Comparative Motility of X and Y Chromosome–Bearing Bovine Sperm Separated on the Basis of DNA Content by Flow Sorting

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ABSTRACT A combination of flow cytometric sperm sorting of X and Y chromosome–bearing sperm (X and Y sperm) and computer-assisted sperm analysis (CASA) for measuring sperm motility allows assessment of motion parameters in the two populations. Bull sperm were separated into X and Y populations by flow cytometry following staining with the DNA-binding dye Hoechst 33342. The motion parameters differed depending on sperm concentration. Decreasing sperm concentration resulted in higher velocities and straighter trajectories. The concentrations of control (stained-unsorted and unstained-unsorted) and flow-sorted sperm were therefore adjusted to similar numbers (5 \times 10^6 sperm per milliliter). Samples of sorted X and Y sperm and control sperm were transferred to prewarmed slides on a heated stage (37°C) and their motion video recorded for 2 min using a magnification of \times 100 and a high-resolution camera. The sperm analysis was carried out on a Hobson Sperm Tracker (HST) using HST 7 software. The following motion parameters were measured: curvilinear, straight-line, and average path velocity; mean angular displacement (MAD); beat cross-frequency; amplitude of lateral head displacement; linearity (LIN); and straightness of path (STR). Sperm movement was unaffected by staining with Hoechst 33342, excitation by ultraviolet (UV) light, or the physical process of cell sorting. Significant differences were seen between X and Y sperm for MAD, LIN, and STR. No difference was observed for the other parameters. The results indicate that in a simple salts solution, Y bull sperm do not swim faster than X sperm but may be distinguished from X sperm on the basis of LIN and STR. Mol. Reprod. Dev. 50:323–327, 1998. r 1998 Wiley-Liss, Inc.

Key Words: CASA; sperm; flow cytometry; X and Y chromosome–bearing sperm

INTRODUCTION Gender preselection has been a desire of humankind for generations. Numerous research studies have been conducted to achieve this goal for livestock production as well as for the human population (Aman, 1989).

Among the methods devised to separate X and Y sperm by apparent “physical characteristics” is the albumin gradient method described by Ericsson et al. in 1973 and applied by various clinics in human medicine for the past 15 years (Beernink et al., 1993). Supposedly, the faster swimming speed of the smaller Y sperm enables those sperm to reach the bottom of the gradient before the X-bearing sperm. No conclusive proof has ever been put forth that this view is indeed true, since no method previously existed by which sperm could be separated into nearly pure X and Y sperm populations (Johnson, 1992). This is so despite the fact that the albumin method has been offered to clinicians for many years as an effective method for preselecting sex.

The X chromosome is larger than the Y chromosome and therefore contains more DNA. It might be expected that differences in DNA mass between X and Y chromosome–bearing sperm (Johnson et al., 1989) would influence swimming velocity. However, it also might be expected that any difference in motion parameters between X and Y sperm would be subtle and that experimental conditions to date may not have been sufficiently sensitive to determine these differences. With the development of a validated sperm sexing method (Johnson, 1991; Johnson et al., 1989, 1993) based on the only known difference in X and Y chromosome sperm (DNA content) and the ability of computer-assisted sperm analysis (CASA) to measure motility in a semiquantitative manner, it became possible to test the hypothesis. CASA has been used extensively in the past 10 years with increasing usefulness for assessing sperm quality (Holt, 1996).

Separation of X and Y sperm based on a difference in DNA content has been validated by the birth of offspring for the rabbit (Johnson et al., 1989), boar (Johnson, 1991), bull (Cran et al., 1993), and sheep...
referred to as 35°C in the absence of HO42 stain (control; hereafter at 35°C (Johnson et al., 1989) and incubated for 35 min
Behring Corp., La Jolla, CA) and incubated for 35 min
Hoechst 33342 fluorochrome (HO42; Calbiochem-
semen were prepared as follows: stained with 5 µg/ml of
5-W 90-5 Argon Inova laser (Coherent, Inc., Palo Alto,
CA) operating at 175 mW, and fluorescence was de-
tected through 418-nm long-pass filters. A 76-µm jet-in-
airflow tip was used. Data were collected as 256-
channel histograms. Sheath fluid was 0.1 M phosphate-
buffered saline (PBS) containing 0.1% BSA. All viable
sperm sorting (USDA Beltsville Sperm Sexing Technol-
ogy) was carried out at room temperature (−22°C), as
described by Johnson et al. (1989).

Reanalysis of Sorted X and Y
Chromosome-Bearing Sperm
Sorted sperm were collected during the sampling
process into 500-µl presiliconized microfuge tubes. Af-
ter completion of the video recording of X- and Y-sorted
sperm, the remainder of the sorted sample was soni-
cated for 10 sec to remove the sperm tails. Additional
(0.5 µg/ml) HO42 was added to the sample before
incubating at 35°C for 15 min (Johnson et al., 1987).
Samples were then reanalyzed at room temperature on
the modified flow cytometer for DNA content to deter-
mine the purity of the collected sperm.

Computer-Assisted Analysis of Sperm Movement
Aliquots of 7 µl from each sperm concentration in
Experiment 1 and each treatment group in Experiment
2 (X-sorted sperm, Y-sorted sperm, stained-unsorted
control, and unstained control) were transferred to
PBS) containing 0.1% BSA. All viable
channel histograms. Sheath fluid was 0.1 M phosphate-
airflow tip was used. Data were collected as 256-
HST version 7 software and a frame rate of 25 Hz
recorded for 2 min, and care was taken to ensure
left for 30 sec on the stage for the sample to equilibrate
and minimize drift. Sperm motion was continuously
recorded for 2 min, and care was taken to ensure
similar light levels for each sample. Motion parameters
(Boyer et al., 1989) were examined using HST with
HST version 7 software and a frame rate of 25 Hz
(Hobson Tracking Systems, Ltd., Sheffield, UK). Defini-
tions of each motion parameter are as follows:
Curvilinear velocity (VCL): velocity over the total
distance moved, i.e., including all deviations of sperm
head movement.
Straight-line velocity (VSL): velocity calculated using
the straight-line distance between the beginning and
end of the sperm track.
Average path velocity (VAP): velocity over a calcu-
lated, smoothed path, i.e., a shorter distance than that
used for calculating VCL.
Mean angular displacement (MAD): the mean angu-
lar displacement of the sperm head around the curviline-
ar path.
Beat cross-frequency (BCF): the frequency with which
the actual track crosses the smoothed track.
Amplitude of lateral head displacement (ALH): the
average value of the extreme side-to-side movement of
the sperm head in each beat cycle.

MATERIALS AND METHODS
Semen Collection and Preparation
Ejaculates from three bulls were collected three
times over 9 days in Experiment 1, and ejaculates from
two bulls were collected four times over 8 days in
Experiment 2. The semen was diluted in HEPES-
buffered medium (130 mM NaCl, 4 mM KCl, 14 mM
fructose, 1 mM CaCl2, 0.05 mM MgCl2, and 1 mM
HEPES) containing 0.1% bovine serum albumin
(HEPES-BSA) (Johnson et al., 1989) to a concentration
of 15 × 106 sperm per milliliter. Samples of diluted
semen were prepared as follows: stained with 5 µg/ml of
Hoechst 33342 fluorochrome (HO42; Calbiochem-Behring Corp., La Jolla, CA) and incubated for 35 min
at 35°C (Johnson et al., 1989) and incubated for 35 min
35°C in the absence of HO42 stain (control; hereafter
referred to as unstained). After initial incubation, prop-
idium iodide (PI) was added to all samples to give a
concentration of 25 µg/ml, and the samples were fur-
ther incubated for 5 min at 30°C in order to stain the
dead sperm in each population to increase sorting
efficiency (Johnson et al., 1994).

Flow Cytometric Sorting of Sperm
Stained sperm were sorted with a modified Epics V
flow cytometer/cell sorter (Coulter Corporation, Miami,
FL) modified for the analysis of X and Y chromosome-
bearing sperm based on DNA content (Johnson and
Pinkel, 1986). The fluorochrome-stained sperm were
excited in the ultraviolet wavelength (351, 364 nm) of a
5-W 90-5 Argon Inova laser (Coherent, Inc., Palo Alto,
CA) operating at 175 mW, and fluorescence was de-

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TABLE 1. Means of Velocity Head Movement and Trajectory Variables for Sperm Concentrations of 1, 5, and 10 × 10^6 sperm/ml

<table>
<thead>
<tr>
<th>Sperm × 10^6/ml</th>
<th>No. of sperm counted</th>
<th>Motion parameter (µm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 9)</td>
<td></td>
<td>VCL</td>
</tr>
<tr>
<td>SE ±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>645</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>1793</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>3364</td>
<td>11</td>
</tr>
</tbody>
</table>

a,b Column means followed by different superscripts are significantly different (P ≤ 0.05).

Linearity (LIN): ratio of distances (as a percentage) of straight-line track length to actual track length (this value is 100% for a completely linear track).

Straightness of path (STR): straight-line velocity divided by the average path velocity.

Conditions for HST Setup
Filter settings were 1,1,1 and 2 for 1, 2, 3, and 4, respectively. Predict was off and search radius was 33.2 µm. Minimum track points were 15 frames. Contrast thresholds were +12/-8. Tracking time was 2 min.

Experiment 1: Effect of Sperm Concentration on Motility
Since the process of flow cytometry and cell sorting dilutes sperm samples from their initial concentration, the effects of concentration only on motion parameters were examined initially. Bull semen (n = 9 total ejaculates) was diluted in HEPES-BSA to concentrations of 1, 5, and 10 × 10^6 sperm per milliliter and incubated at 37°C for the duration of the motility analysis. Three aliquots from each concentration were video recorded for CASA in a 3 × 3 Latin square design.

Experiment 2: Motility of X and Y Chromosome-Bearing Sperm
Bull semen (n = 8 total ejaculates) was diluted in HEPES-BSA and stained with HO42 as described above. Stained samples were flow sorted into X and Y chromosome-bearing sperm according to established protocols (Johnson et al., 1989). Sperm were sorted directly from the modified cell sorter into 500 µl of Test-yolk (20% v/v) buffer (Johnson et al., 1989) before sorting. Control samples of stained and unstained sperm that were not subjected to sperm sorting were diluted 1:15 with PBS containing 0.1% BSA to mimic the effects of dilution that occur during routine flow sorting of sperm. After dilution, 400 liters of each of the control samples was transferred to 500-µl presiliconized microfuge tubes that had been rinsed with HEPES-1% BSA (−22°C) also containing 50 µl of Test-yolk buffer at room temperature (−24°C). After additional to the Test-yolk, control stained and unstained samples were kept at room temperature until approximately 300,000 (400 µl) sorted sperm had been collected to comprise an X and Y chromosome-bearing sperm population (~1 hr). From the results of Experiment 1, an effect of concentration on motility was observed for some motion parameters. Therefore, samples in Experiment 2 were adjusted to a standard concentration before motility was video recorded. Following collection of sorted samples, all tubes, including controls, were centrifuged at 350g for 4 min. Each pellet was resuspended in 66 µl of HEPES-BSA to a concentration of approximately 5 × 10^6/ml. The resuspended sperm were transferred to a heat block at 37°C for the duration of the video recording (45 min). Four aliquots from each treatment group were video recorded for CASA in a 4 × 4 Latin square design to eliminate time effects.

Statistical Analysis
Weighted means were taken into account for differences in the numbers of sperm analyzed. A mixed-model ANOVA (SAS, 1994) was used to avoid underestimating the variance of the group means and where bull and day were considered random effects. Differences in motility parameter means for X and Y sperm populations were compared using the least significant difference test.

RESULTS
Reanalysis of Separated X and Y Chromosome-Bearing Sperm
Aliquots of the sorted X and Y chromosome-bearing sperm were reanalyzed for DNA content. Flow cytometric analysis confirmed purities of ≥85% for X and Y sperm, respectively.

Experiment 1: Effect of Sperm Concentration on Motility
No differences in motion parameters were seen between concentrations of 1 × 10^6 and 5 × 10^6 sperm per milliliter (Table 1; P = 0.05). Lower velocities (VSL, VAP) and lower measurements of trajectory (LIN, P = 0.01; STR, P = 0.05) were recorded for concentrations of sperm at 10 × 10^6 sperm per milliliter compared with 1 × 10^6 and 5 × 10^6 sperm per milliliter (see Table 1).

Experiment 2: Motility of X and Y Chromosome-Bearing Sperm
For clarity of presentation, data are presented in two tables. The staining of sperm with HO42 had an effect of velocity in VSL and MAD in stained samples, but excitation of the stain with ultraviolet light and the
The effect of sperm concentration on velocity, previously described for ram and human sperm (Yi Lui et al., 1991; Suttiyotin and Thwaites, 1992), also was confirmed for bull sperm in this study. From the results in Experiment 1, the importance of standardization of sample concentration between control and flow-sorted samples was confirmed. Samples collected after flow sorting have a low concentration (<1 x 10^6 sperm per milliliter); thus concentrations of all samples were adjusted to 5 x 10^6 sperm per milliliter in this study to standardize video recording of sperm. Higher VCL was seen in sperm samples from Experiment 2. Sperm samples in Experiment 2, including controls, were diluted as a process of or to mimic flow cytometry/cell sorting, and samples were recorded 1 hr after flow sorting or dilution of control samples. The increase in velocity may have been indicative of the onset of the capacitation process, with sperm exhibiting some hyperactivated motility (Suarez et al., 1991) and thus increased velocities, although no increase in velocity or hyperactivated motility was observed visually.

In both experiments, the results for VCL are much greater than those of VSL. This is in contrast to velocities for ram and boar (Holt, 1995) sperm, which have more similar velocities. In this study, values of 13–16 µm were measured for ALH. This magnitude of head movement was further verified by subjective assessment of video-recorded sperm revealing wide side-to-side head movement, while at the same time moving forward linearly, which would account for these discrepancies in velocity.

In this study, CASA was carried out on sperm samples that had been diluted in a buffered medium. In the female reproductive tract, the fluids encountered by the sperm would be significantly more viscous. This increased viscosity would also be true of the albumin procedure for human sperm published by Ericsson et al., 1993. Elevated fluid viscosity has been shown to affect the motility of the sperm (Suarez et al., 1991). It is possible that differences in motility between X and Y sperm may be more or less pronounced in a more viscous medium. Differences in swimming patterns have been observed in Ficoll and cumulus matrix, possibly due to macromolecular differences in structure (Suarez et al., 1991). Interestingly, serum albumin in medium has been shown to have a “shear-thinning” effect, reducing the effects of viscosity as the shear rate increases and altering the efficiency at which a sperm swims (Suarez et al., 1991).

Under the conditions of this study, we conclude that bull Y sperm do not swim faster than bull X sperm. Instead, the difference between X and Y sperm with regard to LIN and STR, which are highly correlated with velocity, indicate that bull X sperm may even swim faster than bull Y sperm. If this were the case, then the

### Table 2. Means of Velocity and Head Movement Variables for Unstained, Hoechst Stained-Unsorted, and X and Y Separated Sperm

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of sperm counted</th>
<th>VCL</th>
<th>VSL</th>
<th>VAP</th>
<th>MAD</th>
<th>BCF</th>
<th>ALH</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE ±</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Unstained</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Stained, unsorted</td>
<td>4145</td>
<td>268b</td>
<td>91b</td>
<td>123b</td>
<td>87a</td>
<td>6.8a</td>
<td>16a</td>
</tr>
<tr>
<td>X sorted</td>
<td>3191</td>
<td>263b</td>
<td>84b</td>
<td>118a</td>
<td>87a</td>
<td>6.8a</td>
<td>16a</td>
</tr>
<tr>
<td>Y sorted</td>
<td>4116</td>
<td>256a</td>
<td>72a</td>
<td>105a</td>
<td>85a</td>
<td>6.8a</td>
<td>16a</td>
</tr>
<tr>
<td>Stained, unsorted</td>
<td>4145</td>
<td>268b</td>
<td>91b</td>
<td>123b</td>
<td>87a</td>
<td>6.8a</td>
<td>16a</td>
</tr>
<tr>
<td>X sorted</td>
<td>3191</td>
<td>263b</td>
<td>84b</td>
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<td>6.8a</td>
<td>16a</td>
</tr>
<tr>
<td>Y sorted</td>
<td>4116</td>
<td>256a</td>
<td>72a</td>
<td>105a</td>
<td>85a</td>
<td>6.8a</td>
<td>16a</td>
</tr>
</tbody>
</table>

### Table 3. Means of Trajectory Variables for Unstained, Hoechst Stained-Unsorted, and X- and Y-Sorted Sperm

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of sperm counted</th>
<th>LIN</th>
<th>STR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE ±</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Unstained</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Stained, unsorted</td>
<td>4145</td>
<td>31b</td>
<td>64a</td>
</tr>
<tr>
<td>X sorted</td>
<td>3191</td>
<td>31b</td>
<td>65a</td>
</tr>
<tr>
<td>Y sorted</td>
<td>4116</td>
<td>27b</td>
<td>58b</td>
</tr>
</tbody>
</table>

a,bColumn means followed by different letters are significantly different from each other at the P = 0.05 level.
overall mass of the sperm is not exerting a significant affect on motility. It would be interesting to consider whether structures such as the flagellum or other sperm factors are modified after the second meiotic division in a way that could influence sperm motility. Future development of a system that enabled analysis of flagellar movement together with head movements may provide more information on differences in motion parameters between X and Y chromosome-bearing sperm. The findings of this study continue to leave open the possibility that the difference in motility of X and Y chromosome-bearing sperm may be related to the presence of the Y or X chromosome; however, it is doubtful that any subtle difference would be useful for separating X and Y chromosome-bearing sperm.

ACKNOWLEDGMENTS

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REFERENCES


