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# Post-Thaw Bovine Spermatozoal Quality Estimated from Fresh Samples

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**ABSTRACT:** Comparisons were made among flow cytometrically quantified populations of fresh and cryopreserved bovine spermatozoa, tri-stained with fluorophores rhodamine 123 (R123), 5- (and 6-) carboxy-4',5'-dimethylfluorescein diacetate (CMFDA), and propidium iodide (PI), and analyzed by dual parameter flow cytometry. The purpose was to find parameters in fresh semen samples that were potentially predictive of frozen sample parameters so that bulls with marginal cryopreservation capacity could be identified. Fresh and cryopreserved aliquots of semen from two sets of six bulls were semen processed in either milk (bulls 1–6) or egg yolk citrate (bulls 11–16). Membrane-damaged red (PI) and intact green (CMFDA + R123) populations were evaluated as percentages of 10,000-cell samples or numbers per straw. In milk, gated central subsets of membrane-damaged sperm cells in fresh samples and moribund cells in post-thaw samples were significantly correlated for sample percentages ($r = 0.90$, $P = 0.014$) and cell numbers per straw ($r = 0.94$, $P = 0.006$). In egg yolk citrate, fresh and frozen membrane-damaged populations were correlated (percentages: $r = 0.81$, $P = 0.048$; numbers: $r = 0.88$, $P = 0.019$). Additionally, post-thaw motility estimated by a photographic method was correlated with the number of sperm cells per straw in the intact central green subset ($r = 0.98$, $P = 0.0006$). These findings suggest that partitioning red and green populations into smaller, central subset populations reveals significant relationships between fresh and cryopreserved bull ejaculates. The proportion of membrane-damaged spermatozoa in fresh semen seems to be predictive of the proportion of post-thaw membrane-damaged or moribund spermatozoa. The data consistently showed ejaculates of bulls 1 and 12 as having the greatest cryopreservation potentials, and bulls 4 and 16 the least. Thus, flow cytometric evaluation of fresh semen may be useful for identifying young sires with relatively poor fertilizing potential.

Key words: Sperm, flow cytometry, cryopreservation, fluorescence, artificial insemination.

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Evaluation of post-thaw spermatozoal quality is important in the artificial insemination (AI) industry. It directly relates to fertility and exhibits much variability among and within bull ejaculates (Elliott, 1978a). One seminal quality parameter on which fertility depends is the number of motile sperm cells present in the thawed insemination dose (Sullivan, 1970; Elliott, 1978a; Pickett and Berndtson, 1978; Gérard and Humblot, 1991; Nadir et al, 1993). Because this is a highly variable factor, the best way to handle semen production is to evaluate each ejaculate on an individual basis. Flow cytometry can quickly and accurately measure fertility-related spermatozoal characteristics.

Flow cytometry has certain advantages over classical laboratory tests that evaluate sperm cells. It is difficult to see the cells in an opaque medium, such as milk. However, sperm cells in milk can be stained with fluorescent dyes, seen under a microscope and assessed with a flow cytometer (Van Demark et al, 1959). Also, this technique can provide information on the quality and metabolic states of the cells on a nearly on-line time scale (Al-Rubeai and Emery, 1993). This is an important feature because the evaluator cannot completely control timing in conventional biochemical and microscopic appraisals. Flow cytometry bypasses the resulting problem of unmeasurable variability. It also improves repeatability because of the large number of cells analyzed (10,000 per sample) relative to traditional testing (about 200 per sample).

Recently, investigators have evaluated spermatozoal quality by flow cytometrically quantifying the percentages of intact, motile sperm cells exhibiting a transmembrane mitochondrial potential (Evenson et al, 1982; Garner et al, 1986, 1988; Auger et al, 1989; Ericsson et al, 1989; Graham et al, 1990; Karabinus et al, 1991; Kramer et al, 1993). Flow cytometric evaluations have also related spermatozoal quality and fertility (Evenson et al, 1980; Ballachey et al, 1987, 1988; Ericsson et al, 1989; Karabinus et al, 1991) as well as the effects of milk and egg yolk extension media on sperm quality (Garner et al, 1988; Karabinus et al, 1991).

To date, there have been no published studies of flow cytometric analyses estimating the post-thaw quality of bovine spermatozoa from fresh, 24-hour extended samples. The utility of a reliable predictive factor becomes

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clear when one considers the maintenance cost of a sire candidate during the 3½-year period of progeny testing. Flow cytometric identification of young sires with poor fertility potential would be highly cost effective if these sires were then eliminated from the lengthy progeny testing program. Therefore, using the protocol described by Ericsson et al (1993), the purpose of this study was to evaluate flow cytometric relationships between fresh and cryopreserved bovine spermatozoa extended in milk and egg yolk citrate. Specific objectives were to find those parameters in fresh semen samples that are potentially predictive of frozen sample parameters, so that bulls with marginal cryopreservation potential could be identified.

## Materials and Methods

### Materials

The stains used to evaluate spermatozoal characteristics were 5-(and 6-) carboxy-4',5'-dimethylfluorescein diacetate (CMFDA), rhodamine 123 (R123), and propidium iodide (PI). The CMFDA and R123 were obtained from Molecular Probes, Incorporated (Eugene, Oregon) and prepared in anhydrous dimethylsulfoxide (DMSO) from Aldrich Chemical Company (Milwaukee, Wisconsin). The PI, purchased from Calbiochem (La Jolla, California), was put into Tyrode's salt solution that was obtained from Sigma Chemical Company (St. Louis, Missouri). Solution concentrations for CMFDA, R123, and PI were 1 mg/ml, 1 mg/5 ml and 2 mg/ml, respectively.

### Samples

Bovine semen samples from two sets of six bulls were collected and processed according to Certified Semen Service Standards (CSS). American Breeders Service (ABS; DeForest, Wisconsin) and Atlantic Breeders Cooperative (ABC; Lancaster, Pennsylvania) both generously donated fresh and cryopreserved samples packaged in 0.5-ml French straws. Fresh, 24-hour processed samples were packed in ice and shipped to Reno, Nevada by next-day Federal Express. Cryopreserved samples from the same ejaculates were shipped by United Parcel Service in small liquid nitrogen tanks. Samples obtained from ABC were processed in whole homogenized milk and 7% glycerol; bulls were identified as numbers 1 through 6. Samples from ABS were extended in 20% egg yolk–sodium citrate and 7% glycerol and identified as numbers 11 through 16.

### Experimental Design

Two experiments were analyzed separately involving either milk or egg yolk citrate-extended samples. Each consisted of three straws (replications) from each of six ejaculates arranged in a randomized complete block design (RCBD). These included (1) three straws of milk-extended semen from each of six bulls collected approximately 24 hours prior to analyses, (2) three cryopreserved straws from each of the same six milk-extended ejaculates, (3) three 24-hour straws of egg yolk-extended semen from a different set of six bulls, and (4) three cryopreserved straws from each of those same six egg yolk-extended ejaculates. To determine differences among bulls and among straws, both for arcsine-transformed percentages and actual numbers per straw, analysis of variance (ANOVA) of arcsine-transformed data was conducted using the least squares procedure and the general linear models procedure of the Statistical Analysis System (SAS, 1985). The interrelationships among variables were evaluated using correlation coefficients generated by SAS as well as plots of regression equations. Variables compared were post-thaw motility, red- and green-stained populations from fresh and cryopreserved samples, and window versus quadrant parameters. Values were compared both for percentage of 10,000-cell samples and numbers of stained cells per straw. Results are presented as least squares means. Mean post-thaw motility estimates were made using time-exposure darkfield photomicrography at ABS ($n = 2$; S. P. Lorton, personal communication).

### Fluorogenic Staining

Each 0.5-ml straw was warmed or thawed in a 37.5°C waterbath for 60 seconds and then diluted 1:3 in microcentrifuge tubes containing prewarmed Tyrode's solution. The tubes were mixed by inverting three times. Each sample was stained in sequence at 2-minute intervals using Pipetman pipettes equipped with FluoroPel® processed pipette tips (Ulster Scientific, Incorporated, New Paltz, New York). These specially coated tips were used to eliminate stain droplets on the outside of the tips, thus reducing variability in the volume of stain applied to the samples. A volume of 0.8 μl R123 was dispensed into samples. After 75 minutes of incubation, 0.8 μl CMFDA and 6.0 μl PI were pipetted into samples and incubated for another 15 minutes. Sample volumes of 75 ml were put into 1 ml of filtered sheath fluid (American Scientific Products, Sunnyvale, California) in polystyrene tubes (Fisher, Pittsburgh, Pennsylvania). Sheath fluid was put into the tubes just before analyses to avoid leaching of plasticizers from the tubes (Garner and Thomas, unpublished data). Diluted samples were then analyzed flow cytometrically at 2-minute intervals.

### Flow Cytometry

Information on the tri-stained cells was collected in list mode with a FACS Analyzer flow cytometer (Becton-Dickinson, Sunnyvale, California). The generated data were then analyzed using a Hewlett Packard Consort 30, a 200-series computer using C30 Version D software. The flow cytometer was equipped with (1) a standard fluorescein isothiocyanate and phycoerythrin dichroic filter set, (2) LP 400 long pass and DF 485/22 bandpass excitation filters at 485 nm, (3) a DM-560 dichroic mirror to separate fluorescent signals, (4) a photomultiplier tube 1 (FL1) collecting 530-nm light through a DF 530/30 bandpass filter, and (5) a second photomultiplier tube (FL2) collecting light through an LP 570 filter. Compensation was used to minimize spillover of green fluorescence into the red channels.

### Histogram Analyses

Contour histograms of 10,000-cell samples were generated, and populations were partitioned for each replicate using window rather than quadrant analyses. Figure 1 illustrates these two methods of isolating particular segments of the histograms.

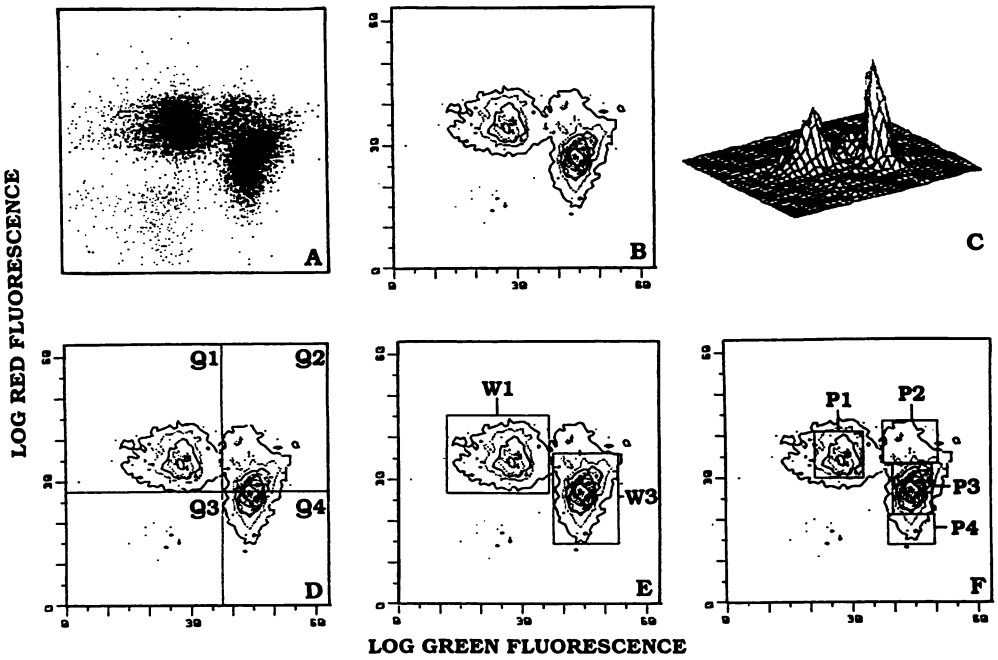



**FIG. 1.** A comparison of quadrant and window analyses of thawed, cryopreserved spermatozoa in milk (bull 2). Red- and green-stained populations indicate membrane-damaged cells and those with intact membranes, respectively. The dot plot (A), contour histogram (B), and net plot (C) illustrate three ways of looking at the same sample. Contour plots can be subdivided into populations and quantified by three methods. The plot can be partitioned into four quadrants (D). Quadrants Q1 and Q3 contain red, propidium iodide (PI)-stained membrane-damaged spermatozoa. Quadrants Q2 and Q4 segregate the green carboxydimethylfluorescein-diacetate (CMFDA)- and rhodamine 123 (R123)-stained population into two halves. In contrast, window analyses (E and F) partition the contour plots of each sample individually. Windows gate and quantify the whole red population, labeled W1, or the green population, labeled W3 (E). Central subsets of these main populations can be quantified and labeled for more specific analyses (F). These are labeled P1 for the central red subset; P3 for the central green subset; P2 for the transitional population of moribund spermatozoa containing both red and green fluorescence; and P4 for the population with the least amount of red fluorescence.

Quadrant analysis partitions the histogram into four quadrants that segregate the red and green populations, whereas window analysis quantifies smaller subsets of cells within the main populations.

Microscopic examination revealed that intact spermatozoa in P3 and P4 retained green fluorescence from CMFDA throughout the cell, with the mitochondria fluorescing green slightly more intensely. The cells in W1 or P1 had red fluorescent nuclei and were considered to be membrane-damaged. Moribund cells in P2 exhibited a red nucleus covered by a green acrosomal compartment and some green in the tail, and they were seen only in cryopreserved, milk-extended samples. Usage of the terms membrane-damaged, intact, and moribund in reference to stained populations is appropriate based on the results of many flow cytometric investigations of sperm cells (Evenson et al, 1980, 1982; Garner et al, 1986, 1988; Ballachey et al, 1987, 1988; Auger et al, 1989; Ericsson et al, 1989; Graham et al, 1990; Karabinus et al, 1991). Therefore, these terms will be used interchangeably with red- and green-stained populations.

Using window analyses, the red-stained populations were analyzed in two ways for both the milk and egg yolk citrate-extended samples. One way partitioned the entire group of membrane-damaged sperm cells that carried red fluorescence. This population was labeled W1FR or W1CR, for fresh or cryopreserved samples, respectively. Alternately, a centrally located subset of this population was quantified separately and termed P1FR and P1CR, for fresh or cryopreserved samples, respectively.

The central subsets of the green-stained populations, representing the majority of cells with intact membranes and functional mitochondria, were quantified as percentages and labeled P3FR and P3CR. The percentages of peripheral cells around the central portions were not included in the analyses. The green-stained populations projecting below P3 and having the least amount of red fluorescence were designated P4FR and P4CR. Moribund cells that carried both red and green stains were observed only in cryopreserved samples in milk. These appeared as transitional populations and were quantified separately as P2CR.

Numbers of spermatozoa per straw carrying red or green stain were evaluated separately from percentages of 10,000-cell samples. These values were calculated for each bull by multiplying flow cytometric percentages times the cell concentration per straw.

## Results

### *Experiment 1: Milk-Extended Samples*

To detect differences among bulls numbered 1 through 6, or among straws (replicates) within each red or green fluorescent population, ANOVA of the arcsine-transformed percentages was conducted. In fresh samples, the mean percentages of membrane-damaged spermatozoa within populations W1FR and P1FR, and percentage of intact

cells in P3FR, differed among bulls ($P = 0.043$ to 0.0001) The population P4FR, which contained minimal PI, did not differ among bulls ($P = 0.784$). Analyses of number of sperm cells per straw revealed that bulls differed, but straws did not within all parameters.

In contrast to fresh samples, those that were cryopreserved did not differ among bulls in W1CR and P1CR ($P = 0.306$ to 0.693). However, the moribund, transitional population (P2CR), as well as P3CR and P4CR, differed among bulls ($P = 0.015$ to 0.0001) using window analyses. Differences were not observed among straws in any of the populations (fresh: $P = 0.385$ to 0.917; frozen: 0.084 to 0.318) except for P2CR, which differed among straws ($P = 0.047$). Quadrant analyses produced very different results. When peripheral cells were included with P3CR, bulls did not differ ($P = 0.340$).

*Rankings of Bulls*—The percentage means per sample and numbers per straw within populations are listed in Table 1. Those bulls that were significantly different from all others are noted. Bulls are ranked from high to low means in fresh and cryopreserved samples. Rankings are reflected by the highly significant correlations between fresh and frozen populations P1FR and P2CR (fresh: $r = 0.90$, $P = 0.014$; frozen: $r = 0.94$, $P = 0.006$). That is, bull 4 ranked first and bull 2 ranked second in P1FR. After cryopreservation, these bulls maintained their first and second place ranks within P2CR. Likewise, bull 1 ranked sixth in both P1FR and P2CR. Translated into numbers of spermatozoa per straw within each window, bulls 4, 5, and 2 ranked first, second, and third, respectively, in both P1FR and P2CR, whereas bull 1 ranked sixth. The changes in percentages of membrane-damaged and intact spermatozoa after cryopreservation showed a decrease in intact cells (P3CR minus P3FR) that ranged from −20.6% in bull 1 to −29.1% in bull 2. The increase in membrane-damaged cells (P1CR minus P1FR) ranged from 21.3% in bull 1 to 16.0% in bull 4.

*Correlations*—A comparison of correlations using quadrant versus window analyses showed that the green populations in cryopreserved samples (Q2 + Q4; Fig. 1D) were not correlated with the fresh populations P1FR ($r = -0.31$, $P = 0.548$). If, rather, the green populations were partitioned closely around the central subsets and quantified as distinct populations (window analyses), P3CR was highly negatively correlated to P1FR ($r = -0.97$, $P = 0.002$). This distinct central subset was apparent in all replicates and all samples. Therefore, all analyses of green-stained cells were performed using window analyses. The central subsets of red-stained cells were not quite as distinctive as the green central subsets, so correlation analyses were performed on both the central (P1) and entire (W1) populations for both experiments.

Correlations among sample percentages and straw numbers in flow cytometric populations in milk are listed in Table 2. Significant correlations existed between fresh and frozen populations. Percentages of red-stained cells in fresh samples (W1FR and P1FR) were correlated with both the transitional populations P2CR and the green-stained populations P3CR. The negative correlation coefficients produced from W1FR and P1FR with P3CR were the same ($r = -0.97$, $P = 0.002$). In contrast, the central red-stained populations P1FR produced a slightly higher positive correlation coefficient with P2CR ($r = 0.90$, $P = 0.01$) than when peripheral red cells were included (W1FR; $r = 0.88$, $P = 0.02$).

Numbers of cells per straw showed significant correlation coefficients between fresh populations W1FR, P3FR, and P4FR and cryopreserved populations W1CR, P2CR, and P3CR ($r > 0.90$, $P < 0.04$). Correlation coefficients for cell numbers per straw had higher values, in general, than those for sample percentages per 10,000 cells.

*Regression*—Regression analyses were performed on mean arcsine-transformed percentages and number of spermatozoa per straw in P1FR and P2CR. The regression line for percentage values is defined by the equation $Y = -0.08 + 1.39X$ (Fig. 2A), where Y is the estimated percentage of transitional, moribund cells carrying both red and green fluorescence in cryopreserved samples (P2CR), and X is the percentage of membrane-damaged cells in the central subsets (P1FR) of the red-stained populations in fresh samples. The regression line for numbers of sperm cells per straw is defined by the equation $Y = -0.42 \times 10^6 + 1.12X$ (Fig. 2B), where Y is the estimated number of moribund, transitional cells carrying both red and green fluorescence in thawed straws (P2CR), and X is the number of membrane-damaged cells located in the central subsets (P1FR) of the total red-stained populations in fresh straws.

### *Experiment 2: Egg Yolk–Citrate-Extended Samples*

As with the milk-extended samples, egg yolk–citrate-extended ejaculates from a second set of six bulls, numbered 11 through 16, were evaluated with ANOVA for differences among bulls and among straws within each parameter. All populations of sperm cells in egg yolk are referred to with the same notations as those in milk except for P2CR. This population was not apparent on any histograms of egg yolk samples. An additional variable was included in these evaluations. Mean percentage of post-thaw motility for each bull ejaculate, estimated by a photographic method, was provided by ABS, thus enabling potential relationships with flow parameters to be analyzed.

Bulls differed within W1, P1, and P3 for fresh and cryopreserved samples ($P = 0.005$ to 0.0001), but not within P4FR ($P = 0.175$) or P4CR ($P = 0.393$). Straws differed within P3CR ($P = 0.029$) but not within any of the other populations.

Table 1. *Rankings of bulls according to mean percent or number of spermatozoa (milk) in populations identified by flow cytometric window analyses*

| Percentage of fresh sperm per sample* | | | | | | | | Percentage of cryopreserved sperm per sample* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| W1FR† | | P1FR‡ | | P3FR§ | | P4FR¶ | | W1CR† | | P1CR‡ | | P2CR‖ | | P3CR§ | | P4CR¶ | |
| Bull | % | Bull | % | Bull | % | Bull | % | Bull | % | Bull | % | Bull | % | Bull | % | Bull | % |
| 4 | 21.7 | 4 | 13.1 | 1 | 68.4 | 1 | 6.2 | 4 | 40.3 | 4 | 29.2 | 4 | 12.2 | 1 | 47.9[a] | 4 | 5.3 |
| 2 | 18.9 | 2 | 10.3 | 2 | 67.5 | 5 | 5.4 | 5 | 39.6 | 5 | 28.6 | 2 | 10.2 | 5 | 44.4[a] | 3 | 4.2 |
| 3 | 17.4 | 5 | 8.5 | 6 | 65.7 | 4 | 5.0 | 2 | 38.9 | 2 | 27.6 | 6 | 9.0 | 6 | 42.5 | 1 | 3.6 |
| 5 | 17.3 | 3 | 8.3 | 5 | 65.1 | 2 | 4.6 | 1 | 38.7 | 1 | 27.2 | 5 | 7.8 | 3 | 42.0 | 2 | 3.1 |
| 6 | 16.6 | 6 | 8.1 | 3 | 63.2[a] | 3 | 4.6 | 6 | 38.5 | 6 | 26.6 | 3 | 7.5 | 2 | 38.4[a] | 5 | 2.5 |
| 1 | 14.8 | 1 | 5.9 | 4 | 56.5[a] | 6 | 4.5 | 3 | 35.3 | 3 | 25.2 | 1 | 2.8[a] | 4 | 34.7[a] | 6 | 2.2 |

| Number of fresh sperm per straw** ($\times 10^6$) | | | | | | | | Number of cryopreserved sperm per straw** ($\times 10^6$) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| W1FR† | | P1FR‡ | | P3FR§ | | P4FR¶ | | W1CR† | | P1CR‡ | | P2CR‖ | | P3CR§ | | P4CR¶ | |
| Bull | No. | Bull | No. | Bull | No. | Bull | No. | Bull | No. | Bull | No. | Bull | No. | Bull | No. | Bull | No. |
| 5 | 5.0[a] | 4 | 2.6 | 5 | 18.7[a] | 5 | 1.5 | 5 | 11.4[a] | 5 | 8.2[a] | 4 | 2.4 | 5 | 12.8[a] | 4 | 1.0 |
| 4 | 4.2 | 5 | 2.5 | 2 | 13.1[a] | 1 | 1.1 | 4 | 7.8 | 4 | 5.7 | 5 | 2.2 | 1 | 8.8[a] | 3 | 0.9 |
| 2 | 3.7 | 2 | 2.0 | 1 | 12.6 | 4 | 1.0 | 2 | 7.6 | 2 | 5.4 | 2 | 2.0 | 3 | 8.2 | 5 | 0.7 |
| 3 | 3.4 | 3 | 1.6 | 6 | 12.5 | 2 | 1.0 | 6 | 7.3 | 6 | 5.0 | 6 | 1.7 | 6 | 8.0 | 1 | 0.7 |
| 6 | 3.2 | 6 | 1.5 | 3 | 12.2 | 3 | 0.9 | 1 | 7.1 | 1 | 5.0 | 3 | 1.4 | 2 | 7.4[a] | 2 | 0.6 |
| 1 | 2.7 | 1 | 1.1 | 4 | 11.0[a] | 6 | 0.8 | 3 | 6.6 | 3 | 4.9 | 1 | 0.5[a] | 4 | 6.7[a] | 6 | 0.4 |

* Bull identification numbers listed from high to low according to mean percent ($n = 3$).
† Entire population of red-stained spermatozoa (fresh, W1FR; cryopreserved, W1CR).
‡ Central subset of red-stained population (fresh, P1FR; cryopreserved, P1CR).
§ Central subset of green-stained population (fresh, P3FR; cryopreserved, P3CR).
¶ Subset of green-stained population exhibiting the least amount of red fluorescence (fresh, P4FR; cryopreserved, P4CR).
‖ Transitional population of cells exhibiting both red and green fluorescence (P2CR).
** Bull identification numbers listed from high to low according to mean number of sperm cells per straw ($\times 10^6$). Values are calculated from flow cytometric percentages (percentage times spermatozoa per straw, $n = 3$).
[a] Bull differs from all others within the column ($P < 0.05$).

Table 2. *Correlation coefficients between fresh and cryopreserved populations of red- and green-stained spermatozoa extended in milk*

| Percentage of cryopreserved spermatozoa* | Percentage of fresh spermatozoa* | | | |
|---|---|---|---|---|
| | W1FR† (*r*) | P1FR‡ (*r*) | P3FR§ (*r*) | P4FR¶ (*r*) |
| W1CR† | 0.37 | 0.44 | −0.18 | 0.33 |
| P1CR‡ | 0.53 | 0.57 | −0.37 | 0.31 |
| P2CR‖ | 0.88[a] | 0.90[a] | −0.64 | −0.79 |
| P3CR§ | −0.97[b] | −0.97[b] | 0.74 | 0.64 |
| P4CR¶ | 0.53 | 0.46 | −0.71 | 0.01 |
| **Number of cryopreserved spermatozoa**** | **Numbers of fresh spermatozoa**** | | | |
| | W1FR† (*r*) | P1FR‡ (*r*) | P3FR§ (*r*) | P4FR¶ (*r*) |
| W1CR† | 0.85[a] | 0.63 | 0.92[b] | 0.94[b] |
| P1CR‡ | 0.88[a] | 0.66 | 0.91[a] | 0.94[b] |
| P2CR‖ | 0.83[a] | 0.94[b] | 0.27 | 0.24 |
| P3CR§ | 0.54 | 0.19 | 0.96[b] | 0.92[a] |
| P4CR¶ | 0.36 | 0.47 | −0.20 | 0.05 |

* Mean percent spermatozoa per population from 10,000-cell samples ($n = 6$).

† Entire population of red-stained spermatozoa (fresh, W1FR; cryopreserved, W1CR).

‡ Central subset of red-stained population (fresh, P1FR; cryopreserved, P1CR).

§ Central subset of the green-stained population (fresh, P3FR; cryopreserved, P3CR).

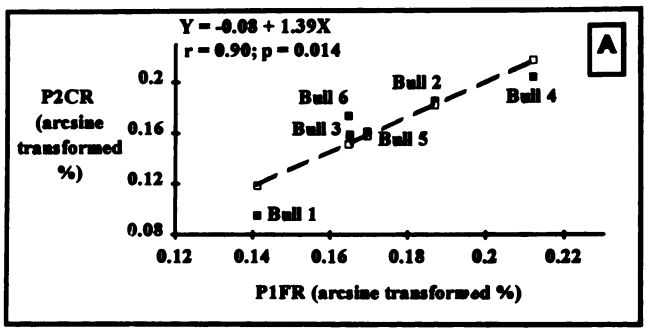
¶ Subset of the green-stained population exhibiting the least amount of red fluorescence (fresh, P4FR; cryopreserved, P4CR).

‖ Transitional population of cells exhibiting both red and green fluorescence (P2CR).

** Mean number of spermatozoa per straw exhibiting red or green fluorescence, calculated as percentage of 10,000-cell sample times cell concentration per straw ($n = 6$).

[a] Significant ($P < 0.05$).

[b] Highly significant ($P < 0.01$).



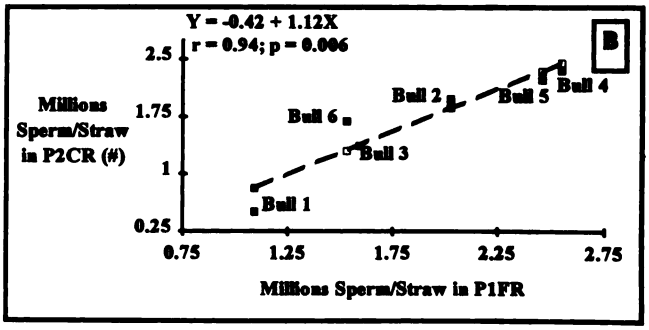




FIG. 2. Percentage (A) and number (B) of cryopreserved bovine spermatozoa predicted from fresh, 24-hour samples processed in milk. For each bull the predicted values are shown as open boxes (□), whereas the actual data points from which the regression equation was derived are shown as closed boxes (■). The predicted transitional population of moribund sperm cells that carry both green and red stain in cryopreserved samples P2CR (□) and the data points from which the regression was derived (■) ($n = 6$) are shown. (A), Predicted percentages in thawed samples (□) are defined by the regression equation $Y = -0.08 + 1.39X$, where Y is the estimated percentage of cells in P2CR, and X is the percentage of cells in P1FR determined flow cytometrically. (B), Predicted numbers of cells in thawed straws are defined by the regression equation $Y = -0.42 + 1.12X$, where Y is the estimated number of moribund cells containing both red and green stain ($\times 10^6$) in a thawed straw (P2CR), and X is the number of cells in the central red subset in a fresh straw (P1FR).

*Rankings of Bulls*—Bull percentage means and numbers per straw, ranked from high to low for motility and flow cytometric parameters, are listed in Table 3. Those bulls that differed significantly from all others ($P < 0.05$) are noted. Bulls 12 and 14 showed the highest percentages of post-thaw motility as well as the highest percentages of intact, green-stained cells in cryopreserved samples (P3CR); bull 16 had the lowest. Conversely, bulls 12 and 14 had the lowest percentages of membrane-damaged, red-stained cells in cryopreserved samples (W1CR and P1CR) and bull 16 the highest.

The percent change within bulls between fresh and cryopreserved samples showed smaller decreases of green-stained cells in egg yolk than in milk samples. In population P3, the percent change ranged from a 6.6% decrease for bull 12 to a 32.3% decrease for bull 16. Bull 11 also had a large percent change of 28.3%, whereas the remaining three bulls all showed similar changes after cryopreservation (about 20%).

The top 3 bulls with the highest number of motile sperm cells per thawed straw were bulls 14, 12, and 11 with 15.5, 9.8, and 9.5 $\times 10^6$, respectively. These same bulls had 12.9, 8.3, and 7.7 $\times 10^6$ green-stained spermatozoa per straw in P3CR. Bull 16 had the least number of motile cells (7.1 $\times 10^6$) and green-stained spermatozoa (6.4 $\times 10^6$).

*Correlations*—Fresh sample parameters in egg yolk did not produce correlation coefficient values as large as those in milk (Table 4). However, significant correlations were observed between the red-stained populations in fresh and frozen samples W1FR and P1CR (percentages: $r = 0.81$, $P = 0.048$; numbers: $r = 0.88$, $P = 0.019$). Post-thaw motility was negatively correlated with the mean percentages of post-thaw red populations (W1CR; $r = -0.90$, $P = 0.013$) and positively with the green-stained populations (P3CR; $r = 0.89$, $P = 0.018$), whereas none of the fresh sample percentages were correlated with postthaw

Table 3. *Rankings of bulls according to mean percent or number of spermatozoa (egg yolk citrate) in populations identified by flow cytometric window analyses*

| | | Percentage of fresh sperm per sample* | | | | | | | | Percentage of cryopreserved sperm per sample* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MOT† | | W1FR‡ | | P1FR§ | | P3FR¶ | | P4FR‖ | | W1CR‡ | | P1CR§ | | P3CR¶ | | P4CR‖ | |
| Bull | % | Bull | % | Bull | % | Bull | % | Bull | % | Bull | % | Bull | % | Bull | % | Bull | % |
| 12 | 65 | 15 | 20.7 | 15 | 10.6 | 14 | 66.6* | 11 | 4.3 | 16 | 41.7* | 16 | 26.4* | 12 | 55.2* | 13 | 6.2 |
| 14 | 62 | 16 | 19.5 | 16 | 8.6 | 13 | 60.5 | 14 | 3.5 | 15 | 31.8 | 15 | 20.9* | 14 | 51.5* | 11 | 5.4 |
| 15 | 56 | 13 | 16.5 | 13 | 6.8 | 12 | 59.1 | 12 | 3.2 | 13 | 31.1 | 13 | 16.7 | 13 | 46.7* | 14 | 5.4 |
| 11 | 53 | 11 | 16.4 | 11 | 6.3 | 11 | 58.4 | 16 | 3.1 | 11 | 28.6 | 11 | 14.6 | 15 | 43.3 | 16 | 5.1 |
| 13 | 49 | 12 | 15.2 | 12 | 5.4 | 16 | 55.9* | 15 | 3.1 | 14 | 27.8 | 14 | 11.6 | 11 | 42.7 | 15 | 4.8 |
| 16 | 42 | 14 | 11.2* | 14 | 3.7 | 15 | 53.7* | 13 | 3.0 | 12 | 23.6* | 12 | 10.1 | 16 | 37.8* | 12 | 4.7 |
| | | Number of fresh sperm per straw** ($\times 10^6$) | | | | | | | | Number of cryopreserved sperm per straw** ($\times 10^6$) | | | | | | | |
| MOT† | | W1FR‡ | | P1FR§ | | P3FR¶ | | P4FR‖ | | W1CR‡ | | P1CR§ | | P3CR¶ | | P4CR‖ | |
| Bull | No. | Bull | No. | Bull | No. | Bull | No. | Bull | No. | Bull | No. | Bull | No. | Bull | No. | Bull | No. |
| 14 | 15.5 | 16 | 3.3 | 15 | 1.7 | 14 | 16.7* | 14 | 0.9 | 16 | 7.1 | 16 | 4.5* | 14 | 12.9* | 14 | 1.3* |
| 12 | 9.8 | 15 | 3.3 | 16 | 1.5 | 11 | 10.5* | 11 | 0.7 | 14 | 7.0 | 15 | 3.3 | 12 | 8.3* | 11 | 1.0 |
| 11 | 9.5 | 11 | 3.0 | 11 | 1.1 | 16 | 9.5* | 16 | 0.5 | 11 | 5.2 | 14 | 2.9 | 11 | 7.7* | 13 | 0.9 |
| 15 | 9.0 | 14 | 2.8 | 13 | 1.0 | 13 | 9.1 | 15 | 0.5 | 15 | 5.1 | 11 | 2.6 | 13 | 7.0 | 16 | 0.9 |
| 13 | 7.4 | 13 | 2.5 | 14 | 0.9 | 12 | 8.9 | 12 | 0.5 | 13 | 4.7 | 13 | 2.5 | 15 | 6.9 | 15 | 0.8 |
| 16 | 7.1 | 12 | 2.3 | 12 | 0.8 | 15 | 8.6 | 13 | 0.4 | 12 | 3.5* | 12 | 1.5* | 16 | 6.4* | 12 | 0.7 |

* Bull identification numbers listed from high to low according to mean percent ($n = 3$).
† Post-thaw motility (MOT) determined photographically at ABS.
‡ Entire population of red-stained spermatozoa (fresh, W1FR; cryopreserved, W1CR).
§ Central subset of red-stained population (fresh, P1FR; cryopreserved, P1CR).
¶ Central subset of the green-stained population (fresh, P3FR; cryopreserved, P3CR).
‖ Subset of the green-stained population exhibiting the least amount of red fluorescence (fresh, P4FR; cryopreserved, P4CR).
** Bull identification numbers listed from high to low according to mean numbers of sperm cells per straw ($\times 10^6$). Values are calculated from flow cytometric percentages: (percentage times spermatozoa per straw; $n = 3$).
* Bull differs from all others within the column ($P < 0.05$).

motility. Numbers of motile spermatozoa per straw were highly correlated with the number of green-stained sperm cells ($r = 0.98$, $P = 0.0006$) and are depicted in Figure 3.

*Regression*—The regression lines produced from correlated parameters W1FR and P1CR for both percentages and numbers are shown in Figure 4. The line for percentage values (Fig. 4A) is defined by the equation $Y = -0.10 + 1.43X$, where Y is the estimated percentage of sperm cells in P1CR, and X is the percentage of spermatozoa in W1FR. Likewise, the regression line from numbers of sperm cells per straw (Fig. 4B) is defined by the equation $Y = -2.98 \times 10^6 + 2.06X$, where Y is the estimated number of membrane-damaged, central red subset cells in a thawed straw (P1CR), and X is the population of red-stained cells per straw in a fresh sample (W1FR).

### *Change in Percentage after Cryopreservation*

When fresh samples were cryopreserved and thawed, the increased membrane-damaged cells in milk ranged from 17.9% to 24.0%. The second group of ejaculates from different bulls (in egg yolk citrate) produced a range of increase in membrane-damaged cells of 8.4% to 22.1%. The percentage decrease of intact cells in post-thaw milk samples (P3CR) was −20.6% to −29.1%. Egg yolk samples had a range of decrease of −3.9% to −18.0%. The increase in membrane-damaged cells relative to the decrease in intact cells revealed only bull 12 as having exceptionally good cryopreservation potential. He had the smallest increase in mean percentage of membrane-damaged cells and the lowest post-thaw value. He had the highest percentage of post-thaw intact cells and smallest decrease after cryopreservation. Bull 14 has a 7.9% increase and 15.1% decrease in P1 and P3, respectively. The relative proportions of increased membrane-damaged cells to decreased intact cells seem to indicate that the spermatozoa of bull 14 have the least potential for surviving cryopreservation in this group of bulls. However, mean percentages show bull 16 as having the most substandard ejaculate within this group.

## *Discussion*

### *Experiment 1: Milk-Extended Samples*

One of the goals of this study was to detect differences among bulls known to have good quality semen. The

Table 4. *Correlation coefficients between post-thaw motility (MOT) and fresh and cryopreserved populations of red- and green-stained spermatozoa extended in egg yolk citrate*

| Percentage of spermatozoa | MOT* | Percentage of fresh spermatozoa† (r) W1FR‡ | P1FR§ | P3FR¶ | P4FR‖ | Percentage of cryopreserved spermatozoa† (r) W1CR‡ | P1CR§ | P3CR¶ |
|---|---|---|---|---|---|---|---|---|
| P4CR‖ | −0.43 | −0.23 | −0.22 | 0.42 | 0.04 | 0.15 | 0.02 | −0.15 |
| P3CR¶ | 0.88[a] | −0.72 | −0.72 | 0.61 | −0.03 | −0.87[a] | −0.91[a] | |
| P1CR§ | −0.86[a] | 0.81[a] | 0.83[a] | −0.68 | −0.37 | 0.95[b] | | |
| W1CR‡ | −0.90[a] | 0.61 | 0.62 | −0.45 | −0.32 | | | |
| P4FR‖ | 0.20 | −0.35 | −0.38 | 0.26 | | | | |
| P3FR¶ | 0.44 | −0.97[b] | −0.93[b] | | | | | |
| P1FR§ | −0.57 | 0.98[b] | | | | | | |
| W1FR‡ | −0.61 | | | | | | | |

| Numbers of spermatozoa | MOT* | Numbers of fresh spermatozoa** (r) W1FR‡ | P1FR§ | P3FR¶ | P4FR‖ | Numbers of cryopreserved spermatozoa** (r) W1CR‡ | P1CR§ | P3CR¶ |
|---|---|---|---|---|---|---|---|---|
| P4CR‖ | 0.77 | 0.03 | −0.33 | 0.95[b] | 0.89[a] | 0.66 | 0.13 | 0.82[a] |
| P3CR¶ | 0.98[b] | −0.26 | −0.52 | 0.94[b] | 0.82[a] | 0.34 | −0.24 | |
| P1CR§ | −0.23 | 0.88[a] | 0.77 | 0.02 | 0.05 | 0.81 | | |
| W1CR‡ | 0.32 | 0.64 | 0.32 | 0.59 | 0.57 | | | |
| P4FR‖ | 0.85[a] | 0.13 | −0.31 | | | | | |
| P3FR¶ | 0.92[b] | −0.03 | −0.39 | | | | | |
| P1FR§ | −0.43 | 0.88[a] | | | | | | |
| W1CR‡ | −0.16 | | | | | | | |

* Mean post-thaw motility (MOT; $n = 2$) was determined photographically.
† Mean percent spermatozoa per population from 10,000-cell samples ($n = 6$).
‡ Entire population of red-stained spermatozoa (fresh, W1FR; cryopreserved, W1CR).
§ Central subset of red-stained population (fresh, P1FR; cryopreserved, P1CR).
¶ Central subset of the green-stained population (fresh, P3FR; cryopreserved, P3CR).
‖ Subset of the green-stained population exhibiting the least amount of red fluorescence (fresh, P4FR; cryopreserved, P4CR).
** Numbers of spermatozoa per straw bearing red or green stain were calculated from population percentages (percentage times cell concentration; $n = 6$).
[a] Significant ($P < 0.05$).
[b] Highly significant ($P < 0.01$).

sperm chromatin flow cytometric assay, which evaluates sperm chromatin stability, has successfully differentiated between ejaculates of high and low fertility (Evenson et al, 1980, 1982; Ballachey et al, 1987, 1988). Traditional laboratory testing can easily identify ejaculates of poor fertilizing potential. Bulls that continually produce poor ejaculates are culled when they are young, so in practice, poor ejaculates are not seen in the field. In this study, the objective was to differentiate among good, better, and best within groups of bulls producing quality semen.

There are four separate issues to consider when evaluating spermatozoal populations: (1) the mean proportions of intact and membrane-damaged sperm cells present in fresh and cryopreserved samples of each bull, and which bulls rank high and low; (2) the cryopreservation potential of each bull's sperm cells, that is, the production of cells that have membrane properties which resist destruction during the freezing process; (3) the spermatozoal populations in fresh samples that are predictive of post-thaw quality; and (4) the factors most related to fertility of the samples.

First, to determine if differences existed among bull ejaculates within each population, ANOVA was performed. In this study, the populations consisted of percentages of the 10,000-cell samples, representing groups of similarly stained cells. The arcsine transformation procedure, performed on percentage values, produced data that satisfied the equal variance assumption for ANOVA. Transformed values were used throughout analyses. Mean square error (MSE) terms relative to bull error terms were small, and they indicated that bulls differed significantly within all populations except P4FR. Straws did not differ within any population. Ranked bull means, listed in Table 1, generally showed that the top three ranked bulls differed from the bottom three. Bull rankings of membrane-damaged cells in fresh populations were inversely related to rankings of intact cells in frozen populations. In fresh samples, bull 4 had the highest percentage of membrane-damaged sperm cells and bull 1 the lowest. In post-thaw samples, bull 4 had the lowest percentage of intact cells and bull 1 the highest.

Second, the changes in percentage of membrane-dam-

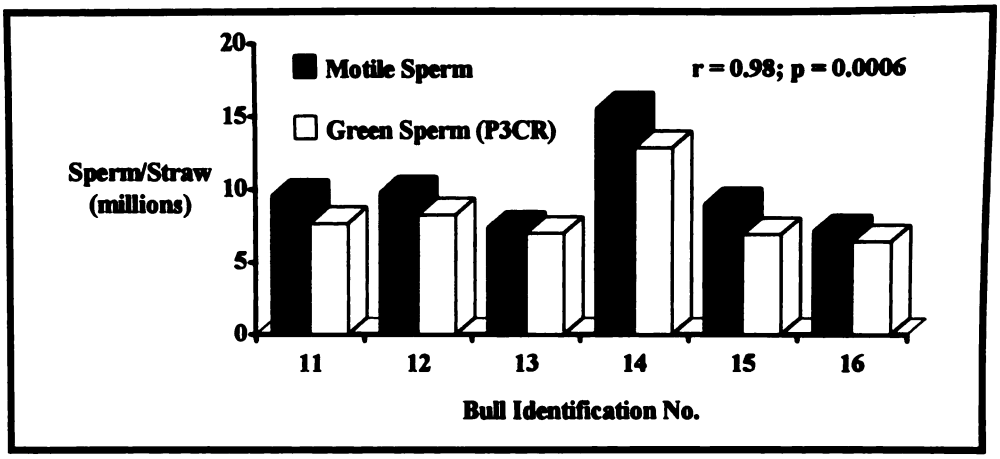
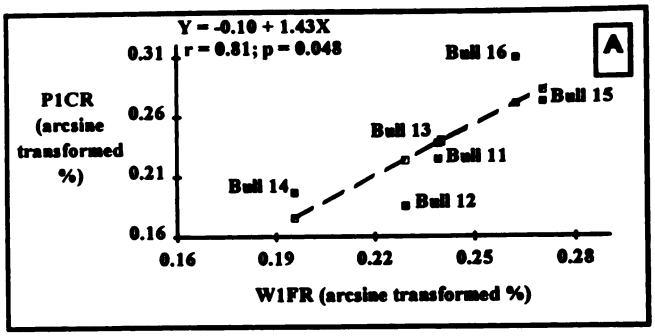



FIG. 3. A comparison of the number of motile spermatozoa per straw and the number of green-stained sperm cells per straw in thawed, cryopreserved samples in egg yolk citrate. Motility and cell concentration were determined using photographic analysis. Numbers of green-stained cells per straw were calculated by multiplying sperm cell concentration times flow cytometric percentages of populations that stained with carboxydimethylfluorescein diacetate (CMFDA) and rhodamine 123 (R123.CR).

aged and intact sperm cells after cryopreservation signified how bulls differed in their ability to withstand the freezing process. In this light, the spermatozoa of bull 2 were more sensitive to the freeze–thaw process than those of bull 4. These had similar increases in membrane-damaged cells, but bull 2 had a 29.1% decrease in intact cells after cryopreservation, whereas bull 4 only had a 21.9% decrease. It appears that all bulls had very similar freezing capacities, but bull 1 seems to have had a slightly better ability to survive than the others. Although he had the greatest increase in membrane-damaged cells after freezing (24.0%), the large increase was more than offset by the small proportion of moribund cells (0.5%) and the small decrease in intact cells (20.6%). Bull 2 seems to have had the lowest cryopreservation potential. His increase in membrane-damaged cells showed a midrange value (20.0%), but he had the largest decrease in percentage of intact cells (29.1%), and he ranked fifth in percentage of intact cells after cryopreservation. However, he displayed fewer moribund cells than did bull 4. Therefore, bulls 1 and 4 appear to have had the best and poorest cryopreservation potentials, respectively.

It is interesting that the P4CR population revealed no differences among bulls. Here, bull means were ranked in a seemingly disorganized way, relative to other populations. For example, bull 4 had the smallest percentage of spermatozoa in P3CR, yet he had the highest percentage in P4CR. In theory, this group of spermatozoa should be the hardiest because they exclude PI to the greatest degree.

When straws were cryopreserved, significance levels for differences among straws changed dramatically relative to fresh samples. For example, significance levels for straw variability went from $P = 0.92$ (P3FR) to $P = 0.11$ (P3CR). This was expected because variability among thawed straws is well known. Both milk and egg yolk samples



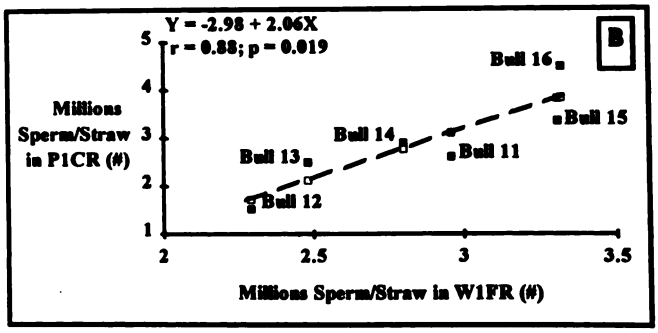




FIG. 4. Percentage (A) and number (B) of cryopreserved bovine spermatozoa predicted from fresh, 24-hour samples processed in egg yolk citrate. For each bull the predicted values are shown as open boxes (□), whereas the actual data points from which the regression equation was derived are shown as closed boxes (■). The predicted red-stained sperm cells (central population P1CR) in cryopreserved samples (□) and the data points from which the regression was derived (■) ($n = 6$) are shown. (A), Predicted percentages in thawed samples (□) are defined by the regression equation $Y = -0.10 + 1.43X$, where Y is the estimated percentage of cells in P1CR, and X is the percentage of cells in W1FR. (B), Predicted numbers ($\times 10^6$) of cells in thawed straws are defined by the regression equation $Y = -2.98 + 2.06X$, where Y is the estimated number ($\times 10^6$) of the central red subset in a thawed straw (P1CR), and X is the number of cells in the primary red-stained population in a fresh straw (W1FR).

showed variability approaching significance among straws after cryopreservation, especially in P2 and P3. The freezing process changes the distribution of phospholipids and produces aggregations of intramembranous particles in the plasma membrane over the postacrosomal region and tail (Holt et al, 1992). Alteration of the membrane structure results in spermatozoa that are less motile than fresh sperm, require a shorter capacitation time, have a more easily induced acrosome reaction, and have reduced fertility (Parks and Graham, 1992).

Finally, correlation and regression analyses indicated that the percentages of membrane-damaged cells in fresh samples were predictive of the percentages of intact cells in thawed sample. In milk (Table 2), fresh red population percentages were negatively correlated with frozen green populations (W1FR/P3CR and P1FR/P3CR; $r = -0.97$). Fresh red populations for both percentages and numbers were positively correlated with moribund sperm cells in frozen samples, with the central subsets showing slightly higher $r$-values. The data points (Fig. 2) from P1FR and P2CR are closely distributed around the regression lines and illustrate the strong relationship between the two populations. These data support the idea that components released from membrane-damaged or dying spermatozoa, possibly the protease acrosin, negatively affect the viability of the remaining live cells.

Using a different staining system than the one described here, Karabinus et al (1990) detected a flow cytometric transitional population in 3 of 20 milk-extended samples. They reported that two of these three had lower nonreturn rates (53.6% and 53.8%) than the mean (67.8%) and suggested that an inverse relationship existed between the presence of a moribund population and fertility. Garner et al (1988), using a dual staining system of PI and carboxyfluorescein diacetate (CFDA), also found transitional populations in a group of bulls having spermatozoa processed in either milk or egg yolk citrate. In the present study, no transitional P2 population appeared in egg yolk samples. The difference between the previous egg yolk sample results (Garner et al, 1988) and the current ones may be due to the different volume of stains applied, the lack of R123, different flow cytometers used for analyses, using Beltsville Thawing Solution versus Tyrode's salt solution, or individual bull differences. Because Karabinus et al (1990) did not reveal a transitional population in all bulls, the possibility exists that this particular group of ejaculates did not just by chance contain a moribund population.

Percentages of 10,000-cell samples were translated into numbers of cells per straw exhibiting red or green fluorescence (percentage times concentration). Although the data were not normally distributed due to differing spermatozoal concentrations among bulls, analyses included all bulls because straws of higher concentration in milk and egg yolk represented true values. They were not considered true statistical "outliers" because the values were not errors in measurement or data recording. As reflected in bull ranks (Table 1), significant correlations existed between P3FR and P3CR as well as P1FR and P2CR. Ratios of numbers of cells per straw in P3CR:P1CR gave similar values, with bulls 1 and 4 continuing to be on the high and low ends.

Conversion of percentages of 10,000-cell samples to numbers of cells per straw produced a change in correlation coefficients (Table 2). In some cases, the $r$-values changed both in value and sign. A change of sign makes no sense biologically. For example, the highly significant negative $r$- value ($r = -0.97$, $P = 0.0015$) between W1FR and P3CR for percentage of cells in milk-extended samples became positive, smaller, and statistically insignificant ($r = 0.54$; $P = 0.2715$) for numbers of cells per straw. A positive correlation means that as the percentage of membrane-damaged cells in a fresh sample increases, the percentage of live cells in a cryopreserved sample also increases. Obviously, this cannot be the case. One can get a visual perspective of the problem from a scatterplot of numbers of cells in P3CR versus W1FR. There was an almost perfect linear relationship among five of the bulls. Bull 5, however, had about a 50% higher cell concentration per straw than the others. The higher concentration of spermatozoa was due to his young age (C. H. Allen, personal communication). His ejaculate produced a percentage value in W1FR that was 0.50% less than the grand mean but 2.8% higher than the grand mean in P3CR. Translated into millions of cells per straw, this appeared as an extreme outlier on the scatterplot well above the others, producing a meaningless $r$-value. Yet bull 5 did, in fact, have significantly more intact cells per insemination dose. It is unlikely that younger bulls have cells that survive cryopreservation better. It is more probable that this is a characteristic of the individual, regardless of age.

### *Experiment 2: Egg Yolk–Citrate-Extended Samples*

Differences among bulls were detected in all populations except P4, both in fresh and cryopreserved samples. Bull 14 had significantly more intact sperm cells in P3FR (66.6%; Table 3) and less membrane-damaged cells in P1FR (3.7%) than all other bulls in fresh samples. However, after cryopreservation, bull 12 had a significantly higher percentage of intact spermatozoa than all other bulls (55.2%) and a lower percentage of membrane-damaged cells (10.1%). Alternately, the ejaculate of bull 16 was significantly lowest and highest in cryopreserved intact (37.8%) and membrane-damaged cells (26.4%), respectively. These two bulls held the middle two ranked positions in population P4CR. The lack of significant differences and correlations when using quadrant analyses

might be explained by the incorporation of P4 into P3. Bull ranks show complementary positions between P1 (or W1) and P3, and between fresh and cryopreserved samples in these populations. Yet P4 did not follow the trend. Within this category, bulls were ranked in a different order. Thus, P3 plus P4 would produce more uniform means among bulls. In a recent study of human spermatozoa, there was a significant correlation ($r = 0.63$, $P = 0.011$) between the fresh and frozen P4 populations (Kramer et al, 1993). The use of quadrant analyses, different staining patterns, or species differences may explain why correlations were significant in the human study, but not in the present study. As in milk samples, the biological significance of this "elite" P4 population of cells, which excludes PI the most, remains to be discovered.

In egg yolk citrate, significant correlations existed between post-thaw motility and P3CR ($r = 0.88$). There were significant negative correlations with P1CR or W1CR ($r = -0.86$ or $-0.90$). These relationships were expected because motile cells are those with intact membranes and functional mitochondria, the very properties on which the fluorescent probes depend. Significant correlations also existed between percentages of P1CR and P1FR ($r = 0.83$) or W1FR ($r = 0.81$). No significant relationships were found for any fresh egg yolk percentages with P3CR. It is not known whether egg yolk samples differed from milk samples because of some difference in stain uptake as a result of medium effects, or whether it was a biological function of differing survival rates of sperm cells in milk versus egg yolk citrate.

It is important to keep in mind that these percentages do not account for the entire 100% of the 10,000 cells analyzed. If all cells were accounted for in the defined populations, an increase in one would automatically produce a decrease of equal magnitude in another population. Here, central subsets of similarly stained cells differed among bulls. They represented the central tendency rather than the total population of cells that stained similarly. It seems analogous to the mean ± 1 standard deviation (SD). In fact, it is questionable whether the increased significance and correlation coefficient values from window analyses over quadrant analyses were due to the biological significance of the subset populations, or whether it was merely a mathematical function due to measures of central tendency.

The number of motile cells per insemination dose is related to fertility up to a critical threshold value. Above this value, increases do not affect fertility (Sullivan and Elliott, 1968; Hafs et al, 1970; Sullivan, 1970; Almquist, 1975; Elliott, 1978b; Ericsson et al, unpublished data; Gérard and Humblot, 1991). However, Nadir et al (1993) studied the effects of sperm cell numbers in fresh and frozen samples on embryo quality and fertility, and found an increase in both when using $100 \times 10^6$ cells versus 20 $\times 10^6$ cells per insemination dose. He suggested that previous studies did not use high enough doses to detect a second level of correlations (a sigmoidal relationship). He also suggested that increased fertility might be due to the presence of more accessory sperm available during fertilization.

Bulls vary greatly in post-thaw motility (Sullivan and Elliott, 1968). Therefore, the minimum number of cells per insemination dose should be determined and used on an individual basis (Pickett and Berndtson, 1978). In the present study, bull 14 had the highest number of motile cells per insemination dose ($15.5 \times 10^6$ per straw). This estimate, using the photographic method, comes very close to that of the flow cytometric estimate. Populations P3 plus P4 show $14.2 \times 10^6$ motile spermatozoa (Table 3). The green-stained cells around the periphery of these two populations were not included in analyses, and they probably account for the difference. Bull 16 had the least number of motile cells in P3CR ($6.4 \times 10^6$) and the largest number of membrane-damaged cells ($7.1 \times 10^6$, P1CR). Bull 14, who had the greatest number of intact cells, did not have the least number of membrane-damaged cells. Rather, he ranked second in W1CR and third in P1CR. Therefore, the ratio of P3CR:P1CR gives bull 12 the highest rank and bull 16 the lowest. The scatterplot of data around the regression line shown in Figure 4B also puts bull 12 in the top position, with bull 14 at the midpoint.

## Conclusions

Although this study has found parameters in fresh semen samples predictive of their frozen counterparts, the utility of using the costly flow cytometric method to evaluate semen remains questionable. Flow cytometry does improve accuracy and precision. This would be extremely important if fresh semen sample analyses determined which young sires were to be culled before the fertility test period. Therefore, the utility of flow cytometry depends on the cost-effectiveness and need for high repeatability and accuracy for each unique situation.

Partitioning red and green windows into smaller subset populations revealed significant relationships between fresh and cryopreserved bull ejaculates. These relationships were not apparent when the entire populations of membrane-damaged, red-stained and intact, green-stained spermatozoa were analyzed. Populations of membrane-damaged sperm cells in fresh samples appeared predictive of cryopreserved populations of membrane-damaged sperm cells in egg yolk citrate, and of moribund and intact populations in milk.

To determine which bull ejaculates were most and least sensitive to cryopreservation, several aspects were considered. Mean percentages of central subsets of red- and

green-stained populations were ranked, both for percentage of 10,000-cell samples and numbers of spermatozoa per straw. Regression analyses were performed on those populations consistently showing significant correlations. Ratios of intact to membrane-damaged populations were considered. The difference between the percentage of fresh spermatozoa and the percentage cryopreserved spermatozoa within each bull was examined. The relationships between post-thaw motility and flow cytometric populations were investigated, and bulls were ranked according to motility. Each of these aspects consistently pointed to the ejaculates of bulls 1 and 12 as having the greatest cryopreservation potentials, and bulls 4 and 16 the least. It is felt that these results provide a reliable data base for later studies on fertility.

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