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INVITED ORIGINAL ARTICLE

Semen Analysis

Morphometry and subpopulation structure of Holstein bull spermatozoa: variations in ejaculates and cryopreservation straws

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Sperm quality is evaluated for the calculation of sperm dosage in artificial reproductive programs. The most common parameter used is motility, but morphology has a higher potential as a predictor of genetic quality. Morphometry calculations from CASA-Morph technology improve morphological evaluation and allow mathematical approaches to the problem. Semen from 28 Holstein bulls was collected by artificial vagina, and several ejaculates were studied. After general evaluation, samples were diluted, packaged in 0.25 ml straws, and stored in liquid nitrogen. Two straws per sample were thawed, and slides were processed and stained with Diff-Quik. Samples were analyzed by a CASA-Morph system for eight morphometric parameters. In addition to the “classical” statistical approach, based on variance analysis (revealing differences between animals, ejaculates, and straws), principal component (PC) analysis showed that the variables were grouped into PC1, related to size, and PC2 to shape. Subpopulation structure analysis showed four groups, namely, big, small, short, and narrow from their dominant characteristics, representing 31.0%, 27.3%, 24.1%, and 17.7% of the total population, respectively. The distributions varied between animals and ejaculates, but between straws, there were no differences in only four animals. This modern approach of considering an ejaculate sperm population as divided into subpopulations reflecting quantifiable parameters generated by CASA-Morph systems technology opens a new view on sperm function. This is the first study applying this approach to evaluate different ejaculates and straws from the same individual. More work must be done to improve seminal dose calculations in assisted reproductive programs.

Asian Journal of Andrology (2016) 18, 851–857; doi: 10.4103/1008-682X.187579; published online: 27 September 2016

Keywords: cryopreservation; Holstein bull; sperm morphometry; subpopulations

INTRODUCTION

The appropriate characterization of male fertility is of highest importance because a bad selection of dairy sires will compromise animal production planning for long periods. The way to evaluate the fertility potential of a male is semen quality evaluation, which is the principal tool for the calculation of sperm doses needed for artificial insemination in most farm animals.

Traditionally, the most commonly used seminal parameter was sperm motility,¹ while morphology analysis had a secondary place because it takes much more time, the definition of universal and clear patterns of normal morphology is highly complex, and the process lacks precision.^{2–4} Nevertheless, morphological characteristics are genetically defined, making its analysis reliable and informative on the genetic quality of the ejaculate.⁵ Obviously, one sample without motility will not be able to fertilize, but motility is affected by many environmental factors,⁶ while morphology is more related to spermatogenesis and epididymal sperm maturation processes.^{7,8}

Despite the presence of extreme forms (perfectly round or greatly elongated heads), the principal problem of morphological analysis is

that it is not easy to discriminate subjectively between similar forms. This fact explains the great coefficients of variation of both intra- and inter-observer, reducing the feasibility of the obtained results.⁹ In heteromorphic sperm species, different efforts have been made to define morphology with universal morphological classifications (bull,^{10–12} cat,¹³ human,¹⁴ llama,¹⁵ and stallion^{16–18}).

The development of CASA-Morph (Computer-Assisted Semen Analysis for Morphology) technology for the study of sperm morphometrical has opened new possibilities for the morphology evaluation of spermatozoa.^{19,20} In the beginning, this technique was used with low-power statistics to perform comparisons following ANOVA, assuming a normal distribution of data or some nonparametric analysis in more accurate work.^{21–23} Since then, multivariate analysis has been introduced for the simultaneous consideration of all the parameters, including their relationships.²⁴ During recent decades, new efforts have been made to define the best analytical approaches by using subpopulation analysis²⁵ and morphological sperm subpopulation structure, based on morphometric data, which has been observed in a great variety of species: dogs,^{26,27} boars,^{28,29} bulls,^{30,31} foxes,³² humans,^{33,34}

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llamas,¹⁵ marmosets,³⁵ pumas,³⁶ rabbits,³⁷ rams,^{38–40} red deer,⁴¹ and stallions.⁴²

The purpose of the present work was to compare different approaches for the evaluation of bull sperm morphometry for the establishment of the most useful and precise method. Moreover, the effect of animal, ejaculate, and straw on both morphometric parameters and subpopulation structure was analyzed.

MATERIALS AND METHODS

Semen collection and processing

Semen was collected, during spring, from 28 Holstein bulls by artificial vagina, under an extraction program of two ejaculates per week. Animals were housed in Xenética Fontao S.A. (Lugo, Spain) installations. For the study, five ejaculates from each animal and two straws from each ejaculate were used, for a total of 280 samples.

Within 5 to 10 min of collection, the semen samples were assessed for volume by conical tube graduated at 0.1 ml, gross motility by placing 20 μ l fresh semen on a prewarmed slide at 37°C, concentration by a bovine photometer Accucell (IMV, L'Aigle, France) at 530 nm wavelength, and sperm viability by flow cytometry with SYBR 14 and PI from a commercially available Live/Dead Sperm Viability Kit (Life Technologies, Carlsbad, CA, USA).

The raw semen was diluted with a commercial egg yolk extender (Optidyl® -IMV, L'Aigle, France) to a final live sperm concentration of 25×10^6 cells/straw. Diluted semen was cooled slowly to 4°C at a linear rate of $-0.3^\circ\text{C min}^{-1}$ in a refrigerator. After cooling of semen, equilibration occurred over 4–5 h at the same temperature.

The semen was then packaged in 0.25 ml straws, which were sealed via automatic filling and sealing machine (MRS 1, IMV Technologies, L'Aigle, France) and frozen by a programmable freezer, Digitcool 5300 (IMV, L'Aigle, France) with the following curve: 4°C to -10°C at 5°C min^{-1} , -10°C to -100°C at $40^\circ\text{C min}^{-1}$, -110°C to -140°C at $20^\circ\text{C min}^{-1}$, and then plunged into liquid nitrogen for storage. All samples were coded in such a way that the technician who performed the morphometric analysis could not deduce the number of the bull, the number of the ejaculate, or which ejaculate belonged to a particular bull.

Sample preparation for morphometric analysis

The semen straws were sent by courier to Proiser R+D, S.L. (Paterna, Spain) in a Dry shipper 3.0 (ST Reproduction Technologies LLC, Navasota, TX, USA). Duplicate samples for morphometric analysis were prepared from two straws per frozen ejaculate. After being thawed at 37°C for 30 s in a water bath, 150 μ l per straw was diluted with 450 μ l emCare (Bodanco, Alkmaar, The Netherlands). After being mixed, 5 μ l of each sample was spread on a glass slide and subsequently air-dried.

The slides were stained by using the Diff-Quik kit (Medion Diagnostics, Düringen, Switzerland), following the instructions of the manufacturer. All the slides were identified and then permanently sealed with Eukitt mounting medium (Kindler & Co, Freiburg, Germany) under a cover slip and analyzed in a double-blind scheme.

Computerized morphometric analysis

Microscope slides were analyzed for sperm head morphometry by the ISAS® v1 (Integrated Semen Analysis System, Proiser R+D, Paterna, Spain). The equipment comprised a microscope (Olympus BH-2; Tokyo, Japan) equipped with a 100 \times bright-field objective and a 3.3 \times photo-ocular. A video digital camera (A312, Basler, Ahrensburg, Germany) was mounted on the microscope to capture the images and transmit them to the computer. The array size of the video frame grabber was 768 \times 576 \times 8 bit, providing digitized images of 442368

pixels and 256 gray levels. Resolution of images was 0.08 μ m per pixel in both the horizontal and vertical axes.

Sperm heads were captured randomly in different fields, rejecting only those that overlapped with background particles or other cells that interfered with subsequent image processing. Initial erroneous definition of the sperm head boundary was corrected by varying the analysis factor of the system. When it was not possible to obtain a correct boundary, the sperm head was deleted from the analysis.

Following the criteria of Boersma *et al.*,⁴³ at least sixty sperm heads were measured on each slide for four primary parameters of head size (length [L, μ m], width [W, μ m], area [A, μm^2], and perimeter [P, μ m]) and four derived dimensionless parameters of head shape (ellipticity [L/W], rugosity [$4\pi A/P^2$], elongation [(L – W)/(L + W)], and regularity [$\pi LW/4A$]). Data from each individual sperm cell were saved in an Excel® (Microsoft Corporation, Redmond, Washington, USA)-compatible database by the software for further analysis.

Statistical analysis

The data obtained from the analysis of all sperm parameters were first tested for normality and homoscedasticity by using Shapiro–Wilk and Kolmogorov–Smirnov tests, respectively. To evaluate the classical statistical analysis approach, repeated measures ANOVA was performed, assuming normal distribution and homogeneity of variances, following the classical approach to the problem. Nevertheless, as morphometric sperm variables did not satisfy the normality requirements, nonparametric Kruskal–Wallis test was performed followed by the Mann–Whitney paired U-test when significant differences were found. The statistical model used was: ($x_{ijk} = \mu + A_i + E_j + S_k + \epsilon_{ijkl}$), where: x_{ijk} = measured sperm morphometry variable, μ = overall mean of variable x , A_i = effect of animal, E_j = effect of ejaculate, S_k = effect of straw, and ϵ_{ijkl} = residual.

Multivariate analysis of variance (MANOVA), based on Wilk's lambda criterion, was performed.⁴⁴ The test used ejaculate and straw as within-bull factors. The multivariate linear model was: ($y_{ij} = \mu + \alpha_i + \beta_j + \epsilon_{ijk} \dots i = 1, \dots, a; j = 1, \dots, b$), where μ is the overall mean, α_i is the additive effect of the level i of factor ejaculate, β_j is the additive effect of the level j of factor, and ϵ_{ijk} is the residual.

Clustering procedures were performed to identify sperm subpopulations from the complete set of morphometric data.⁴⁵ The first step was to perform a principal component analysis (PCA). To select the number of principal components that should be used in the next step of analysis, the criterion of selecting only those with an eigenvalue (variance extracted for that particular principal component) >1 (Kaiser criterion) was followed. The second step was to perform a two-step cluster procedure with the sperm-derived indices obtained after the PCA. All the sperm morphometric measurements within each ejaculate and straw were clustered by shape and size parameters using a nonhierarchical clustering procedure (k-means model and Euclidean distance). This classifies the spermatozoa of the data set into a small number of subpopulations according to their head dimensions, as has been described previously.²⁹ This analysis allowed the identification of sperm subpopulations and the detection of outliers.

The effects of clusters within and between treatments for the measurements of morphometric parameters were analyzed by the generalized linear model. The influence of each ejaculate within bulls on the relative distribution frequency of spermatozoa belonging to each subpopulation was analyzed by Chi-square and Mantel–Haenszel Chi-square tests. After characterizing sperm subpopulations, ANOVA was performed to explore the relationships between the proportions of

each sperm subpopulation in the sample. The results are presented as mean \pm standard deviation (s.d.). Statistical significance was considered as $P < 0.05$. All data were analyzed using InfoStat Software (v. 2008) for Windows.⁴⁶

RESULTS

Traditional analysis

Here, we present a resume of the obtained results. After performing ANOVA analysis (assuming normality and homogeneity of the samples) for each independent morphometric variable, there were statistically significant differences ($P < 0.05$) between 12 (Length), 14 (Width and Regularity), 16 (Ellipticity, Rugosity, and Elongation), and 17 (Area and Perimeter) animals from the total number of 28 animals studied. Following the criterion to evaluate the most useful parameter to differentiate between animals, Perimeter and Area should be considered.

Looking for differences between ejaculates from the same animal, only in one case, there were no differences between the five ejaculates observed. In the other animals, most of the parameters showed differences in at least two of the ejaculates. The most sensitive parameter able to distinguish the ejaculates was the Area, showing differences between all the ejaculates in two animals and between four ejaculates in 11 animals. The less informative parameter was Regularity, with 11 animals not showing differences between ejaculates.

Regarding the differences between straws for one ejaculate, only four animals showed no differences for any straw in the five ejaculates, while for the remaining animals, differences between straws in at least one of the ejaculates were recorded. In this case, the parameter with more differences was Rugosity, showing variation in 16 animals, followed by Area in 11 animals.

The analysis by MANOVA of all the variables showed significant differences between all the animals. Only in one animal (the same as that after ANOVA), differences between ejaculates were not observed, while the remainder showed differences between three (five animals), four (thirteen animals), or even between the five ejaculates (nine animals). In reference to straws, only eight animals presented no differences between straws.

Principal component analysis and subpopulation structure analysis

The PC analysis produced two components, explaining 75.6% of the variance. PC1 was represented by Length, Area, and Perimeter, and called the "size" component; PC2 referred to Width, and negatively, Ellipticity and Elongation, called the "elongation" component (Table 1).

The analysis of subpopulations revealed four well-defined groupings (Figure 1). The characteristics of SP1 showed the lowest size, named "small," comprised 27.3% of the total cells; SP2 comprised low Length with high Rugosity, named "short" cells, and represented 24.1%; SP3 included the cells with higher Area, named "big" cells, being 31.0%; and SP4 was characterized by high Length, Ellipticity, and Elongation, and were named "narrow" cells with a 17.7% of the total (Table 2).

The distribution of subpopulations among animals showed differences after Chi-square analysis. In 18 animals, one of the subpopulations was clearly the most representative: SP1 in five, SP2 in four, SP3 in five, and SP4 in two; in nine animals, two subpopulations presented equivalent values and only one animal showed a similar distribution between three subpopulations (SP2, SP3, and SP4) (Table 3).

Among ejaculates, there were differences in some subpopulations in all animals except one; another showed differences only for SP1, four for two subpopulations, fifteen for three, and seven for all the

subpopulations. The most variable subpopulations were SP1 and SP3 (different among ejaculates for 25 animals), followed by SP2 (for 19 animals), and SP4 (only for 13). Different ejaculates showing different patterns among them were common, but in some cases, there was no difference between ejaculates (Figure 2).

Regarding the differences of subpopulation distributions between straws of the same ejaculate, four animals showed no differences, five differed in only one subpopulation, ten in two, six in three, and three

Table 1: Eigenvalues of each parameter in both PCs for bull sperm head morphometry found in frozen-thawed samples

	PC1 (size)	PC2 (elongation)
Head length	0.53*	
Head width	0.27	0.56*
Head area	0.45*	0.33
Head perimeter	0.44*	
Ellipticity	0.31	-0.53*
Rugosity		0.11
Elongation	0.31	-0.53*
Regularity		-0.05
Explained variation (%)	42.90	32.70

*Expresses the more important variable in each PC. Only eigenvalues >0.3 are presented. PCs: principal components

Table 2: Mean values (\pm s.d.) of each morphometric parameter corresponding to different SPs from frozen/thawed bull spermatozoa

Variable	SP1	SP2	SP3	SP4
n/%	4791/27.28	4233/24.10	5440/30.97	3100/17.65
Head length (μ m)	8.54 \pm 0.35	8.39 \pm 0.32	9.16 \pm 0.29	9.40 \pm 0.46
Head width (μ m)	4.37 \pm 0.18	4.75 \pm 0.19	4.90 \pm 0.18	4.52 \pm 0.20
Head area (μ m ²)	32.46 \pm 2.14	34.40 \pm 2.05	38.31 \pm 1.97	36.23 \pm 2.44
Head perimeter (μ m)	24.33 \pm 0.89	24.36 \pm 0.83	26.07 \pm 0.88	26.34 \pm 1.46
Ellipticity	1.96 \pm 0.09	1.77 \pm 0.08	1.87 \pm 0.08	2.08 \pm 0.14
Rugosity	0.69 \pm 0.04	0.73 \pm 0.03	0.71 \pm 0.03	0.66 \pm 0.05
Elongation	0.32 \pm 0.02	0.28 \pm 0.02	0.30 \pm 0.02	0.35 \pm 0.02
Regularity	0.90 \pm 0.03	0.91 \pm 0.03	0.92 \pm 0.03	0.92 \pm 0.03

SPs: subpopulations; s.d.: standard deviation

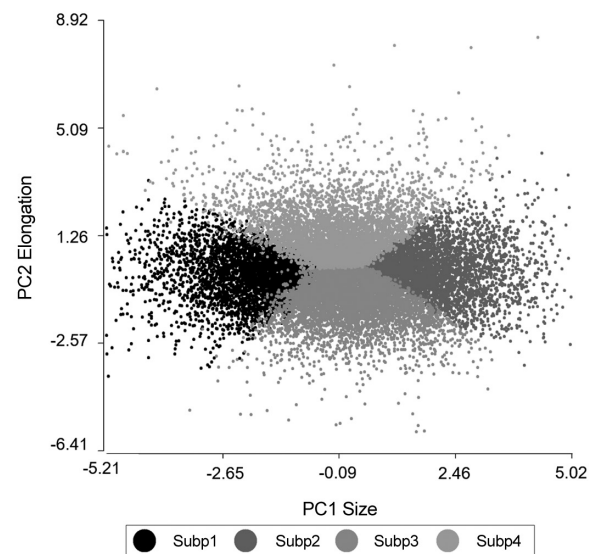


Figure 1: Distribution of subpopulations according to their PC values.

Table 3: Percentage of cells assigned to each SP per animal

Animal	SP1	SP2	SP3	SP4
1	22.50	2.32	28.75	46.43*
2	3.88	4.35	58.39*	33.39
3	40.00*	20.34	18.47	21.19
4	21.78	55.17*	21.14	1.91
5	44.28*	35.95	15.36	4.41
6	18.84	9.89	52.75*	18.52
7	38.52	37.29	17.72	6.47
8	34.72	40.57*	19.70	5.01
9	12.06	3.65	36.19	48.10*
10	23.13	32.57	36.64	7.65
11	26.68	17.94	36.51	18.88
12	26.92	26.38	29.74	16.96
13	14.29	10.05	50.71*	24.96
14	18.71	5.16	39.68	36.45
15	36.38	2.87	25.45	35.30
16	32.01	19.97	32.33	15.69
17	43.56*	19.63	20.25	16.56
18	39.22	45.80*	9.94	5.04
19	34.72	21.04	33.64	10.60
20	23.26	50.95*	23.89	1.90
21	18.66	25.68	44.69*	10.96
22	30.07	22.55	32.84	14.54
23	44.26*	27.05	14.43	14.26
24	15.36	10.08	44.64*	29.92
25	15.41	23.56	38.22	22.81
26	42.70*	16.21	20.55	20.55
27	25.70	31.91	35.68	6.71
28	15.89	51.82*	28.81	3.48

*The most relevant SP in each animal. SP: subpopulation

in four. The most variable subpopulation was SP3, followed by SP1, SP4, and SP2, the latter being the less variable one.

DISCUSSION

The relationship between sperm head morphometry and fertility in the bull is clearly established, indicating that bulls with high fertility produce more elongated and tapered spermatozoa^{47,48} (perhaps our SP1, see below). In addition, some of the differences observed in sperm nuclear shape could be related to the various levels of chromatin stability.^{49,50}

Cryopreservation is a common technique in some species including the bull, but considerable variation in post-thaw semen viability exists.⁵¹ Independent of sperm quality before freezing, the semen of certain individuals will consistently freeze/thaw badly, resulting in poor motility, disrupted acrosomal and plasma membrane,⁵² and thus reduced fertilizing ability, indicating the existence of variation in membrane properties and their response to freeze-thawing between animals. Recent studies suggest that there is a genetic basis for variation in post-thaw semen quality.⁵³ Previous work has analyzed the morphometric characteristics of bull spermatozoa before and after cryopreservation, observing that the results of raw semen differ from that obtained post-thawing, indicating that the cryopreservation process can affect the different types of cells in different ways or modify their previous morphology.³⁰

Osmotic stress is related to differences in osmolality across the plasma membrane, its hydraulic conductivity, and also the cell's volume and surface area. It is likely that subtle differences among spermatozoa in shape and volume or area are responsible for differences in the

speed of water exchanges across the plasmalemma, which could be the origin of different subpopulations of sperm morphology.²⁶ In any case, the sampling processes of smearing and air drying the samples for morphology assessment cause high osmotic stress and membrane damage that can overlap any inherent morphological details in the morphometry analysis. In trying to solve this problem, the use of a new technique for morphological analysis, based on the observation of cells directly on seminal plasma, has been proposed.⁵⁴⁻⁵⁶

In the present work, we have not studied the effect of cryopreservation as such centering the work on frozen samples and comparing different statistical methods for the comparison of morphometric characteristics of spermatozoa. The traditional concept of a sperm population in an ejaculate was to look for "normal cells," those with supposed high-fertility potential. It assumed a more or less unimodal morphological distribution, and so statistical calculations were based on ANOVA (frequently without previous normality and homogeneity analysis) or, in the best case, after this analysis using nonparametric tests such as the Kruskal-Wallis.⁵⁷ This approach has several limitations: (i) each variable is considered independently; (ii) it assumes a uniformity in the population that is not real in heteromorphic species, as the bull; (iii) it cannot be applied to complicated studies such as the present one with a high number of animals, five ejaculates per animal and two straws per ejaculate, as the presentation of the results requires too many tables or graphs.

In the present study, many partial differences were found by following this approach in all the comparisons (individuals, ejaculates, and straws). The use of the MANOVA improved some of the previous limitations because all the variables are considered in a multivariate way. This approach is much better because the morphometric data for each cell are considered simultaneously, increasing the statistical power and reducing the data to work with. Nevertheless, the idea of a homogeneous sperm population remains inherent in this approach.

It has also been used as a new approach to a nonhomogeneous population in species, in which different morphologies have previously been described, following multivariate discriminant analysis.¹⁵ The major limitation of this *a priori* approach is that it is based on subjective classification, even if it is mathematically categorized and provides a mathematical classification matrix to be used for subsequent analysis. The subjectivity is limited to the definition of the canonical cells defined for the matrix calculation.

During the last decade, a *posteriori* subpopulation structure based on principal components and cluster analysis has been introduced. This is the best approximation to the real sperm population in an ejaculate. In a previous attempt, traditional statistics and the new approach to subpopulation structure in stallion were compared.⁴² In future, this kind of work is needed to translate the former results to the new approach to include all the background data.

Taking kinematic data into consideration, four sperm subpopulations were established in Asturiana de los Valles bulls,⁵⁸ suggesting that the presence of four subpopulations could be a common feature of bovine ejaculates. In these studies, differences between animals were only related to the subpopulation with highest velocity and progressiveness, even after cryopreservation.⁵⁸

Sperm morphometric subpopulation structure in the bull has also been described.⁴⁵ In that study, with nuclear fluorescence staining, three PC and four subpopulations were observed. Different animals showed clearly different subpopulations, but only one ejaculate from each bull was analyzed.⁴⁵ Here, we have also found four subpopulations defined by two PCs. Another work has provided evidence of three bull sperm morphometric subpopulations, but these were from mixed data from

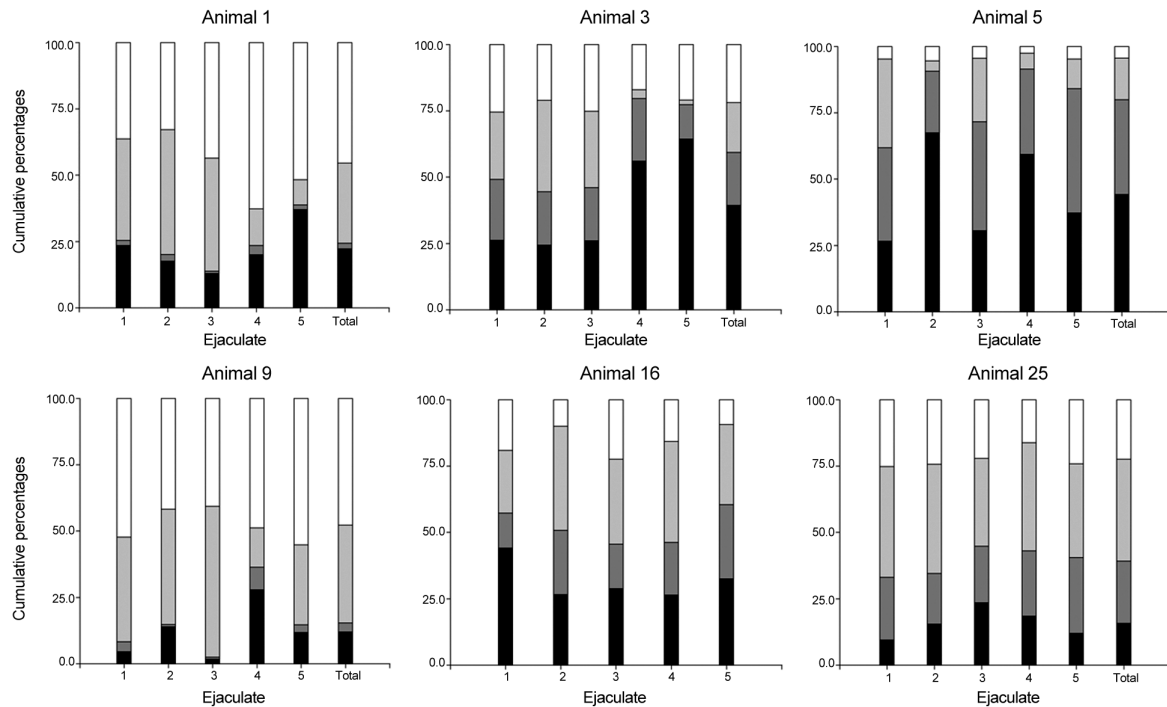


Figure 2: Distribution of subpopulations (SP) per ejaculate in some representative animals. SP1 (black), SP2 (dark gray), SP3 (light gray), and SP4 (white) bars. Animal 1 presents differences for subpopulations SP1, 3 and 4; Animal 3 for SP1 and 3, but the first three ejaculates present equivalent number of cells for each SP; Animal 5 for SP1 and 3, but the distribution of SP in each ejaculate was different; Animal 9 for SP1, 2 and 3; Animal 16 for SP3 and 4; and Animal 25 showed no differences among ejaculates.

five Holstein and five Brahman bulls, not taking into consideration the possible inter-breeding variations.³⁹ Mixing different breeds is common in other species, such as the dog, but it has been demonstrated that different breeds have different sperm subpopulations, so more work is needed on the evaluation of these results.²⁷

Most of the subpopulation studies have shown different distribution between animals, indicating that endogenous factors (genetic, physiological, etc.) are involved.³⁵ The combination of the genetic and physiological status of an individual must be translated into different gamete strategies that reflect the sperm competition context in a particular species.⁵⁹

In a variety of species, a subpopulation structure based on both kinematic and morphometric parameters has been established,⁶⁰ and the differences between animals were considered to be an individual animal strategy. In this work, we have analyzed, for the first time, the sperm morphometry of different ejaculates from the same animal. If the strategy was based on an individual's genetics, the differences between ejaculates should be insignificant or do not exist, but we have found clear differences among ejaculates. In the same direction, mid-piece length is different between bull breeds and also between ejaculates of the same bull.⁶¹ This may indicate that the idea of a subpopulation strategy being just related to the animal strategy on the basis of genetic characters is incorrect or incomplete. More work is needed to understand the real meaning of these results, but we can hypothesize that a combination of genetics and physiological status must be responsible for the final subpopulation strategy of an individual.

As the ejaculate content reflects the sperm reserves available in the distal cauda epididymis at that time, variations in sperm quality in any ejaculate are likely to reflect the balance between distal caudal

emptying (depending on the frequency of copulation or program of semen collection), caudal filling from the proximal cauda (depending on the extent of prior sperm depletion), and mixing of the spermatozoa during seminal emission before ejaculation. In addition, variations in the accessory gland fluid composition could have an effect on the final sperm morphometry.

The result obtained on the differences in sperm subpopulations between straws can be related to the fact that the ejaculate is not homogeneous and it is not possible to take reproducible aliquots, even with thorough mixing before removing a portion. The differences could also be explained by each straw being produced at different times from semen dilution. Either way this needs more study, perhaps by increasing the number of analyzed cells per sample, because if confirmed, these results could have consequences for reproductive success.

CONCLUSIONS

The former approaches to the study of sperm morphometry based on the differences analysis (ANOVA or MANOVA) are not good enough to define the true sperm populations, and it is necessary to use multivariate statistics based on principal component analysis to define subpopulations structure. Differences among ejaculates from the same animal challenge the former idea that the subpopulation structure is an individual characteristic, it could be also related to a physiological response to changes in the environment, even if based on the genetic basis.

AUTHOR CONTRIBUTIONS

AV, HA, AF and CS conceived and designed the experiments; HA, MS, JC, and JY performed the experiments; AV and CS analyzed the data; and CS wrote the paper.

COMPETING INTERESTS

CS is Professor at Valencia University and acts as Scientific Director of Proiser R+D S.L Research and Development Laboratory. Neither he nor the other authors have interests that influenced the results presented in this paper.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Sogol Fereidounfar for her comments and corrections on the manuscript.

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