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Relationship of Bull Fertility to Sperm Nuclear Shape

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ABSTRACT: The relationship between sperm nuclear shape and bull fertility was determined. Two groups of bulls, 3 per group, were selected. Bulls differed in fertility based on lifetime nonreturn rates. Digital images of propidium iodide-stained sperm from each bull were collected and shape-evaluated by Fourier harmonic amplitudes 0 to 5. A discriminant function ($P < .05$) was constructed based on harmonic amplitudes and the 2 fertility groups. When individual sperm were classified as being of high or lower fertility, the percentage of each bull's sperm placed in the high-fertility group had a linear relationship ($r = .89$, $P < .05$) with fertility. To construct a plot of mean sperm shapes, a novel technique to automatically orient and identify the anterior tip of the sperm head was developed. The mean nuclear shape of high-fertility sperm was more elongated and tapered than those of lower fertility. A discriminant function ($P < .05$) was also constructed that separated the 6 bulls into 2 groups based only on the harmonic amplitudes or sperm nuclear shape. The bulls were correctly classified into the 2 fertility groups. A comparison of sperm chromatin structure analysis (SCSA) and harmonic amplitudes found that overall size variance, anterior roundness, and posterior taperedness of sperm nuclei were related to chromatin stability ($P < .05$). Some of the differences observed in sperm nuclear shape between the high- and lower-fertility bulls may be explained by varying levels of chromatin stability. However, sperm nuclear shape appears to contain additional information from chromatin stability alone. In this particular study, with 6 bulls, all with good chromatin quality, sperm nuclear shape was a better predictor of bull fertility.

Key words: Morphology, image analysis, SCSA, chromatin, orientation.

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Whereas differences in fertility have been demonstrated among fertile bulls (Sullivan and Elliot, 1968; Beaty et al, 1969; Saacke et al, 1980; DeJarnette et al, 1992), the causes of these differences remain unclear. Saacke et al (1988, 1994) have suggested that factors associated with semen quality that affect fertility can be classified as either compensable or noncompensable. The effects of compensable factors on fertility were suggested to be sensitive to the number of sperm inseminated, whereas those of noncompensable factors were not. As the number of sperm inseminated increases, fertility increases until a plateau is reached (den Dass, 1992). At this point, compensable factors no longer have an effect on fertility. Commercial insemination of cattle provides at least the plateau number of sperm in an insemination dose, whereas natural matting provides this number of sperm many times over. It is thus the noncompensable factors that contribute most to the fertility level of a bull. A noncompensable defect in sperm would be one in which a sperm reaches the site of fertilization and initiates the process of egg activation, but fails to sustain zygotic, embryonic, or fetal development. We have found evidence of such defects in sperm from bulls that differ in fertility (Eid et al, 1994). Likely candidates for noncompensable factors would be incorrectly assembled chromatin or damaged DNA within the sperm nucleus. A problem remains of how to detect these defects within sperm in a fashion that would allow for commercial or clinical application.

One rapid and objective method that has been successfully used to detect altered chromatin structure associated with fertility potential and toxicant-induced damage is the sperm chromatin structure assay (SCSA; Ballachey et al, 1986, 1987, 1988; Evenson and Jost, 1994, 2000). In this procedure, sperm are first treated for 30 seconds at pH 1.2 to potentially denature DNA in situ. With normal chromatin structure, sperm DNA does not denature under these conditions. The sperm are then stained with the metachromatic DNA-stain acridine orange (AO). When intercalated into native, double-stranded DNA, AO fluoresces green, whereas AO associated with single-stranded DNA fluoresces red. The amount of green and red fluo-

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rescence emitted by each of 5000 sperm is measured per sample and provides an index of the percentage of cells with denatured DNA (% cells outside the main population—%COMP). The extent and variation of DNA denaturation is expressed as alpha t = red/(red + green) fluorescence. SCSA data are expressed as the means of these parameters as well as the variation. SCSA has detected alterations in chromatin that was correlated with fertility in the bull (Ballachey et al, 1987), boar (Evenson et al, 1994), and stallion (Kenney et al, 1995); and exposure to toxicants in the bull (Ballachey et al, 1986), rat (Evenson et al, 1989), and mouse (Wiger et al, 1995). Despite its successes, SCSA has seen limited use in commercial settings due to the cost of a flow cytometer.

Because mammalian sperm heads consist almost entirely of chromatin, their shape should be related to DNA content and chromatin organization (Steinholt et al, 1994; Sailer et al, 1996). The most frequent approach to assessing sperm head shape is subjective evaluation and categorization into normal or abnormal groups (Barth and Oko, 1989). However, it is not uncommon to find sperm classified as normal possessing abnormal chromatin (Dobrinski et al, 1994) or karyotypes (Kishikawa et al, 1999). We predict that potential problems with chromatin or damaged DNA will result in only minor changes to sperm head shape. Traditional, subjective evaluation of sperm morphology is not adequate to assess potential, noncompensable fertility traits. Computer-aided image analysis is a potential alternative for evaluating sperm head morphology. We recently developed an objective and robust approach involving the use of Fourier harmonic amplitudes (Ostermeier et al, 2001). Using this technique, the curvature of sperm perimeters are explained by Fourier functions, which contain multivariate shape measures known as harmonic amplitudes. These measures are able to objectively identify previously reported abnormalities and can differentiate subtle yet distinct differences among sperm likely to be classified as normal using more-conventional approaches to semen analysis.

The primary goal of this research was to determine if sperm nuclear shape is related to bull fertility. Fourier harmonic analysis was used to determine multivariate parameters that describe the shape of sperm nuclei from bulls that differ in fertility. We then used discriminant and regression analysis to determine the relationship of these parameters to fertility and visualized these relationships for the first time by generating plots of average sperm nuclei. Next, the relationship of sperm chromatin structure to nuclear shape was investigated by evaluating sperm for chromatin stability in response to acid exposure and comparing this to the shape of the sperm nuclei as determined by Fourier harmonic analysis.

Methods

Semen Samples

American Breeders Service Global (DeForest, Wisc) graciously provided cryopreserved semen representing one collection from each of 6 bulls along with lifetime, nonreturn rate data for these bulls. Three high-fertility and 3 lower-fertility bulls had mean lifetime nonreturn rates of 78% (SE = 2) and 69% (SE = 1), respectively. Nonreturn rates were determined over the life of bulls and constituted at least 5000 first services per bull. The exact number of ejaculates used in the calculation of nonreturn rates was not provided, however, it likely represents data on hundreds of ejaculates/bull. Characteristics and in vitro fertility of semen from the same collections for these bulls have been previously described and evaluated in our laboratory (Eid et al, 1994). A single straw from each bull was thawed and sperm were separated from extenders on a 45%–90% discontinuous Percoll gradient at 700 $\times$ *g* for 15 minutes (Parrish et al, 1995). The sperm were then resuspended to 1×10^6 sperm/mL in bovine gamete medium 3 (BGM3) without bovine serum albumin (Vredenburgh-Wilberg and Parrish, 1995). Protamine sulfate was added at a concentration of 20 μg/mL to the final sperm suspension to prevent sperm agglutination. Sperm from each bull were then placed on 7 poly-L-lysine coated long coverslips (24 $\times$ 60 mm, No. 1) and air-dried. This treatment helped to ensure that sperm were flat on slides (Ostermeier et al, 2001). All chemicals were obtained from Sigma (St Louis, Mo) unless otherwise noted.

Image Analysis and Data Collection

Fourier harmonic amplitudes were obtained for sperm nuclei as described by Ostermeier et al (2001). Briefly, sperm were stained with propidium iodide (Molecular Probes, Eugene, Ore). Then, epifluorescent microscopy and computer-aided image analysis were used to obtain Cartesian coordinates defining the nuclear perimeters of 100 sperm per bull. The Cartesian coordinates were converted to polar coordinates, then to Fourier functions by multiple trigonometric regression. The Fourier functions contained harmonic amplitudes 0 to 5 and were used as multivariate measures of sperm nuclear shape. Based on the observed variance and number of sperm evaluated, there was a 95% chance that the mean harmonic amplitudes for each bull were within 1% of the true population mean (Snedecor and Cochran, 1989).

To reconstruct the mean shape for a population evaluated with condensed Fourier functions requires both mean harmonic amplitudes and phase angles (Flook, 1982). Although harmonic amplitudes describing sperm nuclear shape were independent of orientation, the phase angles were not (Ostermeier et al, 2001). A method to consistently orient sperm was developed that was both objective and able to be automated. Inspection of polar coordinate graphs for individual sperm nuclei revealed that the longest radius from the centroid always occurred in the anterior region. To enhance this difference, the polar coordinate data were smoothed using the condensed Fourier function (Ostermeier et al, 2001) summed through frequency 2. The angle associated with the largest radial distance was then defined as 0 and consistently bisected the anterior portion of the sperm nucleus.

Table 1. *Effect of bull fertility on harmonic amplitudes (mean ± SE) at Fourier frequencies 0–5**

Bull Label	Nonreturn Rate (%)	Harmonic Amplitude Frequency 0	1	2	3	4	5
				High-Fertility Bulls			
A	80	3.126 ± 0.008	0.148 ± 0.004	1.340 ± 0.008	0.108 ± 0.003	0.389 ± 0.006	0.149 ± 0.004
B	75	3.172 ± 0.008	0.137 ± 0.005	1.240 ± 0.007	0.122 ± 0.004	0.289 ± 0.006	0.131 ± 0.003
C	79	3.046 ± 0.011	0.125 ± 0.005	1.243 ± 0.007	0.098 ± 0.004	0.309 ± 0.006	0.132 ± 0.004
Mean ± SE	78 ± 2	3.115 ± 0.037	0.137 ± 0.007†	1.273 ± 0.033	0.109 ± 0.007	0.329 ± 0.031	0.137 ± 0.006
				Lower-Fertility Bulls			
D	67	3.185 ± 0.010	0.115 ± 0.004	1.199 ± 0.007	0.114 ± 0.003	0.261 ± 0.006	0.101 ± 0.003
E	68	3.030 ± 0.012	0.114 ± 0.005	1.086 ± 0.008	0.119 ± 0.005	0.238 ± 0.006	0.092 ± 0.004
F	71	3.094 ± 0.010	0.115 ± 0.005	1.210 ± 0.007	0.111 ± 0.004	0.313 ± 0.006	0.152 ± 0.003
Mean ± SE	69 ± 1	3.103 ± 0.045	0.115 ± 0.000	1.165 ± 0.040	0.115 ± 0.002	0.271 ± 0.022	0.115 ± 0.019

* Cryopreserved semen from 1 ejaculate/bull was evaluated and corresponded to the same ejaculates and bulls evaluated by Eid et al, 1994. Fertility was based on 60–90 day lifetime nonreturn rates on more than 5000 first-services for each bull. Within each bull, 100 sperm from a single straw were evaluated to determine mean Fourier harmonic amplitudes at frequencies 0–5.

† Analysis of variance found a difference between mean harmonic amplitudes for high- and lower-fertility bulls at the indicated frequency ($P < .05$).

Sperm Chromatin Structure Analysis

SCSA was performed as described by Ballachey et al (1987) and Evenson and Jost (1994, 2000). Briefly, 2 separate straws of semen from each ejaculate studied were independently measured twice by SCSA. Each straw was thawed in a 37°C water bath for 30 seconds and the semen was extruded into a test tube containing enough TNE buffer (0.15 N NaCl, 0.01 M Tris, and 0.001 M EDTA pH 6.8) to bring the sperm concentration to 1–2 × 10^6/mL. Next, 200 μL of this dilution was treated with 400 μL of acid-detergent solution (0.08 N HCL, 0.1% Triton-X 100, pH 1.2) for exactly 30 seconds. Then, 1.20 mL AO staining solution (6 μg/mL electrophoretically purified AO [Molecular Probes] in phosphate citrate buffer) was added and the sample was placed on the flow cytometer. After allowing 2.5 minutes for hydrodynamic equilibration of the sample with the sheath fluid, SCSA data were recorded for a total of 5000 sperm per sample. The parameters evaluated were %COMP (ie, percent of cells with an increased red and decreased green fluorescence indicative of denatured DNA), the mean, and standard deviation of alpha t. The mean ± SE was determined for the samples.

Statistical Analysis

Data were analyzed with Statistical Analysis Systems software (SAS version 7–1; SAS Institute, Cary, NC) using canonical discriminant analysis (Klecka, 1980), linear regression (Lewis-Beck, 1980), and analysis of variance (ANOVA; Jackson and Brashers, 1994).

A canonical discriminant analysis is similar to a multiple regression with the primary difference being that the response variable, fertility group or bull, is measured at the categorical level. In addition, the discriminant analysis constructs multiple orthogonal functions that are formed so that the group means on the resulting canonical variable are as different as possible (Klecka, 1980). Based on the canonical correlation (r*), which assesses the relatedness of the discriminant function with the groups, one can determine if the groups differ (Klecka, 1980). A significant canonical correlation indicates that the function can successfully discriminate observations from the groups. To determine which harmonic amplitudes were most important for separating the fertility groups, the canonical structure coefficients were assessed. These measures are the correlation between the discriminant measure (harmonic amplitudes) and the canonical variable. Thus, a structure coefficient tells us how closely a discriminant measure and the discriminant function are related. When the absolute magnitude of the coefficient is large, the function is carrying nearly the same information as the discriminant measure. When the coefficient is near zero, the discriminant function and discriminant measure have little in common (Klecka, 1980). In this study, 2 canonical discriminant functions were constructed. Both used the individual sperm's harmonic amplitudes, 0 to 5, as the discriminant measures. The categorical data for the first function were the respective bull's fertility group, whereas the second function was constructed to separate the individual bulls. Thus, the first discriminant function was dependent on fertility, whereas the second function was not.

The variation in sperm nuclear shape as described by harmonic amplitudes 0 to 5 is explained not only by the variance of each harmonic amplitude but by the covariances as well. For each bull this information is contained within a 6 × 6 harmonic amplitude covariance matrix. The covariance matrices were evaluated by determining the trace (sum of the variances) and the determinant (product of the variances less the product of covariances). To ensure equal weight among the harmonic amplitude variances, the data from all 6 bulls were combined and the distributions of harmonic amplitudes 0 to 5 were standardized to have a mean of 0 and a standard deviation of 1. Because the underlying distribution of the trace and determinant were unknown, a Mann-Whitney test was used to check for differences (Conover, 1980).

Results

The means and standard deviations of harmonic amplitudes 0 to 5 for sperm from the 2 fertility groups are shown in Table 1. Analysis of variance on individual harmonic amplitudes found that only harmonic amplitude at frequency 1 was different ($P < .05$) between the high-

Table 2. *The discriminant function canonical coefficients and structure*

Harmonic Amplitude	Canonical Coefficient*	Canonical Structure†
0	0.099	−0.198
1	0.193	0.411
2	1.573	0.956
3	0.155	−0.122
4	−0.459	0.704
5	−0.067	0.458

* Coefficients used to compute the canonical variable.
† Correlation of harmonic amplitude with discriminant variable.

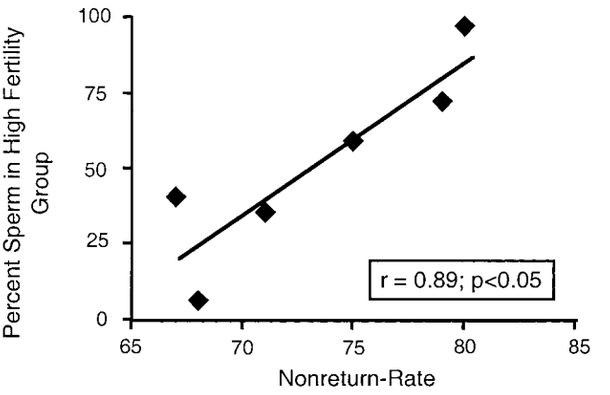

Figure 1. Percent of sperm in the higher fertility group vs non-return rates. The discriminant function, shown in Table 2, was used to classify sperm as either of high or lower fertility. Regression analysis revealed a linear relationship ($r = .89$, $P < 0.05$) between the percent of sperm classified as high fertility and bull lifetime non-return rate.

and lower-fertility bulls. The univariate approach of ANOVA, however, does not consider that multiple y variables, harmonic amplitudes at frequencies 0–5, affect the shape simultaneously. A multivariate approach is needed to take this into account. The approach we chose was discriminant analysis, in which all harmonic amplitudes were evaluated simultaneously and a linear combination was constructed that best placed the bulls in the correct fertility group. There were overall differences ($r^* = 0.55$; $P < .05$) in the shape of sperm nuclei between the 2 fertility groups as demonstrated by the ability to construct the discriminant function of the form shown in Table 2 (canonical coefficients). To determine which harmonic amplitudes were most important in the discriminant function, the canonical structure was examined (Table 2). The greater the absolute value of the correlation, the more important that respective harmonic amplitude is to the separation of the fertility groups. The order from most to least important was harmonic amplitude 2, 4, 5, 1, 3, and 0.

Just as bulls differ in fertility it is likely that individual sperm within a bull also differ in fertility. When a discriminant function is constructed it can be used to place objects into specific groups. The discriminant function shown in Table 2 was thus used to classify individual sperm within each bull as being of either high- or lower-fertility based on their harmonic amplitudes 0–5. This is done on an individual sperm basis by multiplying the specific harmonic amplitude at each frequency by its corresponding canonical coefficient. The terms are then summed to produce a canonical variable, which is a single number. If a canonical variable was positive, then the individual sperm was considered to be in the high-fertility group, and if the canonical variable was negative it was in the lower-fertility group. The percentage of each bull's sperm placed into the high-fertility group was then determined. Regression analysis found a linear relationship ($r = 0.89$; $P < .05$) between the percentage of sperm in the high-fertility group and bull lifetime nonreturn rate (Figure 1). Even though the discriminant function used was constructed from the data on bull fertility groups, it does not necessarily mean that each observation will be placed in the correct group. However, in this study, when each bull's centroid (multivariate mean for harmonic amplitudes 0 to 5) was classified by the discriminant function, 100% were correctly assigned into their respective fertility group (data not shown). Importantly, the discriminant function did not use the nonreturn rate, it used only the fertility group (eg, high or lower). A linear bias was not introduced into the analysis. The results further support that the discriminant function in Table 2 can indeed separate the fertility groups and that sperm in these groups have different shapes.

Although the discriminant function could separate individual sperm into either high- or lower-fertility groups, it was not obvious how the shape of those sperm nuclei differed. A plot of the mean sperm nuclear shape for sperm classified by the discriminate function into the 2 fertility groups was therefore constructed (Figure 2). This required the consistent orientation of sperm and calculation of mean harmonic amplitudes and phase angles. The mean shape of those sperm placed in the high-fertility group was more elongated and tapered than those in the lower-fertility group.

Next, a discriminant function independent of fertility was constructed to separate the 6 bulls into 2 groups (Table 3). Using this approach there was no restriction on how many bulls to place in each group. The discriminant function in Table 3 (canonical coefficients) was used to create a canonical variable value for each sperm. The mean of these values for each bull is shown in Table 4. Bulls in the high-fertility group had canonical variable means greater than 0, whereas bulls of lower fertility had means less than 0 (Table 4). Thus, when supplied only with data on sperm nuclear shape, the discriminant function ($r^* = 0.74$; $P < .05$) separated the high- and lower-

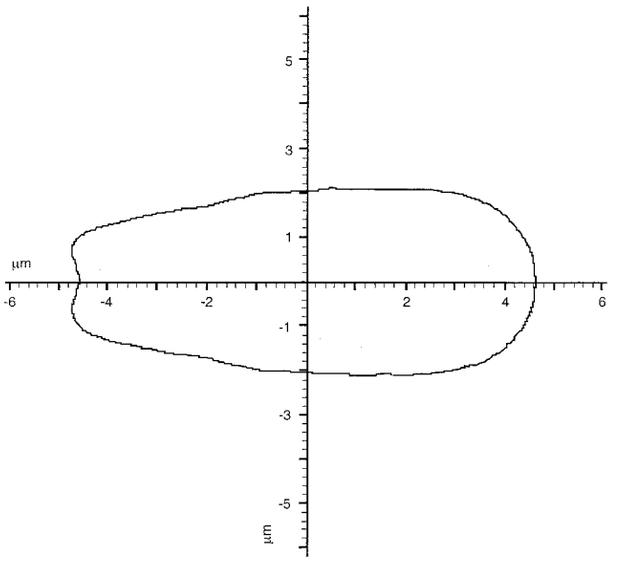

Figure 2. Mean nuclear shape for high and lower fertility sperm. The graphs here are the mean condensed Fourier functions, summed through 20 frequencies, for those sperm placed into the high and lower fertility groups by the discriminant function in Table 2. Note that the high fertility group, shown in black outline, has a mean condensed Fourier function which appears more elongated and tapered than the lower fertility group, shown in gray.

fertility bulls. The canonical structure indicated that correlations were highest for harmonic amplitudes 2, 4, and 5 (Table 3), indicating that these were again the most important factors in separating the bulls into the 2 groups. These results give further support to the argument that sperm nuclear shape differences exist between bulls in the 2 fertility groups.

In addition to mean nuclear shape, the variation in that shape may also explain bull fertility (Parrish et al, 1998). The variation in shape was expressed as the trace and determinant of the harmonic amplitude covariance matrix for each bull. No differences were observed between the high- and lower-fertility groups in either the trace ($P > .05$) or determinant ($P > .05$).

Next, SCSA was performed to determine if sperm chromatin structure was related to nuclear shape. Correlation coefficients between SCSA parameters and harmonic amplitudes are shown in Table 5. Significant correlations ($P < .05$) were found between harmonic amplitude 1 vs $SD_{\alpha t}$ and harmonic amplitude 5 vs $\bar{X}_{\alpha t}$. Based on previous studies (Sailer et al, 1996) it was conceivable that the variance in sperm nuclear shape may show better relationships to SCSA measures than the harmonic amplitude means (Table 6). However, only the standard deviation of harmonic amplitude 0 and $SD_{\alpha t}$ had a significant correlation ($P < .05$). Based on the relationship of each harmonic amplitude to sperm nuclear shape (Ostermeier et al, 2001), the

Table 3. *Discriminant analysis independent of bull fertility group*

Harmonic Amplitude	Canonical Coefficient*	Canonical Structure†
0	0.369	0.377
1	−0.054	0.279
2	1.280	0.955
3	0.188	−0.087
4	0.116	0.748
5	0.125	0.519

* Coefficients used to compute the canonical variable.
† Correlation of harmonic amplitude with discriminant variable.

SCSA results indicate that greater chromatin stability was associated with sperm that were more rounded in the anterior portion and tapered near the most posterior portion of the nucleus. The variation in chromatin stability also increased as the variance in sperm nuclear size increased.

Among the SCSA parameters, $SD_{\alpha t}$ has been shown in 2 studies to be the most reliable predictor of bull fertility (Ballachey et al 1987, 1988) and decreases with increasing fertility. We found that there was a trend ($P = .058$) for $SD_{\alpha t}$ to be smaller for bulls in the high-fertility group (Table 7). In addition, no significant correlations were observed between the SCSA measures and the nonreturn rates of bulls (Table 7). Although no significant differences were found in $\bar{X}_{\alpha t}$ and $\%COMP_{\alpha t}$ (ie, % cells outside main peak identified by increased red and decreased green fluorescence, which is indicative of denatured DNA) between bulls in the 2 fertility groups ($P > .10$), $\%COMP_{\alpha t}$ values were increased by 44% in the lower-fertility group.

Discussion

In the present study, sperm nuclear shape was shown to be related to bull fertility by 3 approaches. First, a dis-

Table 4. *Canonical variable value by bull for discriminant analysis independent of fertility group**

Bull†	Canonical Variable (Mean ± SE)
	High-fertility bulls
A	1.688 ± 0.109
B	0.471 ± 0.009
C	0.053 ± 0.104
	Lower-fertility bulls
D	−0.145 ± 0.091
E	−2.028 ± 0.111
F	−0.040 ± 0.092

* The discriminant function described by the canonical coefficients in Table 3 were used to determine a canonical variable value for each sperm within a bull. The mean of those values, within a bull, is the canonical variable mean reported in this table.
† Bull identification corresponds with Table 1 and Eid et al, 1994.

Table 5. *Correlation coefficients between harmonic amplitudes and sperm chromatin structure assay (SCSA) parameters**

SCSA Parameters	Harmonic Amplitudes					
	0	1	2	3	4	5
$\bar{X}_{\alpha t}$	−0.081	−0.560	−0.655	0.166	−0.755	−0.955†
$SD_{\alpha t}$	−0.393	−0.919†	−0.646	−0.199	−0.557	−0.486
$\%COMP_{\alpha t}$	−0.059	−0.662	−0.325	−0.248	−0.288	−0.420

* The correlation coefficients for the mean of αt ($\bar{X}_{\alpha t}$) where αt = the ratio of red to total fluorescence, standard deviation of αt ($SD_{\alpha t}$) and percent of cells outside the main peak of αt ($\%COMP_{\alpha t}$) are shown.
† Correlations were significant, $P < .05$.

criminant function (Table 2) was constructed based on harmonic amplitudes of nuclear shape and bull fertility. Second, the percentage of high-fertility sperm within a bull's semen, as identified by the discriminant function, had a linear relationship with bull fertility (Figure 1). Third, a discriminant function, which was independent of fertility information (Table 3), correctly separated the bulls into high- and lower-fertility groups. Because we have describe sperm nuclear shape based on Fourier harmonic amplitudes 0–5, a particular nuclear shape is represented by a point in 6-dimensional space. The results suggest that not only do the sperm from high- and lower-fertility bulls occupy unique territories within this 6-dimensional space, but that there is a specific sperm nuclear shape, the percentage of which within a sperm cell population can be used to predict fertility.

In an initial evaluation of the results shown in Table 1, a univariate approach found that harmonic amplitude 1 was the only one related to bull fertility. It is possible that a single harmonic could predict fertility. However, a feature of the harmonic amplitudes at the different frequencies is that they change simultaneously.

Multivariate approaches better address these type of data. When we evaluated the data in Table 1 with the multivariate approach of canonical discriminant analysis, the harmonic amplitudes most important for separating the bulls of the high- and lower-fertility groups were 2, 4, and 5 in both discriminant functions (Tables 2 and 3). These are the harmonic amplitudes associated with elongation and tapering of the posterior head region (Ostermeier et al, 2001). This was verified by constructing the average nuclear shape of sperm (Figure 2) identified by the discriminant function in Table 2. The average shape of sperm identified to be of high fertility was confirmed to be more elongated and tapered than those of lower fertility. Barth et al (1992) observed similar findings when a bull producing high numbers of abnormally tapered sperm was more fertile than a control bull producing high numbers of normally shaped sperm. In the present study, however, preconceived notions of normal and abnormal sperm nuclear shape were ignored. Instead, harmonic amplitudes were used to objectively measure sperm nuclear shape and to determine which nuclear shapes were associated with fertility.

We did not observe differences in the trace or determinant of the harmonic amplitude covariance matrix, suggesting that bull fertility was related to the mean shape of sperm nuclei and not its variation. In contrast, several observations suggest the dispersion of a semen quality parameter may be better related to fertility than its central tendency (Sailer et al, 1996; Parrish et al, 1998). If subtle differences did exist within the variance of specific harmonic amplitudes, they may have been masked by collapsing the variances and covariances into just 2 parameters.

In preparing the semen samples for shape analysis, the sperm were separated on a Percoll gradient. It has been shown that such techniques may alter the morphological distribution of sperm (Georgiou et al, 1998; Gravance et al, 1998), enhancing the percentage of normal sperm. It is unclear whether these changes in morphological distributions are a result of removing dead sperm, which have more abnormalities than live sperm (Fredricsson et al, 1977; Tyler and Crockett 1982), or removing live sperm

Table 6. *Correlation coefficients between harmonic amplitude standard deviations and sperm chromatin structure assay (SCSA) parameters**

SCSA Parameters	Standard Deviation of Harmonic Amplitude					
	0	1	2	3	4	5
$\bar{X}_{\alpha t}$	0.566	−0.100	0.088	−0.161	−0.266	−0.178
$SD_{\alpha t}$	0.859†	0.351	−0.174	−0.108	−0.180	0.072
$\%COMP_{\alpha t}$	0.587	−0.142	−0.314	−0.498	−0.117	−0.061

* The correlation coefficients for the mean of αt ($\bar{X}_{\alpha t}$) where αt = the ratio of red to total fluorescence, standard deviation of αt ($SD_{\alpha t}$) and percent of cells outside the main peak of αt ($\%COMP_{\alpha t}$) are shown.
† Correlations were significant, $P < .05$.

Table 7. *The relationship of the sperm chromatin structure assay (SCSA) parameters to bull fertility group and nonreturn rate (NRR)**

SCSA Parameter	Fertility Group Mean ± SE		Correlation of SCSA vs NRR
	High	Lower	
$\bar{X}_{\alpha t}$	208.7 ± 1.4	215.6 ± 3.9	−0.65
$SD_{\alpha t}$	40.0 ± 3.2†	53.4 ± 0.7	−0.36
$\%COMP_{\alpha t}$	4.3 ± 0.5	6.2 ± 0.4	−0.63

* The SCSA parameters were the mean of αt ($\bar{X}_{\alpha t}$) where αt = the ratio of red to total fluorescence, standard deviation of αt ($SD_{\alpha t}$) and percent of cells outside the main peak of αt ($\%COMP_{\alpha t}$). None of the correlation coefficients between SCSA and NRR were significant, $P > .05$.

† Means between high- and lower-fertility bulls differed, $P = .058$.

with specific abnormalities. The dead and poorly motile sperm removed by the Percoll gradient also represent compensable fertility factors, and in the present studies, we were interested in the noncompensable ones. Furthermore, the ejaculates used here have been extensively analyzed previously in order to determine why they differ in fertility (Hillery-Weinhold, 1991; Eid et al, 1994; Eid, 1995). The percentage of live sperm was enriched for those studies using either the swim-up technique (Hillery-Weinhold, 1991) or Percoll separation (Eid et al, 1994; Eid, 1995). Thus, similar techniques were used in this study for consistency and in order to make comparisons possible.

We have shown previously that the bulls used in the present experiments differ both in in vivo and in in vitro fertility (Hillery-Weinhold, 1991; Eid et al, 1994; Eid, 1995). The bulls of high fertility sire embryos in vitro that are more likely to develop to the morula/blastocyst stage than the bulls of lower fertility. In addition, zygotes sired by the high-fertility bulls start DNA synthesis (S phase) earlier, stay in S phase longer, exit S phase at the same time, have a shorter gap-2 phase, and mitotic phase than zygotes sired by the lower-fertility bulls. It was speculated that the differences observed between these high- and lower-fertility bulls in the zygotic cell cycle were due to varying levels of damaged DNA, unreplicated DNA, or both (Eid et al, 1994; Eid, 1995). These differences would not only explain the discrepancies observed in the zygotic cell cycle, but also the differences observed in this study in sperm nuclear shape.

Several studies in mice (Evenson and Jost, 1993; Kishikawa et al, 1999), and bulls (Ballachey et al, 1986; Karabinus et al, 1997) have shown relationships between chromatin structure, DNA damage, and gross abnormal sperm morphology. It has also been shown that DNA damage and abnormal chromatin structure can occur in sperm commonly classified as normal (Dobrinski et al, 1994; Karabinus et al, 1997; Kishikawa et al, 1999). We have found that Fourier harmonic analysis can quantify variations in nuclear shape that exist within the population of sperm typically classified as normal (Ostermeier et al, 2001). Thus, the small but significant differences in sperm nuclear shape between the high- and lower-fertility bulls may partially reflect DNA damage, differences in chromatin structure, or both.

SCSA was conducted to determine if chromatin structure was associated with differences in sperm nuclear shape. We found that mean harmonic amplitudes at frequencies 1 and 5 and the variation of harmonic amplitude 0 were related to SCSA parameters (Tables 5 and 6). Our results indicate that some of the differences observed in sperm nuclear shape between the high- and lower-fertility bulls can be explained by chromatin structure and its ability to resist DNA denaturation. The results further suggest that alterations to chromatin structure within specific regions of sperm nuclei identified by harmonic amplitudes 1, 5, and 0 may be critical to fertility and worthy of further investigation. However, sperm nuclear shape may contain additional fertility information, as neither the mean or standard deviation of harmonic amplitudes 2 or 4 were related to SCSA measures. These 2 harmonic amplitudes were the most important for differentiating sperm from high- and lower-fertility bulls. This suggests that the shape of the sperm nucleus not only reflects chromatin structure, but additional aspects of noncompensable fertility traits as well.

Two aspects of the methodology were essential to the success of our approach. First, the procedure used an automatic threshold feature of the imaging software (Ostermeier et al, 2001). This provided an objective and repeatable means to determine the perimeter location of the sperm nucleus. Other threshold algorithms could be used (Russ, 1995), but the exact coordinates of perimeter points would be slightly different. The second important aspect of the approach was the development of a novel technique to automatically orient and identify the anterior tip of the sperm head. In these experiments we reconstructed mean sperm shapes for high- and lower-fertility sperm. Orientation of sperm was required to calculate mean phase angles used in the condensed Fourier equations previously described (Ostermeier et al, 2001). Calculation of the mean harmonic amplitudes at each frequency, however, did not require sperm orientation, as these values are independent of orientation (Ostermeier et al, 2001). The approach required no additional time, as it was simply included as a few commands within the SAS statements used to analyze the nuclear perimeter points. Further, the methodology as described should also allow for the orientation of equine, ovine, porcine, human, and nonhuman primate sperm, which have oval shapes. Simple modifications of the procedure would allow its use to orient sperm from any species. The methodology could also be used for any objects or shapes that would need to

be oriented for evaluation. We would simply change which harmonic amplitudes were used to smooth the shape of the object.

We have demonstrated that Fourier harmonic amplitudes can evaluate small differences in sperm nuclear shape (Ostermeier et al, 2001) and predict bull fertility. The approach is objective and does not include preconceived ideas of normal and abnormal sperm morphology. The success of the approach relied on new uses of computer-aided image analysis, the inclusion of sophisticated mathematics to evaluate shape, and statistical methods not commonly used in andrological studies. These types of evaluations may overturn previous concepts that relate sperm morphology to fertility. Indeed, the sperm from the higher-fertility bulls were more tapered and elongated than those from the lower-fertility bulls.

In conclusion, there appear to be relationships between Fourier harmonic amplitudes that describe sperm nuclear shape and lifetime, nonreturn rates of bulls that describe fertility. It appears that bulls of high fertility produce more sperm that are elongated and tapered compared with bulls of lower fertility. In addition, some of the differences observed in sperm nuclear shape may be explained by varying levels of chromatin stability. However, sperm nuclear shape appears to contain additional information from chromatin stability alone. In this particular study, with 6 bulls all with good chromatin quality, sperm nuclear shape was a better predictor of bull fertility.

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References

Ballachey BE, Evenson DP, Saacke RG. The sperm chromatin structure assay. Relationship with alternate tests of semen quality and heterospermic performance of bulls. *J Androl.* 1988;9:109–115.

Ballachey BE, Hohenboken WD, Evenson DP. Heterogeneity of sperm nuclear chromatic structure and its relationship to bull fertility. *Biol Reprod.* 1987;36:915–925.

Ballachey BE, Miller HL, Jost LK, Evenson DP. Flow cytometry evaluation of testicular and sperm cells obtained from bulls implanted with zeranol. *J Anim Sci.* 1986;63:995–1004.

Barth AD, Bowman PA, Gabriel AB, Mapletoft RJ. Effect of narrow sperm head shape on fertility in cattle. *Can Vet J.* 1992;33:31–39.

Barth AD, Oko RJ. *Abnormal Morphology of Bovine Spermatozoa.* Ames: Iowa State University Press; 1989.

Beaty RA, Bennet GH, Hall JG, Hancock JL, Steward DL. An experiment with heterospermic insemination in cattle. *J Reprod Fertil.* 1969;19: 491–502.

Conover WJ. Two independent samples. In: *Practical Nonparametric Statistics.* 2nd ed. New York, NY: John Wiley & Sons; 1980: 215–228.

DeJarnette JM, Saacke RG, Bame J, Vogler CJ. Accessory sperm: their importance to fertility and embryo quality, and attempts to alter their numbers in artificially inseminated cattle. *J Anim Sci.* 1992;70:484–491.

den Dass N. Laboratory assessment of semen characteristics. *Anim Reprod Sci.* 1992;28:87–94.

Dobrinski I, Hughes JPA, Barth AD. Flow cytometric and microscopic evaluation and effect on fertility of abnormal chromatin condensation in bovine sperm nuclei. *Reprod Fertil.* 1994;101:531–538.

Eid LN. *Paternal Regulation of the Zygote Cell Cycle in Bovine Embryos* [PhD thesis]. Madison: University of Wisconsin; 1995.

Eid LN, SP Lorton, Parrish JJ. Paternal influence on S-phase in the first cell cycle of the bovine embryo. *Biol Reprod.* 1994;51:1232–1237.

Evenson DP, Janca FC, Jost LK, Baer RK, Karabinus DS. Flow cytometric analysis of effects of 1,3-dinitrobenzene on rat spermatogenesis. *J Toxicol Environ Health.* 1989;28:81–98.

Evenson DP, Jost LK. Hydroxyurea exposure alters mouse testicular kinetics and sperm chromatin structure. *Cell Proliferation.* 1993;26: 147–159.

Evenson DP, Jost LK. Sperm chromatin structure assay: DNA denaturability. In: Darzynkiewica Z, Robinson JP, Crissman HA, eds. *Methods in Cell Biology. Vol 42, Flow Cytometry.* Orlando, Fla: Academic Press; 1994: 159–176.

Evenson DP, Jost LK. Sperm chromatin structure assay for fertility assessment. In: Robinson P, ed. *Current Protocols in Cytometry.* Vol 1. New York, NY: John Wiley & Sons; 2000.

Evenson DP, Thompson L, Jost L. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. *Theriogenology.* 1994;41:637–651.

Flook AG. Fourier analysis of particle shape. In: Stanly-Wood NG, Allen T. *Particle Size Analysis.* Chinchester, NY: Wiley Heyden Ltd; 1982: 255–262.

Fredricsson B, Waxegard G, Brege S, Lundberg I. On the morphology of live spermatozoa of human semen. *Fertil Steril.* 1977;28:168–174.

Georgiou I, Syrrou M, Stefanidis K, Konstantelli M, Lolis D. Effect of Percoll gradient and swim-up preparation on the chromomycin A3 staining of normal and abnormal semen samples. *Adrologia.* 1998;30: 101–104.

Gravance CG, Champion ZJ, Sax-Gravance SK, Casey PJ. Percentage of normal sperm heads is significantly increased by Percoll separation of semen. *Int J Androl.* 1998;21:116–119.

Hillery-Weinhold F. *Sire Influence on Bovine In Vitro Fertilization and Development* [master's thesis]. Madison: University of Wisconsin; 1991.

Jackson S, Brashers DE. *Random Factors in ANOVA.* Sage University Paper Series on Quantitative Applications in the Social Sciences, 07–098. Thousand Oaks, Calif: Sage Publications; 1994: 1–65.

Karabinus DS, Vogler CJ, Saacke RG, Evenson DP. Chromatin structural changes in sperm after scrotal insulation of holstein bulls. *J Androl.* 1997;18:549–549.

Kenney RM, Evenson DP, Garcia MC, Love CC. Relationships between sperm chromatin structure, motility and morphology of ejaculated sperm, and seasonal pregnancy rate. *Biology of Reproduction Monograph 1: Equine Reproduction VI.* 1995; 647–653.

Kishikawa H, Tateno H, Yanagimachi R. Chromosome analysis of BALB/c mouse spermatozoa with normal and abnormal head morphology. *Biol Reprod.* 1999;61:809–812.

Klecka WR. *Discriminant Analysis.* Sage University Paper Series on Quantitative Application in the Social Sciences, 07–019. Beverly Hills, Calif: Sage Publications; 1980: 7–70.

Lewis-Beck MS. *Applied Regression: An Introduction.* Sage University

Paper Series on Quantitative Applications in the Social Sciences, 07–022. Newbury Park, Calif: Sage Publications; 1980: 9–79.

Ostermeier GC, Sargeant GA, Yandell BS, Parrish JJ. Measurement of bovine sperm nuclear shape using Fourier harmonic amplitudes. *J Androl.* 2001;22:584–594.

Parrish JJ, Krogenaes A, Susko-Parrish JL. Effect of bovine sperm separation by either swim-up or percoll method on success of in vitro fertilization and early embryonic development. *Theriogenology.* 1995; 44:859–869.

Parrish JJ, Ostermeier GC, Pace MM. Fourier harmonic analysis of sperm morphology. In: *Proceedings of the 17th NAAB Technical Conference on Artificial Insemination and Reproduction.* Madison, Wisc; 1998: 25–31.

Russ JC. *The Image Processing Handbook.* 2nd ed. Boca Raton, Fla: CRC Press; 1995:530–533.

Saacke RG, Bame J, Karabinus DS, Mulins J, Whitman S. Transport of abnormal sperm in the artificially inseminated cow based upon accessory sperm in the zona pellucida. In: *Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination.* Dublin, Ireland; 1988;3:292–94.

Saacke RG, Nadir S, Dalton J, Bame J, DeJarnette JM, Defelos S, Nebel RL. Accessory sperm evaluation and bull fertility—an update. In: *Proceedings of the 15th NAAB Technical Conference on Animal Reproduction and Artificial Insemination.* Milwaukee, Wisc; 1994:57–67.

Saacke RG, Vinson WE, O'Connor ML, et al. The relation of semen quality and fertility: heterospermic study. In: *Proceedings of the 8th NAAB Technical Conference on Animal Reproduction and Artificial Insemination.* Milwaukee, Wisc; 1980:71–78.

Sailer BL, Jost LK, Evenson P. Bull sperm head morphometry related to abnormal chromatin structure and fertility. *Cytometry.* 1996;24:167–173.

Snedecor GW, Cochran WG. Sample surveys. In: *Statistical Methods.* 7th ed. Ames, Iowa: Iowa State University Press; 1989:434–459.

Steinholt HC, Chandler JE, Baron RA, Adkinson RW. Chromosome and sperm size of Holsteins with and without bovine leukocyte adhesion deficiency. *J Dairy Sci.* 1994;77:1239–1250.

Sullivan JJ, Elliott FI. Bull fertility as affected by an interaction between motile spermatozoa concentration and fertility level in artificial insemination. In: *Proceedings of the 6th International Congress on Animal Reproduction and Artificial Insemination.* Paris, France; 1969;2: 1307–1310.

Tyler JP, Crockett NG. Comparison of the morphology of vital and dead human spermatozoa. *Reprod Fertil.* 1982;66:667–670.

Vrendenburgh-Wiulberg WL, Parrish JJ. Intracellular pH of bovine sperm increases during capacitation. *Mol Reprod Dev.* 1995;40:490–502.

Wiger R, Hongslo JK, Evenson DP, De angelis P, Schwarze PE, Holme JA. Effects of acetaminophen and hydroxyurea on spermatogenesis and sperm chromatin structure in laboratory mice. *Reprod Toxicol.* 1995;9:21–33.