sperm cells could be cryogenically preserved, samples were separated and frozen in straws. Representative samples were retrieved from liquid nitrogen storage, rapidly thawed to 37°C (~1 min) and analyzed for motility and acrosomal integrity (Table 3). It can be seen that top fractions of frozen and thawed spermatozoa retained sufficient motility and intact acrosomal membranes to be used for insemination. Bottom fraction spermatozoa also successfully survived freezing, although the quantification

of motility was difficult due in part to the low concentration of spermatozoa. The bottom fraction was not analyzed further because only the more easily recovered top fraction contained the spermatozoa to be used for breeding trials.

#### Fertility Trial

Table 4 summarizes the physical parameters, the assessment of semen fractions and the observed conception rates in the fertility trial and later in the test of sex ratio. Most of the fertility data were obtained from donor bull E, with 22 and 33% of the total input spermatozoa remaining above the gel in 2 separations. Only 8.3% of the total input spermatozoa from bull

TABLE 4. Assessment of fractionated and cryopreserved sperm utilized to test fertility and alteration of the sex ratio.

Trial	Semen donor	Separation date	Gel Sp. gr.	Gel fraction	% of input	% motile sperm <sup>a</sup>	% intact acrosomes <sup>b</sup>	No. of heifers inseminated	Conception rate <sup>c</sup>
Fertility	E	2-08-79	1.0751	Top	22.0	35	54	21	0.52
	E	2-08-79	1.0802	Top	33.0	35	71	25	0.58
	K	2-08-79	2.0751	Top	8.3	ND <sup>d</sup>	ND	5	0.60
	K	12-04-78	1.0851	Bottom	44.0	15	ND	14	0.23
Sex ratio	J	5-07-79	1.0818	Top	14.3	40	84	45	0.29
	J	5-10-79	1.0818	Top	14.9	45	80	48	0.56
	J	6-11-79	1.0818	Top	16.9	30	ND	41	0.25

<sup>a</sup>After freezedown and rapid thawing to 37°C.

<sup>b</sup>Expressed as the percent of spermatozoa demonstrating intact, undamaged acrosomes.

<sup>c</sup>Ratio of the total number of fetuses to the total number of insemination attempts.

<sup>d</sup>ND=not determined.



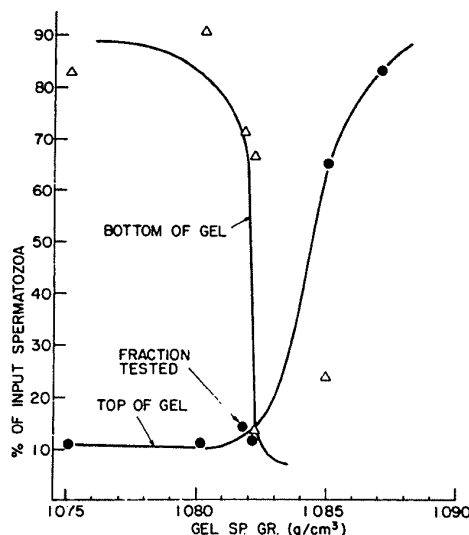


FIG. 6. Newtonian hydrocarbon gel separation of donor bull *J* (5000  $\times$  g  $\times$  1 h at 22°C). The percent of input spermatozoa recovered on the top of the gel surface (●) or in the pellet beneath the gel (Δ) is plotted as a function of gel specific gravity.

*K* were available in the top fraction. The conception rates, with limited numbers, represent reasonable pregnancy rates for commercial artificial insemination (Spalding et al., 1975; Foote, 1978).

A fraction of spermatozoa from bull *K*, representing 44% of the total input at the bottom of the separating gel, yielded the lowest conception rate in the fertility trial (0.23 pregnancies/straw). These data illustrate that a relatively dense sperm fraction, after separation in the gel system, is capable of successful insemination.

#### Sex Ratio Trial

Because the pregnancy rate was acceptable when the top fraction was used, a sex ratio study with this fraction was started. A single donor, bull *J*, with 1909 sexed progeny among single births, was chosen from the active bull stud at Eastern Artificial Insemination Cooperative, Inc. The cell density distribution of semen from donor *J* is given in Fig. 6. The sperm separation profile is similar to the profiles from other donors, differing only in the position of cell crossover. The crossover of cells from the gel surface to tube bottom occurs

sharply between densities of 1.0818 and 1.0851 g/cc. At a specific gravity of 1.0822 g/cc the majority of spermatozoa are entrapped within the gel.

Heifers were inseminated with the sperm fractions remaining above the gel of specific gravity 1.0818 (Fig. 6). Sperm of this specific gravity are on the edge of the crossover profile and this fraction represents the lightest 15%. One might expect that this fraction would be enriched with spermatozoa containing the Y chromosome.

Semen quality and conception rates on 3 semen collections processed identically, frozen, and utilized for evaluating the sex ratio of fetuses 60 days after insemination, are also shown in Table 4. Conception was low in 2 samples from bull *J* when compared with data in the fertility trial. This may be associated with the fact that bull *J* had a below average conception rate in service at Eastern Artificial Insemination Cooperative.

Table 5 summarizes the sex ratios obtained from 51 fetuses. All 51 fetuses were normal based upon gross anatomical observation. Twenty-six male and 25 female fetuses were recovered, representing no significant deviation from 1:1 (0.90 >  $P$  > 0.75). Analyzed individually, no significant deviation from 1:1 was seen in any individual semen lot (14347, 0.25 >  $P$  > 0.10; 24351, 0.75 >  $P$  > 0.5; 54378, 0.5 >  $P$  > 0.75). These data are compared with the sex ratio of 1909 progeny sired by bull *J* utilizing unseparated, untreated semen which yielded 944 males:965 females (0.75 >  $P$  > 0.50).

#### DISCUSSION

The potential for successful separation of X- and Y-chromosome bearing spermatozoa rests on the assumption that slight differences exist in the density of these 2 types of cells (Bahr, 1971). Theoretical distributions of spermatozoa are depicted in Fig. 7A. Although these distributions of cells would have significant overlap, it should be feasible with gels of the proper specific gravities to select cells on the extremes of the distribution. This enrichment should yield a population of sperm enriched for X and Y chromosomes. At correctly chosen gel specific gravities, only the most dense or the least dense cells will pellet through the gel or remain on the gel surface, respectively (Fig. 7B).

The gels used were capable of separating

TABLE 5. Sex ratio data on gel-separated and control spermatozoa from bull J.

Semen used	Lot number	Male	Female	Twins	$\chi^2$ <sup>a</sup>	P value <sup>b</sup>
Gel separated	14347	4	9	0	1.920	0.25>P>0.10 NS <sup>c</sup>
Gel separated	24351	15	12	1 <sup>d</sup>	0.332	0.75>P>0.50 NS
Gel separated	54378	7	4	0	0.818	0.50>P>0.75 NS
	Total separated	26	25	1	0.0196	0.90>P>0.75 NS
Control unseparated		944	965	114	0.231	0.75>P>0.50

<sup>a</sup> $\chi^2 = \sum \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$  Expected refers to the average number expected when the null hypothesis is true (see *Materials and Methods*). Observed refers to the number observed in the trial.

<sup>b</sup>P value as given represents the range of probability that the events observed occurred due to chance alone.

<sup>c</sup>NS=not statistically significant.

<sup>d</sup>Twins were 1 male, 1 female.

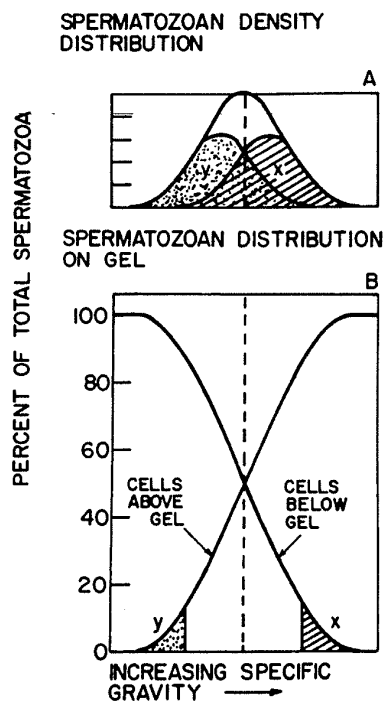


FIG. 7. A) Theoretical density distribution of spermatozoa of a single ejaculate depicted as the sum of the density distribution of X and Y bearing gametes. Speckled area—Y-bearing sperm; X-bearing sperm. B) batched area—Schematicized spermatozoan distribution after separation on gels of known specific gravity. See text for description. At the extremes of the gel separation curve, speckled area—Y-bearing sperm or batched area X-bearing sperm populations should be enriched.

ejaculated spermatozoa into different fractions according to their densities. There was a similar range in specific gravities within a bull, but a marked difference was found among bulls (Fig. 3). No systematic evaluation of a sequence of ejaculates from several bulls was undertaken. Therefore, the cause of this variation is speculative. However, it is inferred from these data that one must characterize an individual bull prior to preparative separation of a defined fraction of spermatozoa. All the animals shown in Fig. 3 were young bulls (2 to 3 years old). The effect of aging and frequency of semen collections of the donors represent unknown additional variables.

The meniscus surface tension of the gels is not believed to have affected, appreciably, spermatozoal separation. A force must be required to overcome meniscus surface tension and this force will be dependent upon the nature of the gel, the ionic composition of the aqueous phase and most likely the size and surface properties of the cell crossing the boundary. One might, therefore, expect a small random fraction of spermatozoa, denser than the gel, to remain at the meniscus due to a lack of energy required to overcome surface tension. This would result in a mixed population of low and high density cells ("male" and "female") above the gel. A reexamination of data given in Table 1 and Fig. 5 suggests this does not take place. When energy is added to the system in the form of higher centrifugal force, no significant removal of cells from the meniscus at any gel specific gravity occurred.

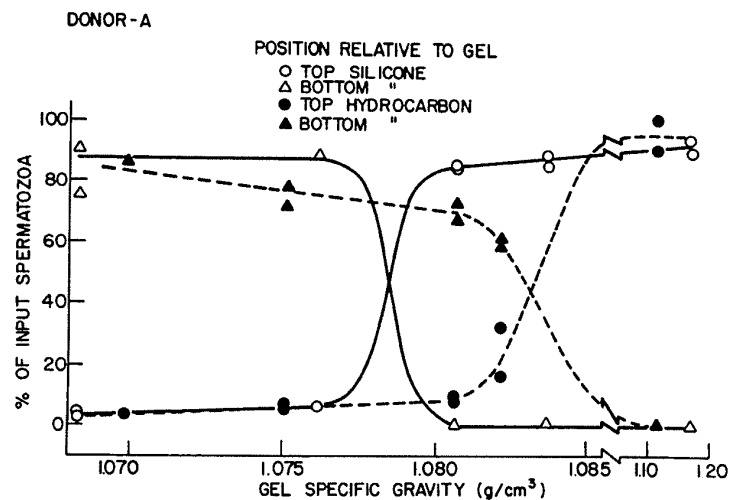


FIG. 8. Comparative abilities of Newtonian silicone and hydrocarbon gels of defined specific gravity to separate spermatozoa from donor A. Separations were performed at  $5000 \times g \times 1 \text{ h}$  at  $22^\circ \text{C}$ . Silicone gel separation: ○— spermatozoa on top of gel; △— spermatozoa on bottom of gel. Hydrocarbon gel separations: ●— spermatozoa on top of gel; ▲— spermatozoa on bottom of gel.

Silicone based gels have about one-half the surface tension of hydrocarbon gels and were included in these studies to address this particular problem. If surface tension were a factor, one would expect lower cell percentages above the gel in silicone systems than in the standard hydrocarbon system. These data (Fig. 8) do not support this hypothesis. In fact, more spermatozoa remained above the gel in the silicone than in the hydrocarbon gels at equivalent specific gravities. The shift could be a consequence of the lower hydrophobicity of the silicone system resulting in less water removal from the spermatozoa during their passage through the gel. Alternatively, the shift to lower apparent specific gravity could result from an increase in gel density as water from the aqueous phase is drawn into the contained silica particle voids with no net change in gel volume. No voids exist in the hydrocarbon system because the smaller molecular weight hydrocarbon oil can totally occupy the contained silica particles. It appears that the gel separation system, as used with our standard protocol ( $5,000 \times g$ , 1 h), fractionates cells on the basis of density alone.

The cell density distribution approximated by the gel centrifugation technique was found to be much narrower (about 0.5%) than would

be expected from the volume distributions obtained by Coulter analysis or the report by Bahr (1971). The reason for this discrepancy is unknown, but may represent influx of fluid into the sperm cells during dilution for sizing with the Coulter Counter.

The technique does not appear to alter the physical characteristics of spermatozoa nor does it appear to alter their physiological state. Separated spermatozoa appeared to be normal after cryopreservation, as judged by their motility and membrane integrity. Fertility of several fractions produced pregnancies at a rate similar to unsexed semen (Spalding et al., 1975). Processing appeared not to have any major effect on fertility, although large numbers are required to estimate fertility accurately (Foote and Oltenacu, 1980). This system may be useful in separating spermatozoa of various densities and for removing extraneous matter.

The sex ratio of 26 males to 25 females is similar to the sex ratio of 50.8% (Foote, 1977) and 50.9% (Cady and VanVleck, 1978) reported from large populations of dairy cattle in the area. It is not different from the sex ratio obtained with unseparated semen from this bull. It would appear that the random variation in sperm cell density overshadows differences imparted by the X or Y chromosome, even at

the extremes of the density distribution. The published literature on sex separation has been reviewed (Beatty, 1974; Anonymous, 1978) and no evidence of consistent success in cattle was found.

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