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Identification of Sperm Morphometric Subpopulations in Two Different Portions of the Boar Ejaculate and Its Relation to Postthaw Quality

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ABSTRACT: A statistical approach using sequentially principal component analysis (PCA), clustering, and discriminant analyses was developed to identify sperm morphometric subpopulations in well-defined portions of the fresh boar ejaculate. Semen was obtained as 2 portions (the first 10 mL of the sperm-rich fraction and the rest of the ejaculate, respectively) and frozen using a conventional protocol. Before freezing, an aliquot was used for computer-assisted sperm morphometry analysis (ASMA). Postthaw quality was evaluated using computer-assisted sperm analysis (CASA), and an annexin-V/PI assay evaluated sperm membranes. The PCA revealed that 3 variables represented more than 78% of the cumulative variance in sperm subpopulations. The clustering and discriminant analyses, based on 5780 individual spermatozoa, revealed the existence of 4 sperm subpopulations. The relative percentage of these subpopulations varied between boar and ejaculate portions. Linear regression models based on measured morphometric characteristics could account for up to 36% of the percentage of intact sperm membranes postthaw. The ASMA protocol used in our study was useful to detect subtle morphometric differences between spermatozoa, and the combination of this analysis with a multivariate statistical procedure gave new information on the biological characteristics of boar ejaculates that is not given by conventional sperm analysis.

Key words: ASMA, sperm subpopulations, cryopreservation, cluster analysis.

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One of the major factors that largely constrain the commercial use of artificial insemination with frozen-thawed semen in the pig industry is the existence of large variations in the ability among individual boars to sustain semen freezability (Holt et al, 2005). Differences in the biochemical characteristics in the sperm membrane, and specifically the relative content and ratio of membrane phospholipids and cholesterol (Darin-Bennet and White, 1997), have been claimed to be the main factors explaining interspecies differences in sensitivity to cryoinjury. However, biochemical characteristics of sperm membranes may show some heterogeneity within a population and are unlikely to substantially differ between individuals of the same species (Thurston et al, 2001).

The coexistence of different sperm subpopulations within the mammalian ejaculate is nowadays widely accepted by the scientific community. The origin of these subpopulations is not clear yet, but it has been hypothesized that they correspond to differences in the assembly of individual spermatozoa during spermatogenesis, as well as to their differential maturational status and age when leaving the cauda epididymides at ejaculation (Abaigar et al, 1999). Characteristics of these subpopulations have been studied by means of flow cytometry and computer-assisted sperm analysis (CASA) and analyzed using multivariate approaches to identify sperm subpopulations in mammals (Abaigar et al, 2001; Martínez-Pastor et al, 2005). Although sperm morphology can be considered a good indicator of semen quality in bull sires (Phillips et al, 2004) and it is recommended as part of the spermiogram for domestic animals (Rodríguez-

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Martínez, 2003), the investigation of morphometric sperm subpopulations in boar semen has received little attention, there being only 2 studies dealing with this issue (Hirai et al, 2001; Thurston et al, 2001), while no data exist regarding sperm morphometric subpopulations in other species.

We have previously demonstrated that sperm quality after cryopreservation of boar semen differs depending on the fraction of the seminal plasma the boar spermatozoa were fortuitously contained in (Peña et al, 2003a,b, 2004). Thus, spermatozoa present in the first 10 mL of the sperm-rich fraction (Portion I) could withstand handling procedures (extension, handling, and freezing-thawing) better than those contained in the latter part of a fractionated ejaculate (second portion of the sperm-rich fraction and the rest of the bulk ejaculate). The reasons for these differences, although not yet disclosed in detail, may be related to differences in electrolyte composition or protein components (Zhu et al, 2000). However other factors may be related to these different abilities, as subtle morphometric differences in sperm head morphology have been related to differences in sperm quality among ejaculates. Most of the studies on sperm subpopulations have been performed using specific software such as the PATN software (Abaigar et al, 1999; Thurston et al, 1999), although a few have used more popular statistical packages (Martínez-Pastor et al, 2004; Quintero-Moreno et al, 2005) such as the SAS software. The aims of the present study were to 1) develop a simple multistep procedure to identify sperm subpopulations within the boar ejaculate based on data gathered with assisted morphology sperm analysis (ASMA), using a commercially available statistical package (SPSS), 2) test the hypothesis that subtle morphometric differences exist among spermatozoa located in different ejaculate portions, and 3) test the hypothesis that the existence of these morphometric sperm subpopulations may at least in part explain differences in the ability of different ejaculates and/or portions within the ejaculate to sustain cryopreservation procedures.

Materials and Methods

Semen Collection

Semen from 5 Swedish Yorkshire boars aged 2–4 years, of proven fertility with fresh semen and showing a minimum of 70% motile and 80% morphologically normal spermatozoa, was collected with the gloved-hand technique and frozen twice weekly for a total of 4 freezing operations per animal. Semen was collected as 2 portions: the first collectable 10 mL of the first sperm-rich fraction (Portion I) was retrieved in a prewarmed test tube, while the rest of the sperm-rich fraction together with the rest of the post–sperm-rich fraction (Portion II) was collected in a prewarmed thermos flask. Sperm concentration and motility were determined. All boars received the same diet and were housed under the same conditions. No significant differences were found between ejaculates and/or ejaculate portions within boars in any of the parameters studied in fresh samples.

Sperm Staining for Morphometric Analysis

Sperm samples were adjusted in phosphate-buffered saline (PBS) to 100×10^6 cells per mL. Thereafter, 10 μL of the sperm suspension was placed on the edge of a slide and extended. Preparations were allowed to dry and were fixed and stained for 10 minutes in an eosin solution (Panreac, Barcelona, Spain) and 10 minutes in a methylene blue solution (Panreac). The excess of stain was removed by washing, and the slide was allowed to dry before being permanently mounted with Eukitt (Panreac).

Computerized Morphometric Analysis

The prepared slides were examined using a Nikon Labophot microscope equipped with a 100× bright field objective lens and a 3.3× photo-ocular lens. The video signal was acquired by a Sony CCD AVC-D7CE video camera (Sony Corporation, Tokyo, Japan) interfaced with a Sperm-Class Analyser® (SCA) version 99 CASMA system (Microptic S.L., Barcelona, Spain). The array size of the video grabber was $512 \times 512 \times 8$ bits, providing digitized images of 262 144 pixels and 256 gray levels. Resolution of images was 0.083 μm per pixel in the horizontal and vertical axes.

At least 200 spermatozoa per sample were captured in 2 slides per ejaculate. Sperm cells were displayed on the monitor at equivalent brightness, and all the cells that did not present any overlap with debris or other cells were considered for analysis. From each sample sperm heads were captured and analyzed using the SCA program as previously described (Buendía et al, 2002). After treatment of the images, some of the sperm images had to be discarded because of defective binarization, as observed by false correspondence between the original image and its mask. Each sperm head was measured for 7 primary parameters (head area [A, μM^2], head perimeter [P, μM], head length [L, μM], head width [W, μM], midpiece width [w, μM], midpiece area [a, μM^2], distance [d, μM] between the major axes of the head and midpiece) and 4 derived parameters of head shape (FUN1 [L/W], FUN2 [$4\pi A/P^2$], FUN3 [$(L-W)/(L+W)$], FUN 4 [$\pi LW/4A$]).

Semen Freezing Protocol

After 60 minutes of holding time at room temperature (20°C to 22°C), the semen was extended (1+1) with Beltsville Thawing Solution (BTS) (206 mM glucose, 20.4 mM Na_3 citrate, 14.9 mM $NaHCO_3$, 3.4 mM Na_2-EDTA, 10 mM KCl, penicillin G Na 0.6 g/L, Dihydrostreptomycin 1.0 g/L). The extended semen was allowed to stand in a cooling centrifuge (Centra MP4R, IEC, Needham Heights, Mass) set at 15°C for 3 hours, after which it was centrifuged at $800 \times g$ for 10 minutes. The supernatant was discarded and the volume (graduated vial) and sperm concentration (Bürker chamber) were measured. The sperm pellet was re-extended with a second extender (Ext II 80 mL [80% v/v 310 mM] β-lactose + 20 mL egg yolk) at a ratio of 1 part of semen to 1 part of extender. After thorough mixing, the semen was further cooled to 5°C for 2 hours in the centrifuge. At this tem-

perature, the semen was slowly mixed with a third extender, consisting of 89.5 mL Ext II, 9 mL glycerol, and 1.5 mL of Equex STM (Nova Chemicals Sales Inc, Scituate, Mass), at a ratio of 2 parts of semen to 1 part of extender, giving a final glycerol concentration of 3%. The final sperm concentration was 1×10^9 spermatozoa, checked in a Bürker Chamber. The work at 15°C and 5°C was done in a cooled cabinet (IMV, L'Aigle France) where semen was loaded in 0.5 mL straws (IMV). After sealing, the straws were transferred to the chamber of a programmable freezer (Mini Digitcool 1400, IMV) and frozen horizontally in racks. The cooling rate was as follows: −3°C/min from 5°C to −5°C; thereafter, −40°C/min from −5°C to −140°C. The frozen straws then were plunged into liquid nitrogen (LN_2, −196°C). After 4 weeks of storage, samples were removed from the LN_2 and thawed in a water bath at 50°C for 12 seconds.

Motility Analyses

Semen samples were diluted at 20°C to 22°C, 1:20 in an extender consisting of 95 mL BTS and 5 mL lactose-egg yolk solution to prevent the spermatozoa from sticking to the glassware during motility analysis. The extended semen, containing approximately 50×10^6/mL, was held in an incubator at 38°C for 30 minutes, and then 5μL of the sample was placed into a 10 μm deep Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) for motility analysis using a CASA system (Strömberg-Mika-CMA, Windows version 1.1, MTM Medical Technologies, Montreux, Switzerland). The setting parameters for the SM-CMA program were 32 frames in which spermatozoa had to be present in at least 16 to be counted, and time resolution, 20 ms (50Hz). Spermatozoa with an average path velocity (VAP) less than 10 μm/s were considered immotile, and spermatozoa with a VAP greater than 25 μm/s were considered motile. A minimum of 8 predetermined fields all around the central reticulum of the chamber were evaluated, counting a minimum of 200 spermatozoa in duplicates. Spermatozoa deviating less than 10% from a straight line were designated linear motile spermatozoa, and those having a circular motion of radius less than 25 μm were classified as circularly motile. The analysis yielded the following motility parameters: motile (% of motile spermatozoa), linearly motile (% of spermatozoa moving linearly), circle (% of spermatozoa with circular motility), VSL (straight linear velocity, μm/s), VAP (average path velocity, μm/s), VCL (curvilinear velocity, μm/s) and ALH (lateral head displacement, μm).

Annexin-V/PI Flow Cytometry Analysis

Staining for annexin-V/PI was performed using the annexin-V(A) conjugated with fluorescein isothiocyanate (FITC)-apoptosis detection kit II (Pharmingen, San Diego, Calif) and Propidium Iodide (PI, Molecular Probes, Eindhoven, The Netherlands) as previously described for boar semen (Peña et al, 2003a). The samples were analyzed by a triple-laser LSR cytometer (Becton Dickinson Immunochemistry Systems, San José, Calif) equipped with standard optics. An Ar-ion laser (INNOVA 90, Coherent, Santa Clara, Calif) tuned at 488 nm and running at 200 mW was used as light source. From each cell, forward light scatter (FSC), orthogonal light scatter (SSC), A-FITC fluorescence (FL1), and PI fluorescence (FL3) were evaluated using Cellquest version 3.3 (Becton Dickinson) software. A gate was applied in the FSC/SSC dot-plot to restrict the analysis to spermatozoa. For the gated cells, the percentages of annexin-V-positive (A+), PI-positive (PI+), and double-positive cells were evaluated, based on quadrants determined from single-stained and unstained control samples. Cells in the lower left quadrant were not fluorescent (A−/PI−) and were recorded as live cells, (eg, without membrane dysfunction). Apoptotic but viable spermatozoa (A+/PI−) were labeled with annexin-V but not with PI and fell in the lower right quadrant. Early necrotic spermatozoa (A+/PI+) that bound both annexin-V and PI (upper right quadrant) are assumed to maintain some degree of membrane integrity although having damaged permeable membranes, and thus still bind Annexin-V. Late necrotic spermatozoa (A−/PI+), however, were labeled by PI but not annexin-V (upper left quadrant). It is assumed that these latter spermatozoa have completely lost sperm membrane integrity and are thus unable to bind annexin-V (Peña et al, 2003b).

Statistical Analysis

The main objective of the analysis was to identify sperm subpopulations using the morphometric data obtained from each boar and ejaculate portion by means of clustering procedures (Martínez-Pastor et al, 2005). The first step was to perform a principal components analysis of the morphometric data. The purpose of the first step was to derive a small number of linear combinations (principal components) that retained as much of the information in the original variables as possible. This allowed us to summarize many variables in few jointly uncorrelated principal components. A good result was considered when few principal components accounting for a high proportion of the total variance were obtained. The second step was to perform a nonhierarchical analysis using the k-means model that uses euclidean distances to calculate the center of the clusters. We used the selected principal components as variables. The third step was to perform a step-wise discriminant analysis of the clusters obtained. This kind of analysis is often used to reduce the number of clusters and to help in the interpretation of the data obtained in the k-means procedure (Hair et al, 1998). To study the distributions of observations (individual spermatozoa) within portions and within subpopulations, a χ^2 test was used.

Data regarding postthaw sperm quality (CASA and annexin-V assay) were first tested using a Kolgomorov-Smirnov test to determine the normality of data distribution. In view of the Gaussian distribution of the data gathered, an analysis of variance was used to determine the motility and membrane integrity values in each boar and ejaculate portion considered. Linear regression analyses were used to investigate relationships between morphometric parameters in fresh semen and sperm quality measurements postthaw. The level of significance was set to $P < .05$. All analyses were performed using the SPSS ver 11.0 for Windows software (SPSS Inc Chicago, Ill).

Results

Identification of Sperm Subpopulations

The data matrix consisted of 5780 observations (morphometric analysis on individual spermatozoa). First, we per-

Table 1. *Classification of boar and ejaculate portions according to their sperm membrane intergrity, motility, and velocities postthaw**

Boar-Ejaculate Portion	Membrane Integrity Postthaw (A−PI−)(%)	Motility Postthaw (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)
1352-II	56.8 ± 7.82^{ac}	55.8 ± 14.53^{a}	114.7 ± 3.85^{a}	65.2 ± 4.83^{a}	75.3 ± 3.12^{a}
684-I	47.7 ± 9.40^{a}	56.1 ± 9.47^{a}	143.3 ± 6.84^{b,c}	61.1 ± 8.21^{a}	81.6 ± 8.72^{b,c}
1277-II	44.7 ± 8.40^{a}	60.8 ± 9.29^{a}	126.3 ± 8.19^{b,c}	69.7 ± 3.29^{a,c}	82.7 ± 4.92^{b}
684–II	38.3 ± 10.61^{b}	61.1 ± 8.02^{a}	148.7 ± 8.91^{b,c}	71.4 ± 3.90^{a,c}	92.4 ± 5.59^{b}
407-II	39.8 ± 8.84^{a,b}	49.6 ± 6.69^{a}	152.6 ± 6.83^{b}	73.1 ± 6.61^{b,c}	87.9 ± 4.93^{b}
1352-I	38.6 ± 8.64^{b}	44.6 ± 16.72^{b,c}	121.9 ± 4.75^{a,c}	63.7 ± 7.03^{a}	76.1 ± 63.7^{a,c}
1277-I	44.1 ± 10.76^{a,b}	40.0 ± 12.23^{b,c}	127.6 ± 9.88^{b,c}	61.2 ± 4.70^{a}	76.9 ± 4.31^{a,c}
1044-II	48.4 ± 8.17^{a,b}	36.4 ± 14.63^{b,c}	127.3 ± 8.29^{b,c}	60.1 ± 4.75^{a}	76.8 ± 4.32^{a,c}
1044-I	42.8 ± 13.10^{a,b}	32.5 ± 6.64^{b}	127.4 ± 8.65^{b,c}	55.6 ± 9.12^{b}	72.9 ± 8.04^{a,c}
407-I	53.2 ± 9.65^{a,b}	28.5 ± 6.50^{b}	143.0 ± 11.97^{b}	51.3 ± 6.13^{b}	72.4 ± 5.15^{a,c}

* Membrane integrity was evaluated as revealed by the annexin-V/PI assay. Motility data are derived from computer-assisted sperm analysis. Results are derived from 5 different experiments repeated on 5 different days. Values are mean ± SD. Within a column, values with different superscript letters differ significantly, $P < .05$. VCL indicates curvilinear velocity; VSL, straight linear velocity; and VAP, average path velocity.

formed a principal component analysis (PCA) to reduce the number of variables (12 derived morphometric data) into a few informative ones. This analysis revealed 3 components with eigen values over 1, representing more of the 78% of the cumulative variance (Table 2). In base of this PCA we selected 3 variables. These variables were head length (L), head width (W) and head area (A). Most of the variance observed among individual spermatozoa was explained by these 3 variables. The second step was performing a k-means clustering analysis using these 3 principal components as variables. For this, we used a k-means cluster procedure followed by a discriminant analysis, resulting in the identification of 4 sperm subpopulations (Table 3). The k-means procedure allowed us to detect outliers, because the clustering procedure is very sensitive to them. Less than 3% of the measurements were dropped because they were outliers. These outliers were clusters with only 1 or 2 members, which were identified and removed before repeating the procedure. The disclosed subpopulations were characterized by different values of sperm head length (L), width (W) and area (A). The subpopulation 7 was characterized by spermatozoa with the smallest L, W, and A. The next subpopulation (15) was also characterized by small A, W, and L, though it was bigger than the subpopulation 7. The analysis revealed 2 subpopulations of more elongated spermatozoa, ie, subpopulations 11 (comprised spermatozoa with the highest values of L, W, and A) and 10 (also formed by long and wide spermatozoa). The average values of L, W, and A were 8.0 ± 0.29 μM, 4.0 ± 0.17 μM, and 27.5 ± 1.12 μM^2, respectively.

Table 2. *Results of the principal component analysis (PCA) performed on the ASMA data**

	Initial Eigen Values		
Component	Eigen Values	% of Variance	Cumulative %
Head length,			
width, and area	3.957	35.97	35.97
by 1, 2, and 3,	3.168	28.80	64.77
respectively	1.510	13.73	78.51

* The eigen vlaues of the first 3 principal components are given. The percentage of variance is the proportion of the total variance explained by each principal component. The eigen vectors are a measure of association of the original parameters with the resulting principal components. ASMA indicates assisted sperm morphometry analysis.

Sperm Subpopulations Within the 2 Portions of the Boar Ejaculate

Within each subpopulation (cluster) the percentage of spermatozoa represented by portion I or II was more or less the same, namely 50% ± 5%, except for cluster 15 where it was 44% in portion I versus 56% in portion II. Additionally, within portion I, the representation of the 4 subpopulations varied from 13% to 33%; in portion II, from 13% to 36%. Clusters 10, 11, and especially cluster 15 were relatively more present in portion II (Table 4).

Sperm Subpopulations Within Each Portion of Individual Boars

Except in boar 1352, where the proportion of the sperm subpopulations did not differ within each portion of the boar ejaculate, there were significant differences within both boar and ejaculate portion (Figures 1 and 2).

Sperm Quality Postthaw

Between boar and ejaculate portion variability was observed in both sperm kinematics and membrane integrity postthaw; however, no significant variation was observed between ejaculates or portions within boars. The 5 boars and ejaculate portions were classified according to the postthaw motility and membrane integrity of their spermatozoa (Table 1). There were boars and/or ejaculate portions showing high percentages of either sperm motility or of sperm membrane integrity postthaw.

Boars and ejaculate portions 1352-II, 407-I, and 1044-II showed the best membrane integrity postthaw ($P <$

Table 3. *Summary of the selected morphometry parameters (L[μ], W[μ], A[μ²]) obtained in this study**

Cluster		Mean	Standard Deviation	Number of Spermatozoa
7	L	7.8	0.24	1325
	W	3.9	0.15	
	A	26.1	0.38	
10	L	8.12	0.26	1692
	W	4.1	0.16	
	A	28.2	0.35	
11	L	8.22	0.27	746
	W	4.2	0.16	
	A	29.4	0.42	
15	L	8.0	0.24	2017
	W	4.0	0.15	
	A	27.1	0.34	

* This table represents the mean values for each subpopulation (cluster). Data were derived from the analysis of 5780 boar spermatozoa. L indicates sperm head length; W, sperm head width; and A, sperm head area.

Table 4. *Relative percentages of spermatozoa within each subpopulation (cluster) and ejaculate portion**

Subpopulations (Clusters)	7	10	11	15	Total Within Portion
Portion I n	669	767	348	885	2669
Within portion (%)	25.1[a]	28.7	13.0	33.2[a]	100%
Within cluster (%)	50.5	45.3[a]	46.6[a]	43.9[a]	
Portion II n	656	925	398	1132	3111
Within portion (%)	21.1[b]	29.7	12.8	36.4[b]	100%
Within cluster (%)	49.5	54.7[b]	53.4[b]	56.1[b]	
Total within cluster	100%	100%	100%	100%	

* Subpopulations included 4 clusters defined by 3 morphometric variables derived from assisted sperm morphometry analysis after a principal component analysis (as described in Table 2 [L(μ), W(μ), A(μ²)]). Portion I indicates the first 10 mL of the sperm-rich fraction; Portion II, the rest of the sperm-rich fraction and the rest of the bulk ejaculate; n, number of spermatozoa. Data were derived from the analysis of 5780 spermatozoa. Within a column, values with different superscript letters differ statistically, $P < .01$ (χ^2 on raw data). Comparisons are made within ejaculate portions and within clusters.

.05). The best motility was observed in 684-I, 1277-II, and 684-II. In respect to sperm velocities, VCL was better in 684-I, 684-II, and 407-II, while VSL and VAP were better in 1277-II, 684-II, and 407-II.

Relationship Between Sperm Morphometry and Sperm Quality Postthaw (Table 5)—Linear regression analysis revealed significant relations among different parameters of sperm quality postthaw and sperm head morphometry in fresh samples. The percentage of intact sperm membranes postthaw (A−/PI−) was explained by 2 models. The first one included the sperm head shape factor FUN2 ($R^2 = 0.257$, adjusted $R^2 = 0.257$, $P < .01$). The second model included 2 sperm head factors, FUN2 and FUN4, ($R^2 = 0.367$ adjusted $R^2 = 0.312$ $P < .01$). This model nominally explains 36.7% of the variation. In relation to sperm kinematics postthaw, midpiece width was a predictor of motility ($R^2 = 0.06$, adjusted $R^2 = 0.047$, $P < .05$). Two models explained the percentage of linear motile sperm postthaw; the first included midpiece width ($R^2 = 0.131$, adjusted $R^2 = 0.119$, $P < .01$), and the second one included midpiece width and the distance between the major axes of the head and midpiece ($R^2 = 0.210$, adjusted $R^2 = 0.190$, $P < .01$). The VAP model contained the terms distance between the major axes of the head and midpiece, FUN4, midpiece width, head area, midpiece area, FUN3, FUN 2, FUN1, head width, head length, and head perimeter ($R^2 = 0.273$, adjusted $R^2 = 0.155$, $P < .05$). VCL was explained by a model including the terms distance between the major axes of the head and midpiece, FUN4, midpiece width, midpiece area, FUN3, FUN2, FUN1, and sperm head width, length, and perimeter ($R^2 = 0.275$, adjusted $R^2 = 0.157$, $P < .05$). Finally, VSL was explained by a model containing the terms distance between the major axes of the head and midpiece, FUN4, midpiece width, head area, midpiece area, FUN3, FUN2, FUN1, head width, length, and perimeter ($R^2 = 0.194$, adjusted $R^2 = 0.064$, $P < .05$).

Discussion

We have hereby developed a simple method (including the staining procedure) combining computerized morphometric analysis and a multivariate analysis procedure using a commercially available, commonly used statistical package to identify morphometric sperm subpopulations in 2 different portions of the boar ejaculate. The development of standardized protocols for computerized analysis of sperm morphology has been considered a high priority for the investigation of human semen (ESHRE, 1998). The procedure employed in our study gave relevant information on the characteristics of boar ejaculates. In the present study, the ASMA system was able to detect subtle morphometric differences among spermatozoa present in ejaculates from different boars. The data derived from the morphometric analysis could be then successfully used to discriminate sperm morphometric subpopulations within 2 different portions of the boar ejaculate. Although there are other studies (Hirai et al, 2001; Ostermeier et al, 2001; Thurston et al, 2001) mainly using fourier shape descriptors to classify sperm heads, this is, to the best of our knowledge, the first description of sperm subpopulations based on morphometric data directly derived from an ASMA analysis.

The statistical procedure hereby used was simple and appeared useful to detect sperm subpopulations. In our study we have used the SPSS software and a different approach from that used in studies on sperm subpopulations derived from motility data (Abaigar et al, 1999; Quintero-Moreno et al, 1999, 2002; Martínez-Pastor et al,

Table 5. *Linear regression of sperm characteristics postthaw and computer-assisted sperm morphometry analysis derived morphometric parameters in fresh samples**

Sperm Characteristics Postthaw	Variables in the Statistical Model	Significance	R^2
Live sperm (A−PI−)	Model 1; FUN2	$P < .05$	0.257
	Model 2; FUN2, FUN4	$P < .05$	0.367
Motile sperm	Midpiece width	$P < .05$	0.060
Linear motile sperm	Model 1; midpiece width	$P < .01$	0.131
	Model 2; midpiece width, distance between the major axes of the head and midpiece	$P < .01$	0.210
VAP	Distance between the major axes of the head and midpiece, FUN4, midpiece width, head area, midpiece area, FUN3, FUN2, FUN1, head width, head length, head perimeter	$P < .05$	0.273
VCL	Distance between the major axes of the head and midpiece, FUN4, midpiece width, head area, midpiece area, FUN3, FUN2, FUN1, head width, length, and perimeter	$P < .05$	0.275
VSL	Distance between the major axes of the head and midpiece, FUN4, midpiece width, head area, midpiece area, FUN3, FUN2, FUN1, head width, length, andperimeter	$P < .05$	0.194

* A−/P− indicates annexin V negative, propidium iodide negative; VAP, average path velocity (μm/s); VCL, curvilinear velocity (μm/s); VSL, straight linear velocity (μm/s); FUN1, L/W; FUN2, $4\pi A/P^2$; FUN3, (L−W)/(L+W); FUN4, πLW/4A; L, head length (μM); W, head width (μM); A, head area (μM^2); P, head perimeter (μM).

2005); these authors used the PATN or SAS software. Like Martínez-Pastor et al (2005), we included in our approach a PCA as a first step to reduce the number of variables to few informative ones. This first step also facilitated the further management of the data. As a second step, we performed a k-means cluster procedure, a kind of clustering indicated when there is a large set of data, as was the case in our study (5780 individual spermatozoa). This procedure has the relative disadvantage that the operator must set the number of clusters a priori. Such cluster numbers were determined in a series of preliminary tests, until the optimal number of clusters was found.

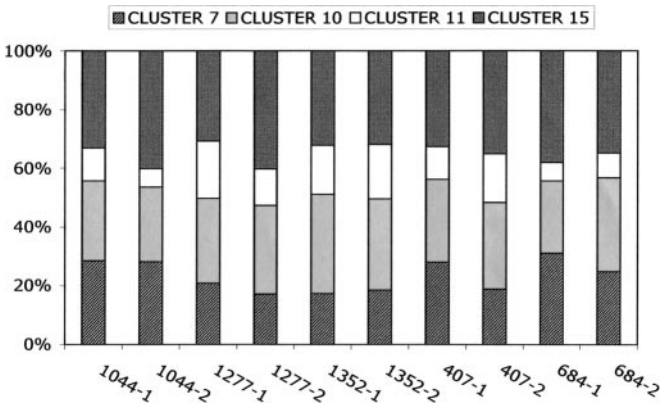

Figure 1. Representation of absolute number of spermatozoa within each cluster (as defined after the clustering analysis of assisted sperm morphometry analysis [ASMA] data) present in the ejaculate portion of the individual boars. Pattern codes for each cluster are given on the right. χ^2 analysis revealed significant differences in the percentage of spermatozoa within each cluster for the different boars and ejaculate portions ($P < .001$).

On the other hand, a major advantage of the k-means cluster procedure is the easy detection of outliers. As a third step, to further reduce the number of clusters and to help in the interpretation of data, we performed a discriminant analysis, using the approach described by Davis et al (1995) to study sperm kinematics in human semen. These authors carried out a multistep iterative procedure combining the k-means model with multivariate discriminative analysis.

In our study we used as a first step a PCA to reduce the number and select the type of variables to be included in the analysis. This first step facilitated the management of the data; in fact in a number of studies on sperm subpopulations based on sperm kinematics, one of the major critical points was the selection of variables to enter in the analysis. The first objective of our study was to use a simple method using SPSS software to identify sperm morphometric subpopulations. The statistical tool is not new, but its use for this type of data is new. Since the number of variables obtained from the ASMA analysis was high, the inclusion of a PCA as a first step was considered as essential to simplify the statistical procedure.

The origin of these subpopulations is not clear. Genetically derived variation on sperm morphology has been demonstrated as the base for phenotypic differences observed between spermatozoa of different strains of mice (Beatty, 1972). Studies in animal species other than pigs seem to indicate that it is plausible that variation in sperm morphology arises during spermatogenesis, when genotypic effects influence sperm structure. Sperm morphology phenotype appears to be controlled by genes tran-

scribed in the pre-meiotic phase of development (Burgoyne, 1975). Inbreeding coefficients have been related to poor ejaculate quality, further demonstrating the genetic control of sperm morphology (Roldan et al, 1998). This fact, together with the easy identification (albeit few boars were used) of differences on sperm ejaculates between boars and ejaculate portions within the population of normal spermatozoa, points out the possibility of identifying those boars—or sperm portions—more suitable for biotechnological procedures such as sperm cryopreservation or sorting. However, the results may not necessarily apply to other boars, as 5 boars from 1 breed were investigated. The fact that the results were significant although only 5 boars were included can be misleading because the unit of measurement was not the boar.

Relationship Between Sperm Subpopulations and Sperm Quality Postthaw

Both portions of the ejaculate varied in the percentage of spermatozoa within each subpopulation except in boar 1352, where no significant differences were observed in the size of the sperm subpopulations. It is noteworthy that these 2 sperm subpopulations showed a different ability to sustain freezing-thawing procedures (Peña et al, 2003a,b, 2004). Cryopreservation implies many insults to the spermatozoa (Mazur, 1984). Perhaps the 2 main ones are the osmotic stress and the formation/reshaping of intracellular ice during freezing and again during thawing. It is noteworthy that when comparisons are made among species for their ability to sustain cold shock, clear sperm differences are evident (Watson and Plummer, 1985); the spermatozoa of those species less sensitive to cold shock are smaller and more rounded in shape. Obviously, many other factors are involved in cryoresistance, but we hypothesized that sperm shape influences sperm area, thus causing differences in heat exchange as well as in movements of water and ions. It is, therefore, plausible to think that spermatozoa may vary in their physical properties depending on their shape. Although many other factors can also be related, the importance of shape factors is that these are probably inherited traits (Thurston et al, 2001), which points to the possibility of identifying boars with ''good'' or ''bad'' sperm freezability through the morphometric study of the ejaculates. Considering conventional cryopreservation of boar semen, while 50% of the original spermatozoa remain motile postthaw, not more than 2.5% of the motile sperm remain fully competent for fertilization (Holt et al, 2005). Therefore, approaches such as selection of ''good freezers'' (either as individual boars or as well-defined ejaculate portions) could have a tremendous impact on the success of cryopreservation. In fact, in our study, regression analysis models were able to predict up to the 36% of the variance in the percentage of sperm membranes postthaw, and other models predicted sperm velocities and motility after freezing-thawing procedures.

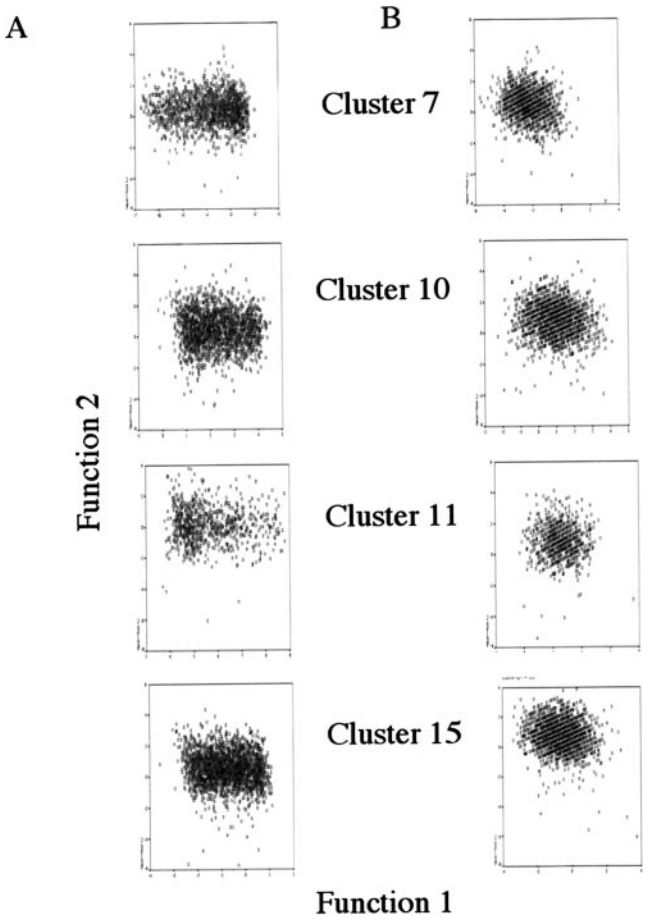

Figure 2. Example of the clusters obtained after the discriminant analysis. Dot plots of the morphometric data defined by the 2 first principal components (PRIN1 and PRIN2). Each event represents and individual spermatozoa. The left part of the figure **(A)** represents the 4 sperm subpopulation found in a boar that can be classified as ''good freezer.'' The right part **(B)** represents the same subpopulations in a boar classified as ''bad freezer.''

We have found in some ejaculate portions a sperm motility rate substantially higher than membrane integrity. This is an unusual finding that, however, can be explained by the technique used to assess sperm membrane integrity. The use of the A/PI assay allows a better discrimination of sperm membranes than a classical combination of probes such as SYBR-14/PI (Peña et al, 2003b). In fact, the subpopulation of live sperm as assessed using SYBR-14/PI is an heterogeneous population in which can be found spermatozoa with intact membranes and spermatozoa showing translocation of the phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the sperm membrane. This is an early change in the process of cryodamage, and sperm motility is still not seriously compromised at this stage, so a proportion of sper-

matozoa classified as damaged using the A/PI assay may still remain motile.

In conclusion, we have developed a simple statistical procedure to identify sperm morphometric subpopulations within the boar ejaculate. The ASMA protocol used was able to detect subtle morphometric differences within different portions of the boar ejaculate. Such a combination of multivariate analysis with ASMA analysis could be considered as a powerful tool to improve the spermiogram of stud boars.

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