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INVITED ORIGINAL ARTICLE

Semen Analysis

Sperm kinematic, head morphometric and kinetic-morphometric subpopulations in the blue fox (*Alopex lagopus*)

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This work provides information on the blue fox ejaculated sperm quality needed for seminal dose calculations. Twenty semen samples, obtained by masturbation, were analyzed for kinematic and morphometric parameters by using CASA-Mot and CASA-Morph system and principal component (PC) analysis. For motility, eight kinematic parameters were evaluated, which were reduced to PC1, related to linear variables, and PC2, related to oscillatory movement. The whole population was divided into three independent subpopulations: SP1, fast cells with linear movement; SP2, slow cells and nonoscillatory motility; and SP3, medium speed cells and oscillatory movement. In almost all cases, the subpopulation distribution by animal was significantly different. Head morphology analysis generated four size and four shape parameters, which were reduced to PC1, related to size, and PC2, related to shape of the cells. Three morphometric subpopulations existed: SP1: large oval cells; SP2: medium size elongated cells; and SP3: small and short cells. The subpopulation distribution differed between animals. Combining the kinematic and morphometric datasets produced PC1, related to morphometric parameters, and PC2, related to kinematics, which generated four sperm subpopulations – SP1: high oscillatory motility, large and short heads; SP2: medium velocity with small and short heads; SP3: slow motion small and elongated cells; and SP4: high linear speed and large elongated cells. Subpopulation distribution was different in all animals. The establishment of sperm subpopulations from kinematic, morphometric, and combined variables not only improves the well-defined fox semen characteristics and offers a good conceptual basis for fertility and sperm preservation techniques in this species, but also opens the door to use this approach in other species, included humans.

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Keywords: integration of motility and morphology; principal component analysis; sperm morphometry; subpopulation

INTRODUCTION

Foxes have been domesticated in some cold countries (Finland, China, Russia, Argentina, Canada, and others), where its breeding is of high economical relevance. Most reproduction of these farmed foxes is by artificial insemination, but the process is not very technical, and only a few trials regarding general reproductive physiology and management have been proposed.[1–5] Furthermore, sperm characteristics have only recently been studied.[6,7] These species are sperm homomorphous with a low level of morphological sperm abnormalities (around 10%). In species with low sperm morpho-abnormalities, it would seem that morphological analysis is of little importance, but for this reason, the use of morphometry by CASA-Morph systems to find possible differences between ejaculates is obviously efficient and necessary.[8–12]

The purpose of the present work, with the aim of offering a scientific basis for this service, was to combine the multivariate analysis of both kinematic and morphometric data and the establishment of subpopulation structure based on all these parameters in the blue fox species.

MATERIALS AND METHODS

Individual semen samples from twenty individual blue Foxes (*Alopex lagopus*) were obtained by masturbation directly by technical personnel on five farms in the area of Vaasa (Finland). Samples were obtained from trained animals used for artificial insemination in a routine program. The whole sample was deposited in nonsterile sample tubes. Samples were analyzed at the same farm where they were obtained.

Kinematic and morphometric analyses were performed with the ISAS®v1 CASA-Mot and CASA-Morph systems (Proiser R+D, S.L., Paterna, Spain), comprising a UOP200i/Proiser microscope, the Proiser 782M video camera, and software. For motility analysis, raw samples were initially observed in the microscope, through a 10× negative phase-contrast objective to estimate the sperm concentration, after

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which 10 µl of raw sample was diluted with extender Safecell Plus (IMV, L'Aigle, France) to a working concentration of 20×10^6 cells ml^{-1}. After thorough mixing, the samples were placed in an ISAS®D4C16 disposable counting chamber (Proiser R+D). Each one has four tracks with seven printed viewing squares, with a constant depth of 16 µm between slide and fixed cover slide. A sample volume of 3 µl is enough to fill the chamber completely, being distributed along the track by capillarity. When full, the slide could be immediately analyzed because time is not needed for stabilization of the fluid inside the chamber. Samples were analyzed with the ISAS®v1 motility module. Eight kinematic parameters were obtained, three velocities (curvilinear [VCL], straight-line [VSL], and averaged-path [VAP], µm s^{-1}), three dimensionless motility indices (LIN [VSL/VCL], STR [VSL/VAP], and WOB [VAP/VCL]) and ALH (µm) and BCF (Hz). For each sample, seven fields, one inside each of the squares on the counting chamber, were captured and analyzed.[7]

For morphological analysis, 5 µl of each sample was deposited on a glass slide, smeared and air dried for one hour. Smears were stained with Diff-Quick (Medion Diagnostics, Düdingen, Switzerland), dipped for 25 s in each solution (fixative, solution I and solution II), washed free of excess colorant with water, air dried, and mounted with Eukitt (Sigma-Aldrich, Saint Louis, MO, USA). In the ISAS®v1 morphology module, about 200 cell images were captured at random and analyzed which generated four size parameters [length (L, µm), width (W, µm), area (A, µm2) and perimeter (P, µm)] and four derived dimensionless parameters of head shape [ellipticity (L/W), rugosity ($4\pi A/P^2$), elongation ((L-W)/(L+W)), regularity ($\pi LW/4A$)].

Statistical analysis

To identify sperm subpopulations, clustering procedures were performed at first by each kinematic and morphometric parameter independently and then by the combination both datasets.[12] In the three cases, the first step was to perform a principal component analysis (PCA) of the morphometric data. To select the number of principal components that should be used in the next step of analysis, the criterion of selecting only those components with an eigenvalue (variance extracted for that particular principal component) >1 (Kaiser criterion) was chosen. The second step was to perform a two-step cluster procedure with the sperm-derived indices obtained after the PCA to determine the subpopulation structure.

All sperm measurements within each ejaculate were clustered by kinematic, morphometric, and combined kinematic + morphometric parameter values using a nonhierarchical clustering procedure (k-means model and Euclidean distance), to classify the spermatozoa of the dataset into a reduced number of subpopulations according to their kinematic and sperm head morphometric values as has been described previously.[9] The relative distribution frequency of spermatozoa belonging to each subpopulation by animal was analyzed by Chi-square and Mantel–Haenszel Chi-square tests.

The results are presented as mean ± standard deviation (s.d.). Statistical significance was considered at $P < 0.05$. All data were analyzed using InfoStat Software (v. 2008, University of Córdoba, Córdoba, Argentina) for Windows.[13]

RESULTS

Principal component analysis

The analysis was performed at three levels: kinematic, morphometric, and a combination of kinematic and morphometrics (**Table 1**).

The eight kinematic parameters were reduced to two PCs. PC1 was related to linear variables (VSL, VAP, and LIN), explaining the 50.1% of the variance. PC2 was related to oscillatory movement (VCL and ALH), explaining 32.8% (**Table 1**).

Table 1: PC analysis of fox spermatozoa based on kinetic (K), morphometric (M), and both sets of (T) data

Variables	*KPC1*	*KPC2*	*MPC1*	*MPC2*	*TPC1*	*TPC2*
VCL (µm s^{-1})		0.50				
VSL (µm s^{-1})	0.48					0.42
VAP (µm s^{-1})	0.43					0.31
LIN	0.37	–0.39				0.43
STR	0.36					0.37
WOB		–0.41				0.34
ALH (µm)		0.54				
BCF (Hz)	0.38					
Length (µm)			0.51		0.40	
Width (µm)			0.32	–0.45		
Area (μm^2)			0.47		0.33	
Perimeter (µm)			0.50		0.40	
Ellipticity				0.51	0.35	
Rugosity				–0.38	–0.36	
Elongation				0.51	0.35	
Regularity						
Explained variation	50.12	32.27	45.05	35.81	34.23	28.62

Only eigenvalues >0.30 are shown. PC: principal component; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: averaged path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency

The eight morphometric variables were also reduced to two PCs, being PC1, referring to size variables (Length, Area, and Perimeter) and explaining the 45.1%, and PC2, referring to elongation shape of the cells (Ellipticity and Elongation) for 35.8% of the total variance (**Table 1**).

Finally, considering all the variables together, again two PCs were found, even though explaining only 62.9% of the total variance. PC1 was related to morphometric parameters while PC2 was related to kinematic parameters (**Table 1**).

Kinematic subpopulation structure

For the kinematic parameters, the whole population was divided into three independent subpopulations (**Figure 1a**). SP1 comprised 40.7% of the cells and was defined by fast and linear movement (with the highest VSL and an STR of 0.91); SP2 was less frequent at 22.2%, characterized by slow and nonoscillatory motility (indicating by the smallest ALH); and SP3, with 37.1% of the cells, was medium in speed and oscillatory (the highest VCL and ALH). The BCF increased from SP1 to SP3 (**Table 2**).

In almost all cases, the subpopulation distribution by animal was significantly different (χ^2, $P < 0.05$) and only two animals (numbers 8 and 16) showed no differences in subpopulations. SP1 was predominant in ten animals, SP2 in two, and SP3 in six. In all cases, one subpopulation was clearly greater than the others (**Table 2**).

Morphometric subpopulation structure

The morphometric data also revealed three subpopulations (**Figure 1b**). SP1 comprised 35.3% of the cells and was characterized by large oval cells; SP2, less frequent at 26.7%, included medium size elongated cells; SP3 with 38.1% referred to small and short cells. The high level of regularity shown in all the subpopulations was remarkable (**Table 3**).

The subpopulation distribution by animal was significantly different (χ^2, $P < 0.05$) in all cases although three animals (numbers

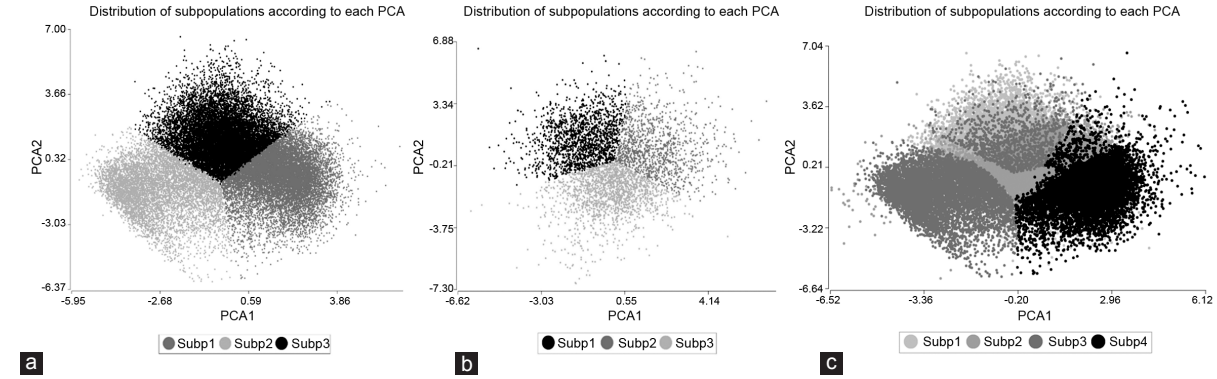

Figure 1: Subpopulation (Subp) distribution according principal component analysis (PCA) for (**a**) kinematics; (**b**) morphometry; (**c**) kinetics and morphometry.

Table 2: Kinematic sperm subpopulations in fox semen in all animals (A) and percentage of subpopulations in each male (B)

Variable	*Subpopulation 1*	*Subpopulation 2*	*Subpopulation 3*
(A) All animals			
n/%	13791/40.73	7498/22.15	12567/37.12
VCL (μm s^{-1})	178.19±36.56	78.02±35.14	208.35±43.32
VSL (μm s^{-1})	96.83±20.77	21.35±14.12	52.59±21.05
VAP (μm s^{-1})	105.93±18.52	37.92±18.15	93.50±18.70
LIN	55.34±12.04	29.10±15.20	26.08±10.28
STR	91.20±9.02	56.71±23.01	57.84±22.29
WOB	61.45±9.11	50.30±12.30	46.42±8.20
ALH (μm)	3.88±0.91	2.28±0.89	5.12±1.15
BCF (Hz)	21.30±5.66	9.26±4.77	15.59±4.79
(B) Individual animal number			
1	62.70#	20.90	16.40
2	1.70	88.20#	10.10
3	0.00	81.60#	18.40
4	59.50#	35.60	4.90
5	65.90#	16.30	17.80
6	20.40	14.20	65.40#
7	38.30	8.50	53.20#
8*	26.10	29.40	44.50
9	53.90#	8.90	37.20
10	84.10#	11.30	4.60
11	52.30#	26.20	21.50
12	70.00#	15.20	14.80
13	43.70#	19.70	36.60
14	47.40#	29.70	22.90
15	37.90	19.60	42.50#
16*	37.60	24.00	38.40
17	8.70	14.30	77.00#
18	4.40	5.70	89.90#
19	13.50	24.90	61.60#
20	65.90#	16.20	17.80

#The most abundant population for an animal; *Pearson's Chi-square test (P>0.05). VCL: curvilinear velocity; VSL: straight-line velocity; VAP: averaged path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency

1, 4, and 9) showed two populations with similar frequency. SP1 was predominant in five animals, SP2 in four, and SP3 in eight (**Table 3**).

Combined kinematic and morphometric subpopulation structure

When both kinematic and morphometric variables were considered together, the total population could be divided into four subpopulations (**Figure 1c**). SP1 included 20.8% of cells assessed for motility and 29.7% of cells assessed for morphometry, with high oscillatory motility, large size, and short heads; SP2 was composed of medium velocity cells with small and short heads and comprising 32.1% of the motile and 26.9% of the morphologically assessed cells; SP3 with 21.0% of motile and 26.7% morphologically assessed cells, with slow motion and small and elongated cells; and SP4 was composed of high linear speed and large size elongated cells for 26.1% of motile and 16.7% of the morphologically assessed cells (**Table 4**).

Subpopulation distribution was different (χ^2, $P < 0.05$) in all animals but one (number 1). SP1 was the least frequent, being the biggest in only one case, whereas SP2 was most frequent in four, SP3 in two and the most frequent was SP4 being the most usual in six animals. Seven animals showed two subpopulations with equivalent frequencies (**Table 4**).

DISCUSSION

Until recently, semen was, and even is now, considered as a group of "equivalent" cells having the same role to win the race toward the oocyte, in something like a marathon. From this point of view, the concept of morphologically "normal" spermatozoa was developed and used for decades.[14–17] The introduction of CASA technology has allowed the acquisition of kinematic and morphometric parameters that can be used for advanced multivariate statistics. By combining both CASA data and multivariate statistics, many publications in recent years have revealed the true structure of the sperm population in the semen is composed by different subpopulations with possible, but unknown, functional significance.[18] These studies have been made using kinematic (boar,[19] dog,[20] fox,[7] rabbit,[21] solea,[22] and stallion[23,24]) or morphometric parameters (boar, bull, goat,[12] llama,[25] ram,[26,27] and red deer[28]). To the best of our knowledge, this study is the first work analyzing combined information of both kinetic and morphometric parameters, and offering a new approach to the evaluation of sperm subpopulations. Some previous papers have been published on the dog using both datasets, but not joining them as here.[29,30] In any case, this holistic approach provides a much more complete explanation of phenomena related to sperm function.

The results obtained in the present study for motility subpopulation structure were completely in accordance with that published previously

Table 3: Morphometric sperm subpopulations in fox semen in all animals (A) and percentage of subpopulations in each male (B)

Variable	*Subpopulation 1*	*Subpopulation 2*	*Subpopulation 3*
(A) All animals			
n/%	1564/35.26	1183/26.67	1689/38.07
Length (μm)	6.06±0.28	6.03±0.32	5.40±0.26
Width (μm)	3.82±0.15	3.45±0.17	3.45±0.16
Area (μm^2)	19.43±1.26	17.04±1.58	15.46±1.23
Perimeter (μm)	17.35±0.64	16.60±0.84	15.43±0.65
Ellipticity	1.59±0.07	1.75±0.08	1.57±0.08
Rugosity	0.81±0.02	0.78±0.03	0.81±0.02
Elongation	0.23±0.02	0.27±0.02	0.22±0.02
Regularity	0.94±0.03	0.96±0.03	0.95±0.03
(B) Individuals animal number			
1*	43.40	10.70	45.90
2	73.20#	18.70	8.10
3	92.30#	2.90	4.80
4*	40.30	20.90	38.80
5	31.30	14.90	53.80#
6	35.30	45.10#	19.50
7	7.00	13.00	80.00#
8	27.80	17.50	54.70#
9*	50.20	45.00	4.80
10	6.90	23.30	69.80#
11	84.10#	11.60	4.30
12	82.20#	9.90	8.00
13	35.10	18.50	46.40#
14	6.30	30.70	63.00#
15	11.10	60.30#	28.60
16	3.90	98.70#	57.40
17	17.00	18.10	64.90#
18	5.40	18.60	76.00#
19	11.40	77.90#	10.60
20	56.10#	33.50	10.30

#The most abundant population for an animal; *Pearson's Chi-square test (P>0.05). VCL: curvilinear velocity; VSL: straight-line velocity; VAP: averaged path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency

for the same species.[7] This fact indicates that, independently of the animal, the structure is constant within the species. The three morphometric subpopulations defined in this species are in agreement with those described for other carnivore species such as the puma,[31] but not with those for the dog, where four subpopulations have been found.[29] This is the first time analysis with both kinds of metric parameters, kinematics and morphometric, have been performed. It revealed four sperm subpopulations, indicating that motility and morphology can be combined to provide a new perspective to assess what an ejaculate is and how it may function.

Even without following the holistic approach used here, it has been shown that sperm size and velocity subpopulations are interrelated, and that both are good indicators of fertility in red deer, when both sets of parameters are independently considered. Males show higher fertility when samples comprise higher percentages of spermatozoa with rapid and linear movement and of elongated shape.[32] However, in another study, a clear correspondence between morphometric and kinematic sperm subpopulations was not observed in the ram.[33] In the boar, it has been demonstrated that spermatozoa back-flowing after artificial insemination are those of low head size and short flagellum.[34] In some birds, it has been shown that a strong positive correlation exists between sperm velocity and sperm flagellar length, the flagellum:head length ratio, tail length, and total sperm length.[35,36] Both sperm length and velocity are heritable traits.[11,37]

Finally, it must be emphasized that in addition to the improvement in farm production of foxes, the information obtained on farmed foxes could be useful for its application to silver fox populations, and not only to this species but also to related ones. This was revealed in other species such as the red deer[38] and brown bear.[39,40]

CONCLUSION

The establishment of sperm subpopulations by the use of kinematic, morphometric, and combined variables not only improves the well-defined fox semen characteristics, and offers a good conceptual basis for fertility and sperm preservation techniques in this species, but also opens the door to use this approach in other species, included humans.

Table 4: Combined kinematic and morphometric sperm subpopulations in fox semen in all animals (A) and percentage of subpopulations in each male (B)

Variable	*Subpopulation 1*	*Subpopulation 2*	*Subpopulation 3*	*Subpopulation 4*
(A) All animals				
n/%	7032/20.77	10874/32.12	7109/21.00	8841/26.11
VCL (μm s^{-1})	214.47±47.10	193.09±37.18	75.33±33.69	171.62±36.80
VSL (μm s^{-1})	39.03±16.67	75.57±17.31	20.54±13.33	103.39±20.43
VAP (μm s^{-1})	94.92±19.69	94.45±18.04	36.46±17.29	109.32±18.82
LIN	18.56±7.05	39.64±7.54	28.32±15.03	61.05±10.20
STR	42.32±17.21	80.10±12.21	56.62±23.09	94.41±7.32
WOB	45.14±9.25	49.63±7.23	50.02±12.42	65.42±9.21
ALH (μm)	5.32±1.28	4.61±0.91	2.23±0.87	3.58±0.84
BCF (Hz)	14.97±5.04	17.84±4.81	9.04±4.67	22.13±6.04
n/%	1319/29.73	1193/26.89	1182/26.65	742/16.73
Length (μm)	5.95±0.23	5.33±0.25	5.75±0.23	6.36±0.25
Width (μm)	3.81±0.15	3.46±0.15	3.37±0.14	3.69±0.16
Area (μm^2)	19.11±1.28	15.33±1.22	15.91±1.11	19.33±1.31
Perimeter (μm)	17.15±0.59	15.32±0.65	15.94±0.58	17.66±0.65
Ellipticity	1.56±0.06	1.54±0.07	1.71±0.07	1.72±0.08

Contd...

Table 4: Contd...

Variable	*Subpopulation 1*	*Subpopulation 2*	*Subpopulation 3*	*Subpopulation 4*
Rugosity	0.82±0.02	0.82±0.02	0.79±0.02	0.78±0.03
Elongation	0.22±0.02	0.21±0.02	0.26±0.02	0.27±0.02
Regularity	0.93±0.03	0.95±0.03	0.96±0.03	0.95±0.03
(B) Individual animal number				
1	11.99	19.00	19.41	49.59#
2	13.03	8.79	67.75#	10.42
3	35.34	7.60	51.06	6.01
4	12.06	18.75	28.49	40.70#
5	12.09	26.83	15.96	45.12#
6	16.02	53.26#	18.12	12.60
7	10.14	69.00#	12.43	8.43
8	27.15	35.76	26.14	10.95
9	20.21	39.20#	9.26	31.34
10	3.63	24.19	18.82	53.36#
11*	17.83	27.12	20.85	34.20
12	16.49	19.55	13.08	50.89#
13	19.28	39.96#	18.72	22.04
14	11.19	28.39	29.48	30.94
15	21.71	37.30	21.85	19.14
16	9.81	37.83	37.30	15.06
17	49.08#	33.45	14.60	2.87
18	32.32	36.46	28.18	3.04
19	36.57	27.64	27.56	8.22
20	12.46	24.77	15.43	47.34#

Columns correspond to subpopulations and rows to males. #The most abundant subpopulation for animal (when two or more values are closed no # was used); *Pearson's Chi-square test (P>0.05). VCL: curvilinear velocity; VSL: straight-line velocity; VAP: averaged path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency

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.

AUTHOR CONTRIBUTIONS

CS and JS conceived and designed the experiments; JC, LB, MS, and AG-M performed the experiments; JC, AV, and CS analyzed the data; CS wrote the paper.

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