

## Structure of Sperm Cell Subpopulations in the Bull Frozen Semen Influences *In Vitro* Fertility

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### Abstract

Sperm morphometric subpopulations and hetero morphism in *Bos taurus indicus* bull semen and their influence on *in vitro* fertilization were investigated. Area, center of mass and width obtained on 200 digitized cells/animal (n=9) were used to determine the subpopulations. The analysis of Ward's grouping with Euclidean distance was used to determine the subpopulations, and number of subpopulations was defined according to the behavior of distance level and similarity. Fertility was evaluated by Pro-Nuclei Formation Rate (PFR) after *In Vitro* Fertilization (IVF). Sperm hetero morphism in one hundred cells considered to be morphologically normal was assessed by Harmonic Fourier Descriptors and Principal Component Analysis (PCA). Four subpopulations (SP1 to SP4) were determined, exhibiting asymmetrical distribution. SP4 was composed by 43.0% of the cells that showed the greatest area ( $39.3 \pm 1.8 \mu\text{m}^2$ ), while SP1 ( $35.3 \pm 2.4 \mu\text{m}^2$ ), SP2 ( $35.9 \pm 2.5 \mu\text{m}^2$ ), and SP3 ( $36.9 \pm 3.0 \mu\text{m}^2$ ) presented similar distribution (21.0%; 17.5%; 18.5%; respectively). A negative correlation ( $r = -0.86$ ;  $P = 0.01$ ) was observed between the SP4 and PFR. Hetero morphism rate did not influence PFR. In conclusion, semen of *Bos taurus indicus* bulls exhibited morphological heterogeneous structure and cells exhibiting large head area presented lower capacity of IVF.

**Keywords:** *Bos taurus indicus*; Spermatozoa; Subpopulation; Morphometry; IVF

### Introduction

Methodologies of sperm cell evaluation have experienced advances in the last years, but it is still not possible to precisely predict the fertility of a semen straw [1]. One reason for this constraint should be the attempt to consider the ejaculate as a homogeneous solution of spermatozoa, but cells present distinct patterns of movement and morphometry [1,2] and different clusters defined by these parameters were described in various species [3-9]. Besides, the use of mean values of seminal characteristics to correlate with fertility may be no representative [2], and currently, a subpopulation structure based on principal components and cluster analysis has been introduced [10].

The semen of the bull exhibits a heterospermic template. Sperm morphometry analysis has shown that variations within and among individuals influence fertility of the ejaculates [11]. Studies have investigated the phenotype of the sperm head and its possible relation to fertility [12,13], but those evidencing influence of morphological sperm subpopulations and fertility are speculative [10].

Fertility may be estimated *in vivo* or by the capacity of spermatozoa to penetrate oocytes *in vitro* [14-16]. However, only one study has addressed the correlation between the influence of subpopulations and fertility [3]. The present study aimed to identify sperm subpopulations and morphological parameters in frozen semen of *Bos taurus indicus* bulls related to *in vitro* fertility.

## Material and Methods

### Experiment 1: Determining Sperm Subpopulations and Semen Heteromorphism

#### Semen samples

One semen straw of nine sexually mature bulls of the Nelore breed ( $11.0 \pm 3.1$  years old) were used. Semen was collected via artificial vagina and frozen according standard protocols in an artificial insemination center. The experiment was conducted in the Veterinary Department - UFPA, and EMBRAPA, Brazil, being approved by the ethics committee on the use of animals (CEUA-UFPA 073/15).

#### Semen processing

Straws were thawed ( $37^\circ\text{C}/30\text{s}$ ) and motility was assessed using computer-assisted sperm analysis (IVOS-Ultimate 12's, Hamilton Thorne Biosciences, Beverly, MA, USA), configured for bovine spermatozoa: number of frames: 30; frames/s: 60Hz; minimum contrast: 50; minimum cell size: 6 pixels; contrast to static cells: 30; straightness: 60%; average path velocity cutoff:  $30\ \mu\text{m/s}$ ; minimum average path velocity:  $40\ \mu\text{m/s}$ ; straight-line velocity cutoff:  $20\ \mu\text{m/s}$ ; cell intensity: 80; static head size: 0.23-1.91; static head intensity: 0.56-1.20; static elongation: 8-92; magnification: X 1.89 and temperature:  $37^\circ\text{C}$ . For the evaluation,  $9\ \mu\text{L}$  of semen diluted in TALP at a concentration of  $25 \times 10^6$  cells/mL were placed in a chamber (Makler, Santa Ana, CA, USA). Three fields were selected for analysis. Parameters evaluated were Total Motility (TM), Progressive Motility (PM) and Linearity (LIN) in %; Average Path Velocity (VAP); Straight Line Velocity (VSL) and Curvilinear Velocity (VCL) in micrometers/second; Lateral Head Amplitude (ALH) in micrometers and Beat Cross Frequency (BCF) in Hz.

A semen aliquot of  $50\ \mu\text{L}$  was added to a tube containing  $100\ \mu\text{L}$  of formaldehyde saline solution. Ten microliters were examined and 200 cells considered to be morphologically normal per straw were selected. Morphology was assessed by light microscopy, Harmonic Fourier Descriptors [17] and Principal Component Analysis [18], using 100 cells per bull in the latter analyses. A phase contrast microscope (Olympus BX41) with a green filter (IF550) coupled to a camera (Charge Coupled Detector - SDC-415, Samsung) was used to acquire the images, stored as .bmp files. Spermatozoa were centered on the screen at 400X magnification and it was assured that the cells were in the same focus plane with none artifacts.

#### Image processing

Sperm images analyses were performed using the Image J platform (National Institutes of Health, USA). Images were transformed to 8-bit and digitized using limits around 65-76 in

gray scale (Threshold tool). After adjustments, the spermatozoon head was cut and transferred to an image with white background. The head contour line was identified (Wand tool) creating a Region of Interest (ROI) to be analyzed. Parameters evaluated were area ( $\mu\text{m}^2$ ), centroid (X and Y), center of mass (XM and YM), perimeter, rectangle, ellipse axis, width, and length ( $\mu\text{m}$ ). To avoid redundancy [19], after statistical analysis, only three measurements were used to determine the subpopulations.

#### Determination of subpopulations

Sperm subpopulations were defined by multivariate analysis of hierarchical and nonhierarchical grouping. Samples were evaluated independently and together. The multivariate cluster analysis K-means was performed to classify spermatozoa in optimum reduced number of subpopulations, according to the head measurements, after applying the Ward's grouping analysis, using Euclidean distance. Cells with similar morphometry were clustered in the same group and different clusters were formed according to the observed mean values of each group. Subpopulation numbers was based on the previous analysis of hierarchical dendrogram and calculated on the estimate of the level of distance and similarity among groups.

### Experiment 2: Fertility Evaluation

#### Semen samples

Semen of three out of the nine bulls were excluded from *in vitro* fertility evaluation, because significant differences ( $P < 0.05$ ) for area after categorization in clusters were not observed, total motility was below 30% and because one was considered an outlier in the residual analysis.

#### Bovine oocytes collection and maturation

All reagents used in IVF were obtained from Sigma-Aldrich Química S.A (São Paulo, SP, Brazil).

Ovaries from slaughterhouses were transported in saline solution with  $100\ \mu\text{g/mL}$  of streptomycin and  $100\ \text{UI/mL}$  of penicillin G at  $37^\circ\text{C}$ . Cumulus-Oocyte Complexes (COCs) aspirated from follicles from 3 to 8 mm were deposited in tubes of  $50\ \text{mL}$  for sedimentation. The supernatant was removed, precipitate was poured in a  $100 \times 200\ \text{mm}$  cell culture plate containing TALP-HEPES for COCs evaluation. COCs with three or more layers of cumulus cells with homogeneous cytoplasm were selected for *In Vitro* Maturation (IVM). Groups of 25-30 COCs were placed in  $400\ \mu\text{L}$  of medium for IVM compounded by TCM 199 with Earle salts, L-glutamine,  $2.2\ \text{g/L}$  of sodium bicarbonate, 10% of estrus cow serum,  $20\ \mu\text{g/mL}$  of follicle stimulating hormone,  $49.4\ \mu\text{g/mL}$  of sodium pyruvate ( $0.45\ \text{mM}$ ),  $100\ \text{UI/mL}$  of penicillin and  $0.1\ \text{mg/mL}$  of streptomycin in four-well plates and cultivated in a chamber during 22-24h at  $38.5^\circ\text{C}$ , with 5% of  $\text{CO}_2$ .

## Preparing semen and *in vitro* fertilization

After maturation, COCs were washed in Phosphate-Buffered Saline (PBS) and transferred into drops of 70  $\mu\text{L}$  of the fertilization medium (FERT-TALP plus heparin, 10 UI/mL). One semen straw of the same ejaculate of each bull used to determine the subpopulations was used for IVF. Straws were thawed at 37  $^{\circ}\text{C}/30\text{s}$  and semen motility was evaluated under light microscopy.

A semen aliquot of 250  $\mu\text{L}$  was centrifuged during 7min at 300 $\times$ g at room temperature. Supernatant was removed and the sediment was re-suspended in 400  $\mu\text{L}$  of medium FERT-TALP and centrifuged again (5min; 300 $\times$ g). Supernatant was removed and the sediment was re-suspended in 70  $\mu\text{L}$  of medium FERT-TALP. Motility and vigor were evaluated once more. Spermatozoa were counted in a Neubauer chamber and diluted in fertilization medium for a final concentration of  $2 \times 10^6$  sperm cells/mL. A semen aliquot of 30  $\mu\text{L}$  was added to each drop of fertilization, achieving a volume of 100  $\mu\text{L}$ . Plates were incubated at 38.5  $^{\circ}\text{C}/20\text{h}$  in chambers with 5% of  $\text{CO}_2$ .

## Evaluation of pronuclei formation rate

Possible zygotes were transferred to 1 mL of TALP 20h after insemination and stirred during 5min to remove cumulus cells, being washed in PBS with polyvinyl alcohol (PVA; 1 mg/mL), fixed during 30min in paraformaldehyde 4% and washed again in PBS-PVA. Specimens were stained with Hoechst 33342 (5  $\mu\text{g}/\text{mL}$ ), placed under a coverslip and PFR was evaluated [3] under 400X magnification in epifluorescence microscopy (Motic BA400, excitation of 330-385 nm and emission of 420-490 nm).

## Statistical Analyses

Principal Component Analysis (PCA) was done using scripts of the Matlab software version R2012 (8.0.783). Statistical analyses of subpopulations were performed in software Minitab 17.0.1. Regression analyses and residues were made in R software version 3.4.2, package MVar.pt version 1.9.9. Data previously standardized because metrics were at different scales were tested regarding normality of residues by the Shapiro-Wilk test. According to Martinez-Pastor, et al. [19], data were submitted to variance group analysis and one parameter of each formed group was selected, and selected parameters were submitted to Pearson's correlation test. Only those presenting  $r$  values  $<0.3$  and  $P < 0.05$  were used.

Hierarchical and nonhierarchical methods were used for grouping 1800 spermatozoa from one straw per bull evaluated

together. For each bull 200 cells were analyzed. A multivariate analysis of Ward's hierarchical grouping based on Euclidean distance was performed to create a hierarchical alignment cluster and then to obtain a dendrogram. The optimum number of clusters was determined by the behavior of similarity level and the distance in the steps of grouping. During the analysis to establish the groups, similarity among conglomerates decreases and distance increases. Sharp variations of these measurements indicate the ideal number of clusters. After defining the number of groups, the K-means method was applied by a nonhierarchical multivariate analysis to determine the proportion of cells belonging to each subpopulation. Validation of groups to verify the mean values difference among each subpopulation was made by the Tukey test.

Each straw was considered one independent observation. Motility at the time of IVF, PFR and proportion of subpopulations were tested regarding normality, homoscedasticity, and independence by means of the Shapiro-Wilk, Bartlett and Box-Pierce tests, respectively. The relationship between the proportion of each subpopulation in the semen samples and IVF was evaluated by regression analysis according to a previous work [3], as well as the relationship between sperm motility before and at the time of IVF and PFR. Differences were indicated by a probability of  $P < 0.05$ .

## Results

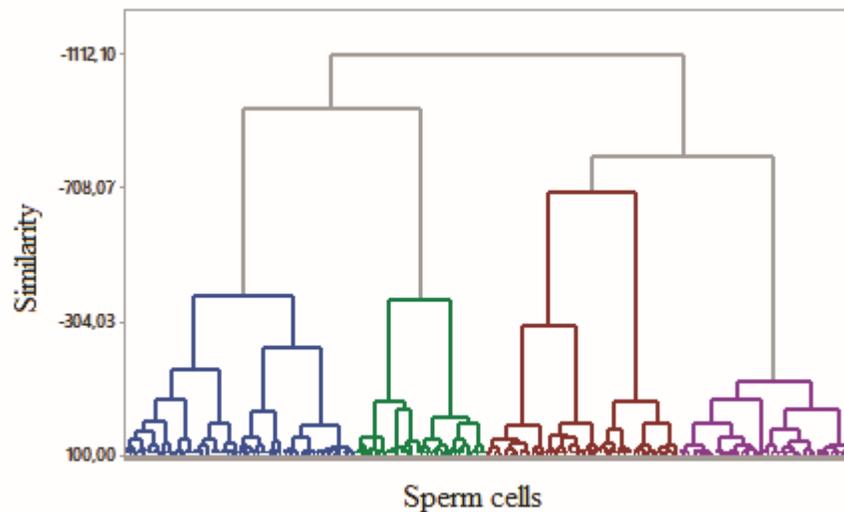
To determine subpopulations, using the variance grouping analysis and Pearson's correlation, it was selected area, center of mass and width. These variables presented the lower correlation values ( $r < 0.3$ ;  $P < 0.05$ ).

Four different sperm subpopulations were defined on the basis of the difference in the index obtained by grouping similarity and distance levels (Figure 1). When the cells of all animals were analyzed together, spermatozoa head area presented the greatest difference among the four subpopulations ( $P < 0.05$ ). Subpopulation 1 was composed by sperm cells with smaller area ( $35.3 \pm 2.4 \mu\text{m}^2$ ), as well as the SP2 ( $35.9 \pm 2.5 \mu\text{m}^2$ ), representing 21.3% and 17.5% of the total cells, respectively. Spermatozoa of these two subpopulations were defined as short and tapered. SP3 was composed by average spermatozoa head area of  $36.9 \pm 3.0 \mu\text{m}^2$  and has 18.5% of the total cells, being defined as short and wider. SP4 was composed by the largest areas ( $39.3 \pm 1.8 \mu\text{m}^2$ ), representing 42.7% of the total of cells, being defined as large and elongated (Table 1).

Subpopulations	Proportion (%)	Area ( $\mu\text{m}^2$ )	XM (coordinate)	YM (coordinate)	Width ( $\mu\text{m}$ )
SP1	21.3	35.32	175.504.0	264.935.0	6.92
	(383/1800)	(35.07-35.56) <sup>d</sup>	(169.178.9-181.829.1) <sup>c</sup>	(260.040.9-269.829.1) <sup>a</sup>	(6.75-7.09) <sup>c</sup>
SP2	17.5	35.9	168.653.6	111.989.0	6.75
	(316/1800)	(35.62-36.18) <sup>c</sup>	(160.570.8-176.736.5) <sup>c</sup>	(107.000.9-116.977.1) <sup>c</sup>	(6.56-6.92) <sup>c</sup>
SP3	18.5	36.85	304.894.7	192.908.2	9.2
	(333/1800)	(36.53-37.18) <sup>b</sup>	(300.551.8-309.237.5) <sup>a</sup>	(186.705.2-199.111.1) <sup>b</sup>	(8.85-9.52) <sup>a</sup>
SP4	42.7	39.31	186.735.6	200.425.2	8.47
	(768/1800)	(39.19-39.45) <sup>a</sup>	(183.550.4-189.920.9) <sup>b</sup>	(198.566.4-202.283.9) <sup>b</sup>	(8.36-8.57) <sup>b</sup>

Same letters in the column did not differ ( $P>0.05$ ).  
Abbreviations: SP: Subpopulation; XM and YM: center of mass;  $\mu\text{m}$ : micrometer.

**Table 1:** Descriptive means of variables observed in sperm cell subpopulations in the Nelore bull frozen semen.



**Figure 1:** Hierarchical cluster analysis of sperm subpopulations defined by morphometric parameters (area, center of mass and width) of nine Nelore bulls. SP1 is marked in red, SP2 in green, SP3 in purple and SP4 in blue.

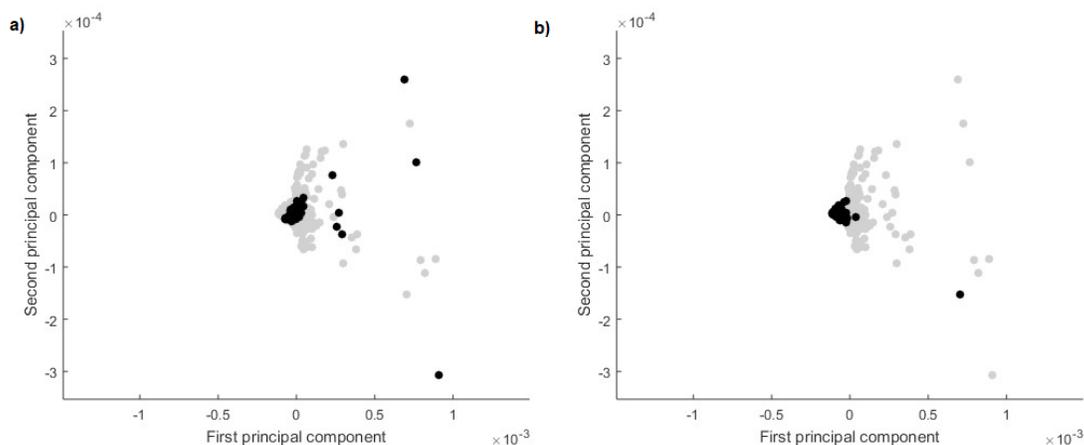
SP4 presented the smallest variations of the center of mass, measured by Coefficients of Variation (CVs), while SP2 showed the greatest. Distribution of subpopulations among all animals was asymmetrical. Four out of nine animals presented normal distribution among the subpopulations. SP1 exhibited normal distribution in six animals, SP2 in seven and SP3 and SP4 in eight of the nine bulls. Six bulls had SP4 as the most representative cluster, SP2 in two and SP1 in one individual (Table 2).

Subpopulations	Bull 1	Bull 2	Bull 3	Bull 4	Bull 5	Bull 6	Bull 7	Bull 8	Bull 9
SP1	15.5 (31/200)	36.5 (73/200)	16.0 (32/200)	17.5 (35/200)	29.5 (59/200)	15.0 (30/200)	25.0 (50/200)	28.0 (56/200)	18.0 (36/200)
SP2	32.5 (65/200)	23.5 (47/200)	33.0 (66/200)	9.0 (18/200)	9.5 (19/200)	26.0 (52/200)	9.5 (19/200)	8.5 (17/200)	20.0 (40/200)

SP3	27.5 (55/200)	24.0 (48/200)	20.5 (41/200)	26.0 (52/200)	28.0 (56/200)	25.5 (51/200)	28.0 (56/200)	22.5 (45/200)	21.5 (43/200)
SP4	24.5 (49/200)	16.0 (32/200)	30.5 (61/200)	47.5 (95/200)	33.0 (66/200)	33.5 (67/200)	37.5 (75/200)	41.0 (82/200)	40.5 (81/200)
Abbreviations: SP: Subpopulation.									

**Table 2:** Sperm subpopulations distribution in Nelore bulls frozen semen. Percentages and the relative numbers of spermatozoa within each subpopulation are shown.

Morphology was evaluated in the nine bulls by PCA and the first two components revealed a variance ranging from 45.5 to 95.6% (Figure 2). No correlation was observed between hetero morphism rate and PFR.



**Figure 2:** Bull sperm cells exhibiting high (a) and (b) low hetero morphism rate. Black dots are cells of one bull over the cells of the other eight bulls (gray dots).

After processing for IVF, all straws had motility above 70% (Table 3). The fertility rate of six straws used to IVF ranged from 54.4% to 64.7% and a negative correlation ( $r = -0.86$ ,  $P < 0.05$ ) was observed between the number of cells in SP4 and PFR. The regression equation ( $PFR = 70.8 - 0.2893SP4$ ) showed  $R^2 = 0.73$  and the model demonstrated that when the relative proportion of SP4 cells increased, PFR decreased. There was no significant correlation between sperm motility before (total and progressive) and at the time of IVF and PFR ( $R^2 = 0.14$ , Figure 3). Negative correlations between PFR and VCL ( $r = -0.27$ ), VAP ( $r = -0.35$ ) and VSL ( $r = -0.50$ ) were observed ( $P < 0.05$ ).

Bull	TM (%)	PM (%)	VAP ( $\mu\text{m/s}$ )	VSL ( $\mu\text{m/s}$ )	VCL ( $\mu\text{m/s}$ )	Motility in IVF (%)	SP4 (%)	PFR (%)
2	48	21	66.7 $\pm$ 30.5	49.4 $\pm$ 25.4	126.3 $\pm$ 54.9	71.6 $\pm$ 2.8	16.0	64.6 $\pm$ 2.1
3	44	19	93.6 $\pm$ 44.6	62.5 $\pm$ 38.4	180.6 $\pm$ 71.1	76.7 $\pm$ 5.8	30.5	62.3 $\pm$ 6.1
4	69	35	88.2 $\pm$ 38.8	62.6 $\pm$ 37.0	163.7 $\pm$ 61.3	81.6 $\pm$ 5.8	47.5	54.4 $\pm$ 1.5
5	39	17	91.9 $\pm$ 44.0	63.2 $\pm$ 41.4	176.4 $\pm$ 67.7	70.0 $\pm$ 5.0	33.0	62.5 $\pm$ 11.8
7	61	29	69.5 $\pm$ 30.6	52.7 $\pm$ 29.7	128.0 $\pm$ 49.6	83.3 $\pm$ 2.8	37.5	62.4 $\pm$ 5.5
9	49	27	76.3 $\pm$ 30.0	57.0 $\pm$ 28.6	145.0 $\pm$ 53.2	70.0 $\pm$ 5.0	40.5	59.3 $\pm$ 11.2
Abbreviations: TM: Total Motility; PM: Progressive Motility; VAP: Average Path Velocity; VSL: Straight Line Velocity; VCL: Curvilinear Velocity; IVF: <i>In Vitro</i> Fertilization; PFR: Pronuclei Formation Rate; SP4: Subpopulation 4.								

**Table 3:** Parameters of computer-assisted sperm analysis and sperm motility after Percoll treatment before IVF assays. Proportion of cells in SP4 and PFR in frozen semen of six Nelore bulls.

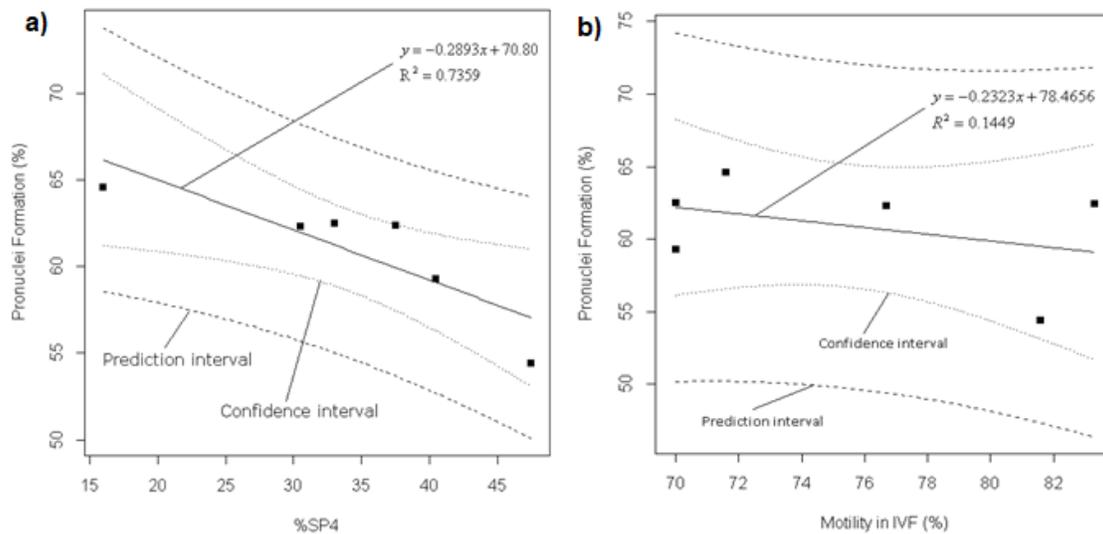


Figure 3: a) Linear regression between the proportion of cells in the subpopulation4 and Pronuclei Formation rate (PFR); b) Regression between the sperm motility at the time of *in vitro* fertilization and PFR; no correlation and a poor coefficient of determination are observed.

## Discussion

As it was observed previously in *Bos taurus taurus* [1,2,10], frozen semen of *Bos taurus indicus* also exhibits morphological heterogeneity and four sperm subpopulations defined by different head area were verified in our study. In *Bos taurus taurus* three to four subpopulations based on morphometric head parameters were observed [8,10,20] and four subpopulations were identified by kinetic attributes [3,5,6]. According to our results, the number of subpopulations determined by sperm head morphometric parameters ranges from three to four between the two subspecies.

Most studies on subpopulations showed a different cluster distribution among the animals involved [4,10,21], like data of the present study, and differences among ejaculates of the same individual [10]. These differences are considered to be a result of genetic and endocrine changes, related to adaptations to the fertilization environment [2,22], that should influence the fertility of the animal.

The average head area of the cells observed in this experiment, varying from 35.3 to 39.3  $\mu\text{m}^2$ , is comparable to other data [12,13,23,24]. Our results demonstrated that the higher proportion of SP4 decreases PFR. Estimation of semen fertility involves several factors [25], and IVF has been used as an indicator for it [3,26]. One study showed that motility could be one factor responsible for PFR [3], and positive correlations among rapid and progressive cells and the number of spermatozoa linked to the pellucid zone ( $r=0.79$ ), penetration rate ( $r=0.66$ ) and PFR ( $r=0.63$ ) were verified. In cervids [27], high fertility males have ejaculates

with high percentages of spermatozoa exhibiting fast and linear movements. Morphologically, these patterns of motility were correlated with a large proportion of spermatozoa having small and elongated heads.

In the present experiment, semen samples with total motility <30% were removed from IVF and statistical analysis. Variations in the relative proportions of cell clusters did not interfere with semen kinetics. No correlation was verified between motility ranging from 39.0% to 69.0% ( $\geq 70\%$  after Percoll treatment) and PFR. Moreover, and negative correlations between PFR and VCL, VAP and VSL were observed. These results suggest that large and wide cells were responsible for the differences observed in fertility among the bulls. However, in the sheep, a positive correlation between fertility and proportion of wide and long cells was observed [28], while a negative correlation was observed between fertility and percentage of spermatozoa with short and wide heads [29].

Some efforts were made to investigate sperm cell morphometric parameters and fertility [12,13,30]. In the filtered and extended semen of the stallion, average measurements of length, area and perimeter of the spermatozoa head were higher in sub fertile animals [30]. In the frozen semen of the bull, no differences were observed among the mean values of length, width, area and perimeter, and the non-return to estrus rate [13]. Nonetheless, these studies used mean values of sperm head variables and heterogeneity of spermatozoa was not considered. In the fresh semen of highly fertile bulls it was observed that small variations in size of the sperm head can exist not interfering with the fertilization capacity of the sperm cells [12].

Sperm morphology [31] was assessed comparing frozen semen of bulls divided in high and low fertility groups. Bulls of high-fertility have sperm nuclear shape more elongated and tapered than those of lower fertility. Our approach consisted in cluster categorization and semen *in vitro* fertility evaluated by PFR, and a negative correlation between cells allocated in SP4 (long and wide) and IVF was observed. High fertility rates among rams [32] were associated to a subpopulation with short and elongated heads, while no relationship between fertility and average values of sperm head dimensions were found. Thus, cluster analysis is relevant in semen evaluation.

Morphology of sperm cells is sensible to physical [23] and physiological stress [24]. PCA in four bulls showed that the first two components explained more than 81.3% of the variance among sperm cells, thus cell morphology in these ejaculates was very similar. In the other five individuals, this variation ranged from 45.5 to 74.5%, which implies a greater sperm cell heteromorphism. In addition, the center of mass CVs indicated that some cell clusters are morphologically more stable than the others. No relationship was observed between heteromorphism rate and PFR. Number of cells showing normal morphology and morphometry increase after Percoll treatment [33], but how these parameters influence the fertilization process and if only the sperm dimensions are involved remain uncertainty. Nonetheless, sperm head morphometry appears to be an independent semen parameter and normal morphology is not necessarily an indicative of morphometric attributes.

The association found in the present study between poor fertility and large sperm head size may be related to chromatin condensation, as it was observed previously [34,35]. Bovine spermatozoa with chromatin condensation abnormalities presented alterations that include larger head area [31,36,37] and bulls that possess spermatozoa with high levels of DNA fragmentation and poor protamination also have lower fertility [35]. Lower rates of PFR verified in this experiment found in semen with more large-head cells could be a consequence of inadequate protamination, resulting in sperm heads exhibiting larger areas.

## Conclusion

In conclusion, heterogeneity among sperm cell clusters must be considered in an accurate semen analysis. The frozen semen of *Bos taurus indicus* bulls is composed by four sperm cell subpopulations, defined by head morphometric parameters. Morphological subpopulation structure and distribution is different among individuals, and a high relative proportion of cells in a cluster exhibiting large sperm head area is related to poor *in vitro* fertility. The heterospermic pattern in cells considered to be normal in light microscopy observed in the ejaculate does not influence fertility evaluated by pro-nuclei formation rate.

## References

1. Rodríguez-Martínez H (2007) State of the art in farm animal sperm evaluation. *Reprod Fertil Dev* 19: 91-101.
2. Ramón M, Jiménez-Rabadán P, García-Álvarez O, Maroto-Morales A, Soler AJ, Fernández, et al. (2014) Understanding sperm heterogeneity: Biological and practical implications. *Reprod Domest Anim* 49: 30-36.
3. Ferraz MAMM, Morató R, Yeste M, Arcarons N, Pena AI, et al. (2014) Evaluation of sperm subpopulation structure in relation to *in vitro* sperm-oocyte interaction of frozen-thawed semen from Holstein bulls. *Theriogenology* 81: 1067-1072.
4. Maroto-Morales A, Ramón M, García-Álvarez O, Soler AJ, Fernández-Santos MR, et al. (2012) Morphometrically-distinct sperm subpopulations defined by a multistep statistical procedure in Ram ejaculates: Intra- and interindividual variation. *Theriogenology* 77: 1529-1539.
5. Muiño R, Tamargo C, Hidalgo CO, Peña AI (2008) Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: Effects of cryopreservation and between-bull variation. *Anim Reprod Sci* 109: 27-39.
6. Muiño R, Peña AI, Rodríguez A, Tamargo C, Hidalgo CO (2009) Effects of cryopreservation on the motile sperm subpopulations in semen from Asturiana de los Valles bulls. *Theriogenology* 72: 860-868.
7. Quintero-Moreno A, Miró J, Teresa Rigau A, Rodríguez-Gil JE (2003) Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. *Theriogenology* 59: 1973-1990.
8. Rubio-Guillén J, González D, Garde JJ, Esteso MC, Fernández-Santos MR, et al. (2007) Effects of cryopreservation on bull spermatozoa distribution in morphometrically distinct subpopulations. *Reprod Domest Anim* 42: 354-357.
9. Thurston LM, Watson PF, Mileham AJ, Holt WV (2001) Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. *J Androl* 22: 382-394.
10. Valverde A, Arenán H, Sancho M, Contell J, Yániz J, et al. (2016) Morphometry and subpopulation structure of Holstein bull spermatozoa: variations in ejaculates and cryopreservation straws. *Asian J Androl* 18: 1-7.
11. Soler C, García-Molina A, Contell J, Silvestre MA, Sancho M (2015) The Trumorph® system: The new univ the morphology of living sperm. *Anim Reprod Sci* 158: 1-10.
12. Beletti ME, Costa LDF, Viana MP (2005) A comparison of morphometric characteristics of sperm from fertile *Bos taurus* and *Bos indicus* bulls in Brazil. *Anim Reprod Sci* 85: 105-116.
13. Gravance CG, Casey ME, Casey PJ (2009) Pre-freeze bull sperm head morphometry related to post-thaw fertility. *Anim Reprod Sci* 114: 81-88.
14. García-Álvarez O, Maroto-Morales A, Martínez-Pastor F, Garde JJ, Ramón M, et al. (2009) Sperm characteristics and *in vitro* fertilization ability of thawed spermatozoa from Black Manchega ram: Electroejaculation and postmortem collection. *Theriogenology* 72: 160-168.
15. Macedo MC, Lucia T, Rambo G, Ferreira Filho EB, Rosa AP, et al. (2010) *In vitro* penetration of swine oocytes by homologous spermatozoa: Distinct systems for gamete's co-incubation and oocyte's cryopreservation. *Anim Reprod Sci* 117: 295-301.

16. Taberner E, Morató R, Mogas T, Miró J (2010) Ability of Catalanian donkey sperm to penetrate zona pellucida-free bovine oocytes matured in vitro. *Anim Reprod Sci* 118: 354-361.
17. Zhang D, Lu G (2002) Generic Fourier descriptor for shape-based image retrieval. *Proc - 2002 IEEE Int Conf Multimed Expo, ICME 2002*: 425-428.
18. Hotelling H (1933) Analysis of a complex of statistical variables into principal components. *J Educ Psychol* 24: 417-441.
19. Martínez-Pastor F, Tizado EJ, Garde JJ, Anel L, de Paz P (2011) Statistical Series: Opportunities and challenges of sperm motility subpopulation analysis. *Theriogenology* 75: 783-795.
20. Vicente-Fiel S, Palacín I, Santolaria P, Yániz JL (2013) A comparative study of sperm morphometric subpopulations in cattle, goat, sheep and pigs using a computer-assisted fluorescence method (CASMA-F). *Anim Reprod Sci* 139: 182-189.
21. Martí JI, Aparicio IM, García-Herreros M (2011) Sperm morphometric subpopulations are differentially distributed in rams with different maturity age in cryopreserved ejaculates. *Theriogenology* 76: 97-109.
22. Maroto-Morales A, García-Álvarez O, Ramón M, Martínez-Pastor F, Fernández-Santos Mr, et al. (2016) Current status and potential of morphometric sperm analysis. *Asian J Androl* 18: 1-8.
23. Gravance CG, Vishwanath R, Pitt C, Garner DL, Casey PJ (1998) Effects of Cryopreservation on Bull Sperm Head Morphometry. *J Androl* 19: 704-709.
24. Révay T, Nagy S, Kovács A, Edvi ME, Hidas A, et al. (2004) Head area measurements of dead, live, X- and Y-bearing bovine spermatozoa. *Reprod Fertil Dev* 16: 681-687.
25. Rodríguez-Martínez H (2003) Laboratory semen assessment and prediction of fertility: Still Utopia? *Reprod Domest Anim* 38: 312-318.
26. Park Y-J, Mohamed E-SA, Oh S-A, Yoon S-J, Kwon W-S, et al. (2012) Sperm Penetration Assay as an Indicator of Bull Fertility. *J Reprod Dev* 58: 461-466.
27. Ramón M, Soler AJ, Ortiz JA, García-Alvarez O, Maroto-Morales A, et al. (2013) Sperm Population Structure and Male Fertility: An Intraspecific Study of Sperm Design and Velocity in Red Deer. *Biol Reprod* 89: 1-7.
28. Yániz JL, Palacín I, Vicente-Fiel S, Sánchez-Nadal JA, Santolaria P (2015) Sperm population structure in high and low field fertility rams. *Anim Reprod Sci* 156: 128-134.
29. Martínez-Rodríguez C, Alvarez M, López-Urueña E, Gomes-Alves S, Anel-López L, et al. (2015) Head morphology of ram spermatozoa is associated with their ability to migrate in vitro and correlates with fertility. *Reprod Fertil Dev (Who)*.
30. Casey PJ, Gravance CG, Davis RO, Chabot DD, Liu IKM (1997) Morphometric Differences in sperm Head Dimensions of Fertile and Subfertile Stallions. *Theriogenology* 97: 575-582.
31. Ostermeier GC, Sargeant GA, Yandell BS, Evenson DP, Parrish JJ (2001) Relationship of bull fertility to sperm nuclear shape. *J Androl* 22: 595-603.
32. Maroto-Morales A, Ramón M, García-Álvarez O, Montoro V, Soler AJ, et al. (2015) Sperm head phenotype and male fertility in ram semen. *Theriogenology* 84: 1536-1541.
33. Gravance CG, Champion ZJ, Sax-Gravance SK, Casey PJ (1998) Percentage of normal sperm heads is significantly increased by Percoll separation of semen. *Int J Androl* 21: 116-119.
34. Nagy S, Johannisson A, Wahlsten T, Ijäs R, Andersson M, et al. (2013) Sperm chromatin structure and sperm morphology: Their association with fertility in AI-dairy Ayrshire sires. *Theriogenology* 79: 1153-1161.
35. Dogan S, Vargovic P, Oliveira R, Belser LE, Kaya A, et al. (2015) Sperm protamine-status correlates to the fertility of breeding bulls. *Biol Reprod* 92: 92.
36. Kipper BH, Trevizan JT, Carreira JT, Carvalho IR, Mingoti GZ, et al. (2017) Sperm morphometry and chromatin condensation in Nelore bulls of different ages and their effects on IVF. *Theriogenology* 87: 154-160.
37. Sailer BL, Tost LK, Evenson DP (1996) Bull sperm head morphometry related to abnormal chromatin structure and fertility. *Cytometry* 24: 167-173.