Comparison of Semen Quality in Young and Mature Holstein Bulls Measured by Light Microscopy and Flow Cytometry

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ABSTRACT

Random samples of cryopreserved, milk-extended semen, collected from 20 Holstein bulls at about 14 mo of age (young) and again at about 4 yr of age (mature), were evaluated at thawing and during 3-h incubation to compare semen quality of young versus mature bulls. Evaluation by differential interference contrast microscopy showed greater proportions of cytoplasmic droplets in semen from young versus mature bulls. Mature bulls exhibited greater proportions of intact acrosomes in freshly thawed semen than did young bulls. Evaluation of sperm chromatin structure by flow cytometry after staining with acridine orange showed lower values for mature versus young bulls, indicating resistance of DNA in nuclear chromatin to acid denaturation increased with age. Correlations between ages for most sperm morphology, acrosome integrity, and flow cytometry variables were high and positive. Nonreturn rate for young bulls was positively related to morphologically normal sperm and acrosomal integrity and negatively related to flow cytometry traits. Results suggest semen quality of young bulls was related to subsequent quality as mature bulls. With flow cytometry, differences were detected between semen samples that were not evident with light microscopy.

(Key words: bull semen quality, light microscopy, flow cytometry)

INTRODUCTION

Artificial insemination organizations acquire numerous young bulls each year, and part of the daily activities involves the collection and evaluation of semen from these potential young sires (28). Early collections from young bulls are often substandard in terms of sperm concentration, motility, or morphology (10, 12, 18); but some qualitative and quantitative improvement occurs with age (1). Young bulls are culled from sampling programs when they fail to perform up to the reproductive standards established by the AI organization; however, semen collection of some young bulls begins as early as 10 mo of age with culling decisions based upon semen production up to 16 mo of age (28).

The extent to which the young bull's semen quality is predictive of that of the mature bull is not well known. Knowing the relationship between the quality of semen produced by the young bull and that produced by the mature bull would improve the ability to evaluate potential AI sires at an early age. Although many semen quality tests are in use for evaluating fertility-related characteristics of a semen sample (12, 15, 21, 25), these tests often are subjective (3), have low repeatability between observers (16), and can be time-consuming. Recently, the results of a flow cytometric technique for analysis of sperm chromatin structure (14) have been shown to correlate with fertility (6) in addition to offering speed (hundreds of cells per second), precision, and objectivity of measurement.

This study was conducted to investigate the relationship between young bulls (sampling sire age) and those same bulls at a mature age (approximating establishment of a proof) for semen quality as determined by light microscopy and flow cytometry.

MATERIALS AND METHODS

Twenty bulls owned by Eastern Artificial Insemination Cooperative, Inc. (EAIC), Ithaca, NY, were used to compare semen quality of young versus mature bulls. At approximately 14 mo of age (young bulls) and again at approximately 4 yr of age (mature bulls), semen from one collection from each bull at each age was randomly selected for evaluation. Semen from young bulls was randomly selected from among the first 1000 units processed for each bull. Semen from mature bulls was randomly selected from each bull's production during its 4th yr. The semen was initially evaluated by EAIC before being processed in milk based extender, frozen in .5-ml polyvinyl chloride straws, and stored in liquid nitrogen. Because of limited semen availability, one straw was evaluated per bull per age. Nonreturn rates (%NR), available for young bulls only, were supplied by EAIC and were based upon 356 ± 29 (range 323 to 417) first services and averaged (± SD) 67.8 ± 10.1% (range 53.0% to 80.3%).

Each straw of semen was thawed at 37°C for 30 s, emptied into a 1.5-ml polyethylene microcentrifuge tube, and incubated at 38.5°C for 3 h. At thawing and at 30, 60, 120, and 180 min thereafter (time 0, 1, 2, 3, and 4), 24 µl semen were pipetted into 300 µl of TNE buffer (.01 M Tris-HCl, .15 M NaCl, .001 M disodium EDTA, pH 7.4) in .5-ml polyethylene microcentrifuge tubes. Diluted samples were gently vortexed, immediately placed in a –20°C freezer overnight, and then stored at –95°C. At each sample time, an additional 20 µl of semen were fixed using 100 µl of 1% glutaraldehyde in .03 M cacodylate buffer, gently vortexed, and stored at 4°C.

Flow Cytometry

The frozen, TNE-diluted samples were evaluated by flow cytometry (FCM) using the sperm chromatin structure assay (SCSA) developed in this laboratory (13). Immediately upon removal from the –95°C freezer, the capped samples were thawed in 37°C water for 60 s and placed in an ice slurry until FCM evaluation. A 200-µl sample of frozen-thawed, TNE-diluted semen was admixed with 400 µl of ice-cold .1% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in .08 N HCl and .15 M NaCl,

pH 1.2. After 30 s, 1.2 ml of ice-cold acridine orange (AO) staining solution [.2 M Na$_2$HPO$_4$, 1.0 mM disodium EDTA, .15 M NaCl, .1 M citric acid monohydrate, 6 µg/ml of chromatographically purified AO (Polysciences, Warrington, PA), pH 6.0] were admixed, and the sample was analyzed by FCM 3 min later. Because sperm concentrations varied, semen samples were diluted such that 150 to 250 sperm/s were evaluated by the flow cytometer. Dilutions were performed to ensure an AO: sperm DNA-phosphate molar ratio ≥2 (11).

All FCM sperm samples were measured using an Ortho Diagnostic Systems Cytofluorograf II equipped with a Lexel 100 mW argon ion laser and interfaced with an Ortho Diagnostic Systems model 2150 computer system (Becton Dickinson Immunocytometry Systems, San Jose, CA). Green (515 to 530 nm) and red (>600 nm) fluorescence emitted by each AO-stained sperm after laser excitation (35 mW, 488 nm) was processed through photomultiplier tubes and quantified. Duplicate FCM analyses of 5000 sperm each were performed on each semen sample. A software protocol in the 2150 computer calculated the ratio of red fluorescence to red plus green (total) fluorescence (alpha t, α_t) for each sperm. In addition to the mean and standard deviation of α_t (Xα_t and SDα_t, respectively), the proportion of cells lying outside the main population of α_t (COMPα_t) was calculated for each sample of 5000 sperm analyzed. Figure 1, which contains a cytogram and a frequency histogram generated from an FCM analysis of 5000 sperm, illustrates the population from which the Xα_t, SDα_t, and COMPα_t were calculated for each semen sample.

Light Microscopy

Sperm in fixed semen samples were coincidently evaluated for morphology and acrosomal integrity. Proportions of morphologically normal sperm and sperm having primary (1°, head), secondary (2°, droplet), and tertiary (3°, tail) abnormalities (26) were recorded in addition to proportions having intact acrosomes. Thus, acrosomal integrity of the morphological types was obtained. Results are reported as the percentage of sperm in each morphological classification in addition to percentage of intact acrosomes (%IA), percentage morphologically

normal sperm having intact acrosomes (%NIA) and percentage morphologically abnormal sperm having intact acrosomes (%AIA). A wet mount was prepared from each fixed semen sample and evaluated at 1250x magnification under oil using differential interference contrast microscopy. Two hundred sperm were randomly observed on each coded wet mount.

Statistical Analysis

Data were analyzed by least squares procedures using the general linear models procedure (GLM) of SAS. Main effects for the three-way ANOVA were bull, age, and time. The age effect and the time effect were tested using the bull by age interaction and the bull by time interaction, respectively, as error terms. The residual was used to test all other effects. Means comparisons were performed using the GLM contrast specification. Results are presented as least squares means and standard errors. Pearson product-moment correlations were obtained using the CORR procedure of SAS. Correlations with %NR pertain only to young bulls.

RESULTS AND DISCUSSION

Sperm Morphology

Sperm morphology in frozen-thawed semen did not differ between young and mature bulls, except 2* abnormalities were more prevalent in young bulls (Figure 2). Young and mature bulls were positively related for %normal sperm (r = .73, $P<.001$), %1* abnormalities (r = .87, $P<.001$), and %3* abnormalities (r = .47, $P<.05$), and unrelated for %2* abnormalities (r = -.03, $P>.80$).

The positive correlations between ages and the lack of differences between ages for normal sperm, 1* and 3* abnormalities, suggest that the young bulls' performance was indicative of that in the mature bulls for those traits. These results support those of Pearson et al. (23), who found moderate among ejaculate repeatabilities in young sires for proportions of specific sperm abnormalities and for proportions of sperm in the abnormality classes (i.e., 1*, 2*, and 3*), indicating that little change in those semen quality characteristics would be expected with time. The reduced proportions of secondary
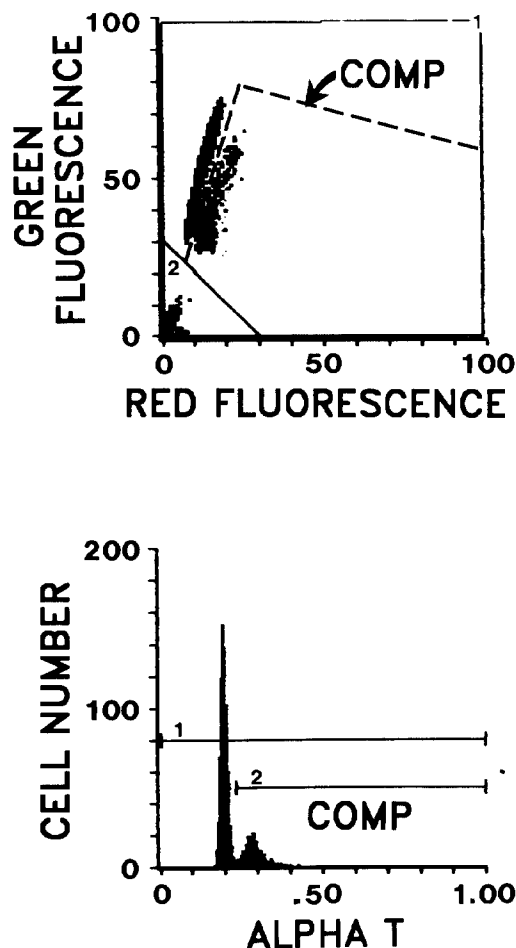
Figure 1. Example of cytogram (top) and frequency histogram (bottom) generated from FCM evaluation of 5000 sperm. Top: diagonal line (2) excludes debris from sperm population to be analyzed. Cells outside the main population of α_t (COMPα_t) separated from main population by broken line. Bottom: entire sperm population indicated by region 1. COMPα_t indicated by region 2.

abnormalities in mature versus young bulls' semen agree with reports of others (8, 19) and suggest that maturation of epididymal sperm (2, 20) may become more efficient (24) with bull maturity. However, frequency of this abnormality was quite low, so differences may not be biologically meaningful.

Nonreturn rate of young bulls was positively related to the percentage of normal sperm (r = .52, $P<.05$), negatively related to %1* abnor-
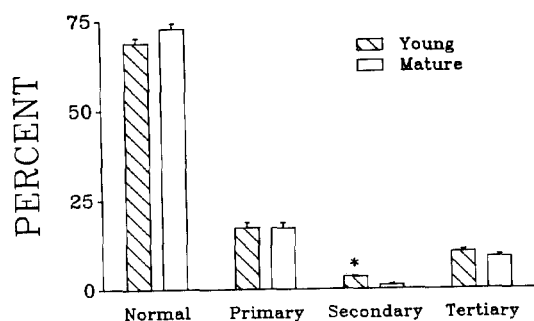
Figure 2. Comparison of sperm morphology from bulls about 14 mo (young) and 4 yr (mature) of age. Bars indicate morphologically normal sperm percentage of primary (head) abnormalities; secondary (cytoplasmic droplet) abnormalities; and tertiary (tail) abnormalities. P<.05.

malities (r = −.54, P<.01), and unrelated to %2° and %3° abnormalities (r = −.11, P>.30, and r = .37, P>.10, respectively). Correlations between %NR and sperm morphology are in agreement with those found for 1° abnormals and 3° abnormals by Saacke and White (26), but are at odds with their results for 2° abnormals, even though percentages of this abnormality were comparable between the two studies. These results suggest that percentages of normal sperm and 1° abnormalities are roughly equal in importance to fertility and indicate the importance of evaluating sperm morphology in assessing fertility potential.

Acrosomal Integrity

Differences between young and mature bulls for acrosomal integrity traits were not significant at most times (Figure 3). Percentage of IA was greater in mature than young bulls at time 0 only (Figure 3a), whereas %NIA was greater in mature than young bulls only at time 0 and time 1 (Figure 3b). Incubation of semen for up to 2 h improved the correlations between young and mature bulls for acrosome integrity traits at time 0 (Table 1). Correlations of %NR with %IA (r = .63, P<.01), %NIA (r = .65, P<.01), and %AIA (r = −.22, P>.35) at time 0 did not improve with incubation.

The similarity between ages and the positive between age correlations for the acrosomal integrity traits suggest the frequency of occur-
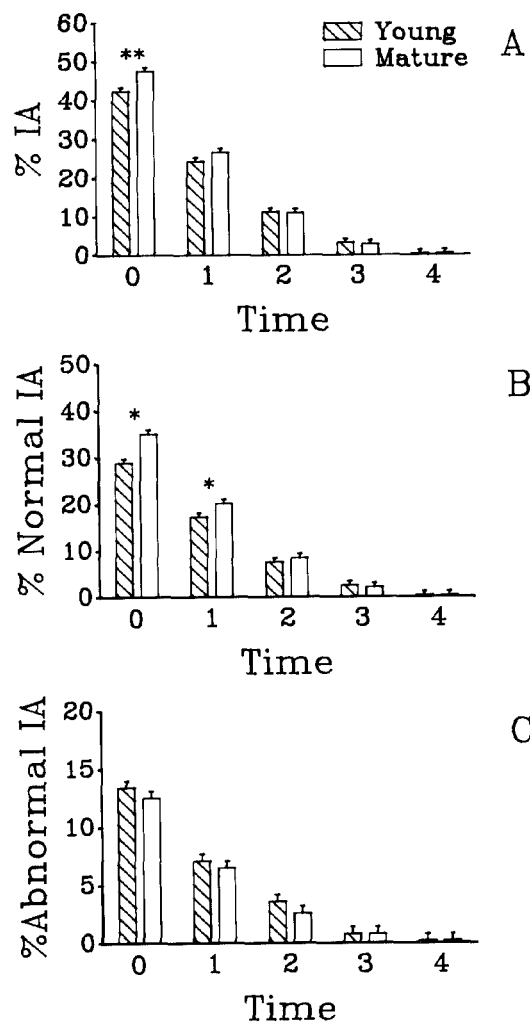

Figure 3. Comparison of acrosomal integrity from bulls about 14 mo (young) and 4 yr (mature) of age after 0, 30, 60, 120, and 180 min of incubation (time 0, 1, 2, 3, and 4) at 38.5°C. (A) Percentage of intact acrosomes, (B) percentage of morphologically normal sperm with an intact acrosome, and (C) percentage of morphologically abnormal sperm with an intact acrosome. *P<.05. **P<.01.

rence of these traits in the young bulls was indicative of that in the mature bulls. Acrosomal integrity improves with age after puberty (17), an observation supported by the present results. The disappearance of differences for acrosomal integrity between ages for incubated semen (Figure 3) indicates that acrosomal performance during incubation was

TABLE 1. Correlations between measurements of sperm acrosomal integrity traits obtained from bulls at a young and a mature age for unincubated and incubated samples of frozen-thawed semen.

Intact acrosomes trait	Incubation time, min				
	0	30	60	120	180
%IA	.53*	.64**	.77***	.75***	-.02
%NIA[1]	.61**	.76***	.72***	.49*	-.16
%AIA[2]	.48*	.54*	.58**	.70***	-.20

[1]Morphologically normal sperm having intact acrosome.

[2]Morphologically abnormal sperm having intact acrosome.

*P<.05.

**P<.01.

***P<.001.

TABLE 2. Correlations between nonreturn rate and sperm flow cytometric (FCM) traits of young bulls for unincubated and incubated samples of frozen-thawed semen.

FCM Trait	Incubation time, min				
	0	30	60	120	180
$X\alpha_t$[1]	-.46*	-.62**	-.66**	-.62**	-.50*
$SD\alpha_t$[2]	-.64**	-.70**	-.76***	-.78***	-.77***
$COMP\alpha_t$[3]	-.60**	-.46*	-.56*	-.49*	-.45*

[1]Mean of alpha t.

[2]Standard deviation of alpha t.

[3]Cells outside the main peak of alpha t.

*P<.05.

**P<.01.

***P<.001.

similar across ages despite initial (time 0) differences. The persistence of acrosomal performance across ages is also illustrated by the improved relationships between ages for acrosomal integrity when sperm, already stressed by the freeze-thawing process, was further stressed by incubation (Table 1).

Flow Cytometry

Mean α_t and $SD\alpha_t$ values were greater for young bulls than for mature bulls at all sample times (Figures 4a, b). Values for $COMP\alpha_t$ were greater for young than for mature bulls at times 0, 1, and 2 (Figure 4c). Correlations at time 0 between young and mature bulls for $X\alpha_t$ (r = .51, P<.05), $SD\alpha_t$ (r = .60, P<.01), and $COMP\alpha_t$ (r = .45, P<.05) were unchanged by incubation. Incubation resulted in generally improved correlations of %NR with $X\alpha_t$ and $SD\alpha_t$ but not with $COMP\alpha_t$ (Table 2). The proportion of the total change in $COMP\alpha_t$ that occurred during the first 30 min of incubation was greater for young bulls than for mature bulls (69.7 ± 2.8% vs. 53.2 ± 2.8%, P<.001).

Lower $X\alpha_t$, $SD\alpha_t$, and $COMP\alpha_t$ values for mature than for young bulls indicate sperm chromatin structure improved with bull maturity. The more rapid rate of change in $COMP\alpha_t$ for incubated sperm from young than for mature bulls indicates greater susceptibility of young bulls' sperm chromatin structure to incubation-induced changes. Lower α_t and

$COMP\alpha_t$ values are consistent with greater resistance to in situ acid denaturation of nuclear DNA in sperm, which corresponds to reduced heterogeneity of chromatin structure. Other studies (6, 7, 14) have shown that increased heterogeneity of sperm nuclear chromatin struc-

TABLE 3. Correlations between bull sperm flow cytometric (FCM) traits and bull sperm morphology and acrosome integrity traits for samples of frozen-thawed semen.

Item	FCM trait[1]		
	$X\alpha_t$	$SD\alpha_t$	$COMP\alpha_t$
%Normal	-.41**	-.50**	-.55***
%Primary[2]	.41**	.50**	.59***
%Secondary[3]	.12	.10	-.01
%Tertiary[4]	-.21	-.21	-.33*
%IA[5]	-.59***	-.60***	-.67***
%NIA[6]	-.56***	-.60***	-.64***
%AIA[7]	.06	.14	.07

[1]Mean α_t, standard deviation of α_t, and cells outside main population of α_t, respectively.

[2]Head abnormalities.

[3]Cytoplasmic droplet abnormalities.

[4]Tail abnormalities.

[5]Intact acrosomes.

[6]Morphologically normal sperm having intact acrosomes.

[7]Morphologically abnormal sperm having intact acrosomes.

*P<.05.

**P<.01.

***P<.001.

ture is associated with spermatogenic distur-
bances, morphologically abnormal sperm and
infertility. In the present study, consistent dif-
ferences between ages for FCM variables but
not for sperm morphology or acrosome integ-
rity suggest that FCM measurements of sperm
chromatin structure may be a more sensitive
indicator of differences among semen samples
than the conventional semen quality tests em-

ployed. Differences between ages for $X\alpha_t$ and
$SD\alpha_t$ were significant throughout incubation
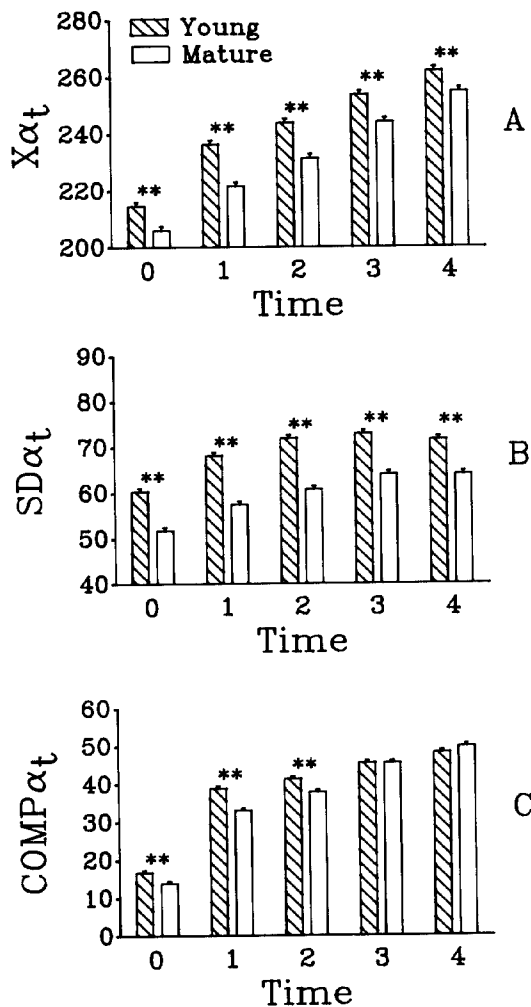and were significant for $COMP\alpha_t$ through time

Figure 4. Comparison of sperm flow cytometric values
from bulls about 14 mo (young) and 4 yr (mature) of age
after 0, 30, 60, 120, and 180 min incubation (time 0, 1, 2, 3
and 4) at 38.5°C. A) Mean α_t of $(X\alpha_t)$, B) standard
deviation α_t $SD\alpha_t$, and C) percentage of cells outside the
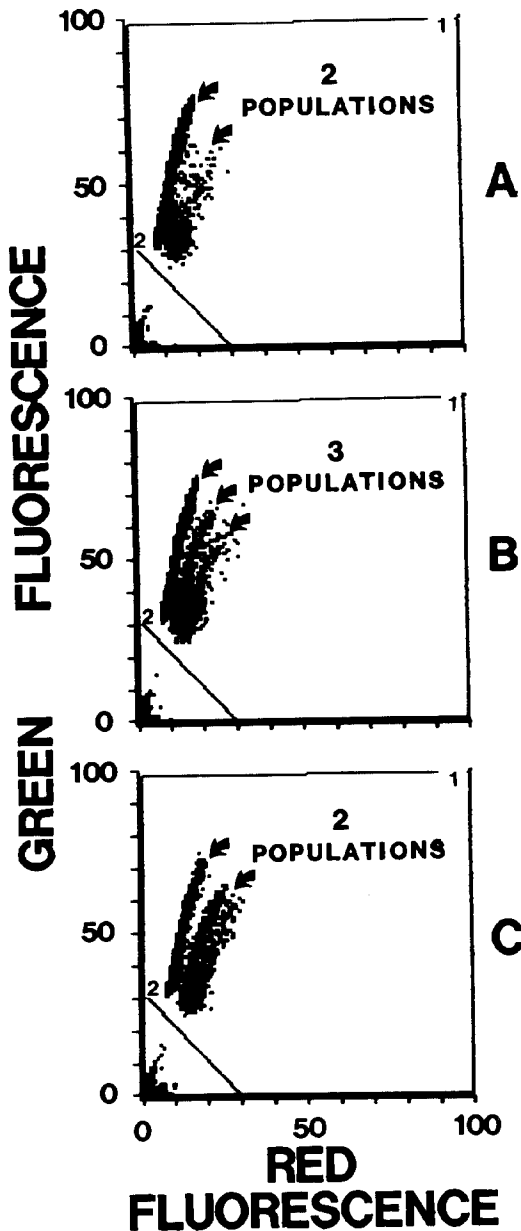main population of α_t $(COMP\alpha_t)$. **$P<.01$.

Figure 5. Cytograms showing examples of A) two
sperm populations normally encountered in semen samples,
B) transient third sperm population observed at 30 min of
incubation, and C) decreased resolution of third population
by 60 min of incubation. Third population observed in 3 of
20 bulls.

2. This indicates that once age differences for FCM traits were established, they tended to persist. The positive correlations between ages for the FCM traits support this view and also show that values for FCM traits of the mature bulls followed those for the young bulls.

Flow cytometric traits were negatively correlated to percentage of normal sperm and positively related to %1° abnormalities (Table 3). Values of 2° and 3° abnormalities were not related to most FCM traits; FCM traits were negatively related to %IA and %NIA and unrelated to %AIA. Incubation did not improve those relationships. The relationships of FCM traits with abnormal sperm morphology and acrosomal integrity in the present study are in general agreement with previous work in the bull (6, 7). They indicate increased susceptibility of sperm nuclear DNA in chromatin to acid denaturation with increased proportions of abnormal sperm, particularly head abnormalities, and decreased percentages of IA. The mechanisms behind these relationships are not clear.

Cytograms of incubated semen samples from 3 of the 20 bulls exhibited a third, transient population of cells where two populations of cells were usually observed. The third population was not apparent at time 0 (Figure 5a), was evident at time 1 (Figure 5b), and was indistinct by time 2 (Figure 5c). The significance of this phenomenon is unclear. The SCSA, sperm morphology, and acrosomal integrity results did not appear to differ from bulls not exhibiting the third population. The nonreturn rates for 2 of the 3 bulls exhibiting the third population was lower than the mean %NR for all 20 bulls (53.6 and 53.8% vs. 67.8%), suggesting a relationship with fertility. One bull exhibited the third population in semen from both ages, suggesting an effect across ages. This evidence indicates that some incubation related changes in sperm chromatin structure may be discontinuous or steplike in nature and may indicate inherent differences between semen samples not previously recognized.

CONCLUSIONS

A randomly selected sample of a bull's semen may be viewed as insufficient to assess semen quality. Semen quality can be adversely affected by factors such as season (27), illness (9), and environmental temperature (4, 22). Al-

though semen quality varies among ejaculates within bulls, greater variation in semen quality occurs among bulls (26). The results of the present study show that the quality of a randomly selected semen sample from the young bull was generally indicative of the quality of a random sample of his semen as a mature bull, when his semen was evaluated using conventional tests. Semen quality of mature bulls in this study was improved only in terms of acrosomal integrity and 2° abnormalities; the other conventional semen quality traits did not differ between ages. However, consistently lower values for all FCM traits in mature versus young bulls indicate improved semen quality in mature bulls and illustrate a greater sensitivity of FCM analysis to differences between semen samples compared with conventional techniques. The results of this study support previously reported relationships of sperm chromatin structure with fertility, sperm morphology, and acrosomal integrity (5, 6, 7). Although this study was conducted as a preliminary investigation of relationships between young and mature bulls for semen quality, it illustrates the potential of the flow cytometer as a tool for use in the evaluation of male fertility.

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