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Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates

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Abstract

The main aim of this study is to assess the influence of freeze/thawing on motile sperm subpopulations in ejaculates from two phylogenetically different mammalian species, boar and donkey. Our results indicate that, whereas boar and donkey sperm respond very differently in their mean motion characteristics to freezing/thawing, this process did not change the existence of a 4-subpopulations structure in the ejaculates in either species when these subpopulations were defined by taking values of curvilinear velocity (VCL) as reference. Moreover, the freezing/thawing-linked changes in mean sperm-motion characteristics in both boar and donkey semen were especially due to changes in the proportion among each concrete subpopulation. In this way, the freezing/thawing-induced mean increase in motion characteristics observed in boar sperm was a result of the decrease in the percentage of sperm in Subpopulation 1 (from 53.9% ± 4.7% to 31.2% ± 3.9% after thawing) and a concomitant increase of sperm from Subpopulations 3 (from 13.3% ± 2.5% to 32.6% ± 3.9% after thawing) and 4 (from 3.4% ± 0.9% to 8.0% ± 1.1% after thawing). On the contrary, changes in mean motility of frozen/thawed donkey sperm were linked to an increase in the percentage of sperm in Subpopulation 1 (from 31.5% ± 4.3% to 58.8% ± 4.9% after thawing) and a concomitant decrease of sperm from Subpopulations 3 (from 32.4% ± 3.2% to 6.6% ± 1.8% after thawing) and 4 (from 12.2% ± 2.5% to 7.3% ± 1.9% after thawing). In conclusion, our results seem to indicate that motility changes induced by the freezing/thawing protocol are linked to concomitant changes in both the specific parameters and, more importantly, to the specific percentage of each of the motile sperm subpopulations. These changes did not affect the overall proportion of motile sperm present in both boar and donkey, which is conserved despite the detrimental effect caused by freezing/thawing in both species. Finally, the presence of some kind of motile sperm subpopulations structure has been described in mammalian species with a very great phylogenetic distance, thus suggesting that this structure could play some role in the maintenance of the overall function of mammalian ejaculates.

Keywords: Freezing/thawing; Sperm subpopulations; Boar; Donkey

1. Introduction

It has been well established that the freezing/thawing-induced decrease in the fertilising ability of mammalian sperm is associated with important changes in both the percentage of motile sperm and their motion quality. In this way, the mean characteristics of motility in frozen/thawed spermatozoa have been described as

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being similar to those observed in capacitated cells. This phenomenon, called ''cryocapacitation'', has been described in several species [1–4], although there is not a general consensus about it. For instance, in boar sperm, whereas several authors have shown that freezing/thawing induced a significant increase in the mean velocity of sperm, concomitantly with a decrease in the mean linearity of the sperm progression [5], others, on the contrary, have shown a general decrease of all of the studied parameters related to sperm velocity [6]. These discrepancies can be due to a myriad of causes, from functional differences among ejaculates to the precise media in which boar semen is diluted prior to its freezing. In any case, and as a general rule in all of the mammalian species studied, it is well known that freezing/thawing modifies sperm function in a way in which sperm motility is greatly altered, although the specific mechanisms by which these modifications are produced (i.e., changes in mitochondrial activity, increase in intracellular, reactive oxygen species levels, etc.; see [2,3]) need to be more precisely defined.

In recent years, it has been reported that ejaculates for an increasing number of mammals are comprised of well-defined subpopulations, which are characterised by precise values of the motion parameters obtained after a computer-assisted motility analysis (CASA; 7–13). Notwithstanding, although the presence of such an organisation seems to be practically universal in mammals, there is no consensus about the physiological role for these motile sperm subpopulations in the ejaculate. In this way, the presence of specific motile sperm subpopulations has been related to semen characteristics, such as cryosurvival and semen quality characteristics in species like boar and horse [10,14,15]. Moreover, the specific sperm selection and competition that is observed after sperm colonisation of oviducts in pigs is modulated by the existence of a specific, motile sperm subpopulation structure pattern, based on the sperm's response to bicarbonate, in boar semen [16]. Hence, the existence of a specific, motile sperm subpopulations structure in an ejaculate would be strongly related, at least in boar, to the semen quality characteristics of this ejaculate. However, the physiological role of sperm subpopulations in other aspects like the sperm's survival ability to freezing/thawing is not well documented, thus leaving many unanswered questions in this area.

The main aim of this study is to determine how a freezing/thawing protocol affects the specific, motile sperm subpopulations structure in a mammalian ejaculate. For this purpose, semen from two mammalian species with great phylogenetic differences and in which semen motility in fresh samples is very different was studied. One species is boar, whose sperm from fresh ejaculates shows low velocity combined with medium-to-low linear movement characteristics [4,15]. The other species is donkey, in which sperm from fresh ejaculates shows very high velocity combined with high linear movement characteristics [12]. Moreover, both species had a 3-to-4 motile sperm subpopulations structure [4,8,12,15], which allows for a direct comparison between them. The freezing/thawing protocol applied to boar is standard, thus allowing for a comparison of our results with those previously published. The results obtained in both species indicate that the freezing/thawing-linked changes in mean motility parameters of both boar and donkey semen samples are strongly related to changes in the specific percentage of sperm included in each subpopulation.

2. Material and methods

2.1. Animals and samples collection

Eleven healthy boars of about 2–3 years of age from a commercial farm were used in this study. The boars were from 3 separate lines (4 Landrace, 3 Large White and 4 Pietrain). All boars had proven fertility after AI using extended, liquid semen. The sperm-rich fraction of each ejaculate utilised in this study was manually collected twice weekly using the gloved-hand method and analysed to ensure the quality and the homogeneity of the ejaculates. Immediately after collection, the ejaculated spermatozoa were suspended (1:2; v/v) in a commercial extender (MR-A, Kubus SA, Majadahonda, Spain). The extended semen samples were cooled and maintained at 17 °C for shipment to the laboratory of the Autonomous University of Barcelona within 24-h post-collection, for further processing and analyses.

Donkey semen was obtained from 7 healthy, mature Catalonian donkeys aged 4–8 years, all of which were previously reported as being fertile. Animals were housed at the Experimental Farm and Countryside Service of the School of Veterinary Medicine of the Autonomous University of Barcelona (Bellaterra, Spain). Semen collection was performed at 2–3-day intervals using an artificial vagina (Hannover model) and an ovariectomised female donkey brought into oestrus with estrogens. The artificial vagina was equipped with an in-line gel filter to permit collection of gel-free semen. Immediately after collection, ejaculates underwent standard analysis for volume, pH, sperm concentration and total sperm number, viability, morphological abnormalities and motility.

Gel-free semen was immediately diluted (proportion 1:5, v/v) with dry skimmed-milk extender (24 mg/mL dry skimmed-milk and 49 mg/mL glucose) kept at 37 °C in a water bath. Diluted semen was immediately sent to the laboratory, where the samples were placed at 20 °C in a water bath until beginning the freezing process.

2.2. Semen cryopreservation

Regarding boar sperm, immediately after receiving the shipped semen samples, an aliquot was taken to perform the appropriate semen quality parameters (fresh semen sample). Only those samples displaying a minimum of 70% progressive motile, and 80% morphologically normal spermatozoa were further processed by adapting a proven protocol [17]. The extended semen was centrifuged in a programmable, refrigerated centrifuge (Medifriger BL-S, JP Selecta, Barcelona, Spain) set at 17 °C, at 600 × *g* for 10 min. After centrifugation, the supernatant was discarded. The remaining pellets were re-extended with a lactose-egg yolk (LEY) extender (80 mL [80%, v/v 310 mM] of β-lactose + 20 mL egg yolk), at a ratio that led to a final concentration of 1.5×10^9 spermatozoa/mL. The sperm concentration was manually assessed in a Thoma or Neubauer haemocytometer. At this point, and after thorough mixing, the semen was further cooled to 5 °C for 2 h in the refrigerator. Afterwards, the semen was slowly mixed with a third extender consisting of 89.5 mL LEY extender, 9 mL glycerol and 1.5 mL of Equex STM (Nova Chemicals Sales Inc., Scituate, MA, USA), which is equivalent to Orvus Es Paste [18], at a ratio of two parts of semen to one part of extender, yielding a final concentration of 3% (v/v) glycerol and a concentration of 1×10^9 spermatozoa/mL at 5 °C, which was verified by counting in a Thoma or Neubauer haemocytometer. Spermatozoa were packaged at 5 °C in a cool cabinet (IMV, L'Aigle, France) in 0.5-mL polyvinyl chloride (PVC) plastic straws (Minitüb, Tiefenbach, Germany), which were sealed with PVC powder and placed on racks for freezing [19]. The racks were transferred to the chamber of a programmable freezer (Ice-Cube 14S, Minitüb) set at 5 °C. The cooling/freezing rate used was: 6 °C/min from 5 to −5 °C, 30 s for crystallisation, and thereafter 50 °C/min from −5 to −140 °C. The samples were then plunged into liquid N_2 (−196 °C) for storage. Frozen samples were stored in liquid N_2 for at least 21 days. After this, samples were thawed by the plunging of samples into a water bath at 37 °C for 20 s. Immediately afterwards, straws were carefully wiped and opened, and samples were immediately analysed to determine the appropriate semen quality parameters (frozen/thawed semen sample).

The donkey-semen freezing protocol was based on [20], including the following modifications. The protocol started with a first centrifugation of the previously diluted samples at 660 × *g* for 15 min at 20 °C. Supernatants were eliminated and sperm was re-suspended with 2 mL of the commercial Gent A® extender containing egg yolk (Minitub Ibérica, S.A., Riudoms, Spain). After this dilution, percentages of viability, sperm concentration, total sperm number and motility were re-evaluated. Only semen samples displaying a minimum of 70% progressive motility, and 80% morphologically normal spermatozoa were further processed. Afterwards, commercial Gent B® extender containing egg yolk and glycerol (Minitub Ibérica, S.A.) was added to obtain a final concentration of 2×10^8 viable sperm/mL. Diluted semen samples were then packaged into 0.5-mL straws and immediately placed in an Ice-Cube 14S programmable freezer. Immediately thereafter, samples were subjected to a three-step cooling/freezing programme. The first step was a cooling phase from 20 to 5 °C at a cooling rate of −0.26 °C/min. The second step was to lower the temperature of the samples from 5 to −90 °C at a freezing rate of −4.75 °C/min. The third and final step was to further freeze samples from −90 to −120 °C at a freezing rate of −11.11 °C/min. Afterwards, frozen samples were stored in liquid N_2 for at least 21 days. Thawing was performed through immersion of the straws in a water bath at 37 °C for 30 s. Thawed semen was immediately evaluated to determine post-thaw sperm motility, viability and concentration (frozen/thawed semen samples).

2.3. Analysis of semen quality parameters

Percentages of viability, altered acrosomes and morphological abnormalities in boar samples were determined by using the Eosin–Nigrosin stain [21]. Similarly, percentages of sperm viability and total morphological abnormalities in donkey samples were determined in samples stained following the same Eosin–Nigrosin technique. In both species, this technique shows viable spermatozoa as being those with a uniform, white stain in all of the cells, whereas the presence of a partial or a totally pinkish stain was indicative of non-viable sperm cells. Moreover, acrosome integrity of boar spermatozoa was evaluated by observing the presence of a regular and intact acrosomal ridge after the Eosin–Nigrosin stain. Any part of the

acrosomal ridge that did not have a regular and intact aspect was considered as being altered acrosomes. In both species, the determinations of the above-mentioned percentages were performed after analysing a minimum of 200 spermatozoa/sample through optical microscopy (magnification: 1000×). Sperm concentration and total sperm number was determined after counting in a haemocytometer chamber. On the other hand, the Osmotic Resistance Test (ORT Test) in boar semen was carried out as described in [22].

In both boar and donkey semen, the computer-assisted analysis of sperm motility (CASA) was performed by using a commercial system (Integrated Sperm Analysis System V1.0, Proiser SL, Valencia, Spain). In this system, samples were previously warmed at 37 °C for 5 min in a water bath and 5-μL aliquots of these samples were then placed on a warmed (37 °C) slide and covered with a 22-mm^2 coverslip. Our CASA system was based upon the analysis of 25 consecutive, digitalized photographic images obtained from a single field at a magnification of 200× on a dark field. These 25 consecutive photographs were taken in a time lapse of 1 s, which implied a velocity of image-capturing of one photograph every 40 ms. Two to three separate fields were taken for each sample. The obtained sperm motility descriptors are described following [10]. Motility descriptors obtained after CASA analysis are:

Curvilinear velocity (VCL): The mean path velocity of the sperm head along its actual trajectory (units: μm/s).
Linear velocity (VSL): The mean path velocity of the sperm head along a straight line from its first to its last position (units: μm/s).
Mean velocity (VAP): The mean velocity of the sperm head along its average trajectory (units: μm/s).
Linearity coefficient (LIN): (VSL/VCL) × 100 (units: %).
Straightness coefficient (STR): (VSL/VAP) × 100 (units: %).
Wobble coefficient (WOB): (VAP/VCL) × 100 (units: %).
Mean amplitude of lateral head displacement (ALH): The mean value of the extreme side-to-side movement of the sperm head in each beat cycle (units: μm).
Frequency of head displacement (BCF): the frequency with which the actual sperm trajectory crosses the average path trajectory (units: Hz).

Finally, total motility was defined as the percentage of spermatozoa which showed a VAP above 10 μm/s.

2.4. Statistical analysis

Data were processed by using the SAS statistical package [23]. Normality of data distributions was assessed by the Shapiro–Wilks Test, which is included in the UNIVARIATE procedure. Afterwards, the FASTCLUS clustering procedure included in the SAS package was utilised to separate motile spermatozoa into specific subpopulations. The FASTCLUS procedure performs a disjointed cluster analysis based on Euclidean distances computed from one or more quantitative parameters. In this case, these variables are the different sperm motility parameters measured by the CASA system. Spermatozoa were divided into clusters such that every observation belonged to a single cluster. Sperm cells that shared similar motility characteristics were assigned to the same cluster, whereas spermatozoa that differed in motility characteristics were assigned to different clusters. A PROC GLM procedure was applied to evaluate significant differences ($P < 0.05$) and the LSMEANS procedure was applied to list these differences. Finally, a Chi-square procedure was applied to determine the subpopulational distribution percentage in every single experiment. Once the distribution percentage per experiment was determined, new PROC GLM and LSMEANS procedures were applied to determine and list, respectively, the differences among the different treatments. The total number of spermatozoa analysed following this protocol was 3744 in boar samples and 1673 in donkey analyses.

3. Results

3.1. Comparison between mean quality parameters of fresh boar and donkey semen and frozen/thawed

As expected, freezing/thawing induced great changes in the values of the mean quality parameters of both boar and donkey sperm. As shown in Table 1, frozen/thawed samples from both species showed a significant ($P < 0.05$) decrease in the percentages of viability and total motility, which was accompanied by a concomitant increase in the percentage of altered acrosomes. Furthermore, boar sperm underwent a significant ($P < 0.05$) decrease in the response to the ORT Test, which fell from 80.6% ± 2.9% in fresh samples to 34.8% ± 2.9% after thawing (Table 1). The response of the mean motility parameters of both boar and donkey spermatozoa to freezing/thawing was not similar, most probably due to the specific motility characteristics that both species showed. Thus, boar sperm from fresh, diluted ejaculates had an overall

Table 1
Mean values of the semen quality analysis of both boar and donkey sperm before and after freezing/thawing

	Boar		Donkey	
	Before freezing	After freezing	Before freezing	After freezing
Viability (%)	85.1 ± 1.2^{a}	51.9 ± 1.2^{b}	86.7 ± 1.0^{a}	49.3 ± 0.9^{b}
Altered acrosomes (%)	13.7 ± 0.4^{a}	47.2 ± 0.9^{b}	14.2 ± 0.4^{a}	51.8 ± 1.1^{b}
ORT Test (%)	80.6 ± 2.9^{a}	34.8 ± 2.9^{b}	N.D.	N.D.
Total motility (%)	75.9 ± 1.2^{a}	46.9 ± 0.9^{b}	83.6 ± 1.3^{a}	48.4 ± 1.0^{b}
VCL (μm/s)	38.8 ± 0.7^{a}	85.2 ± 2.8^{b}	93.9 ± 1.0^{a}	91.7 ± 1.7^{a}
VSL(μm/s)	21.9 ± 0.5^{a}	49.6 ± 2.2^{b}	40.3 ± 0.6^{a}	35.2 ± 1.1^{b}
VAP (μm/s)	28.7 ± 0.7^{a}	62.6 ± 2.7^{b}	58.5 ± 0.7^{a}	50.2 ± 1.7^{b}
LIN (%)	52.6 ± 0.5^{a}	53.0 ± 2.2^{a}	47.4 ± 0.6^{a}	46.2 ± 1.1^{a}
STR (%)	70.8 ± 0.5^{a}	73.8 ± 1.9^{a}	68.1 ± 0.7^{a}	68.7 ± 1.1^{a}
WOB (%)	70.8 ± 0.4^{a}	68.6 ± 1.8^{a}	64.4 ± 0.6^{a}	60.3 ± 0.9^{a}
Mean ALH(μm)	1.65 ± 0.02^{a}	2.98 ± 0.08^{b}	3.65 ± 0.06^{a}	3.72 ± 0.10^{a}
BCF (Hz)	5.8 ± 0.1^{a}	7.0 ± 0.3^{b}	12.2 ± 0.2^{a}	14.4 ± 0.4^{b}

The parameters shown here have been determined as explained in Section 2. Results are expressed as means ± S.E.M. of 15 (boar) and 8 (donkey) different experiments with a total number of analysed spermatozoa of 1981 (boar) and 770 (donkey). N.D. = not determined. Different superscript letters between rows in the same species indicates significant ($P < 0.05$) differences.

motility that was much less rapid, but more linear, than that from donkey samples (VAP: 28.7 μm/s ± 0.7 μm/s in boar vs. 58.5 μm/s ± 0.7 μm/s in donkey; LIN: 52.6% ± 0.5% in boar vs. 47.4% ± 0.6% in donkey; $P < 0.05$). Moreover, boar motile spermatozoa also showed a less intense sperm-head movement than donkey cells, as inferred when the mean values of mean ALH (1.65 ± 0.02 μm in boar vs. 3.65 ± 0.06 μm in donkey) and BCF (5.8 ± 0.1 Hz in boar vs. 12.2 ± 0.2 Hz in donkey cells, see Table 1) were compared. Freezing/thawing induced a great, significant ($P < 0.05$) increase in boar sperm in the mean values of VCL, VSL and VAP, whereas LIN, STR and WOB did not undergo significant changes. In contrast, donkey sperm underwent a slight, but significant ($P < 0.05$) decrease in VSL and VAP, which was accompanied by no modifications in VCL, LIN, STR and WOB values (Table 1). Additionally, both boar and donkey samples increased BCF values after freezing/thawing (boar BCF: from 5.8 ± 0.1 to 7.0 ± 0.3 Hz; donkey BCF: from 12.2 ± 0.2 to 14.4 ± 0.4 Hz, Table 1). Mean ALH was significantly increased in boar semen (from 1.65 ± 0.02 to 2.98 ± 0.08 μm in frozen/thawed samples), while the increase of this parameter after thawing on donkey samples was not significant (Table 1).

3.2. Motile sperm subpopulations structure in boar and donkey samples before freezing

Both boar and donkey samples had a similar, four-subpopulations structure when sperm motility was analysed through CASA. These results were similar to those published before [12,24,25]. In our experimental design, subpopulations were classified by taking VCL as the marker point in both species. Under this point of view, boar motile-sperm subpopulations are characterised as follows (see Table 2):

Subpopulation 1: This subpopulation showed the lowest values of VCL (25.5 ± 2.6 μm/s). Subpopulation 1 was defined by overall low values of velocity, based on the results of VCL, VSL and VAP, low values of linearity, as indicated by values of LIN and STR, and low values of oscillatory movement, as indicated by WOB, mean ALH and BCF values (Table 2). Subpopulation 1 was made up of the highest percentage of cells, since it included 53.9% ± 4.7% of all of motile sperm (Fig. 1A).

Subpopulation 2: This subpopulation was characterised by the second lowest values of VCL (43.9 ± 2.1 μm/s). Sperm included in Subpopulation 2 showed middle-to-high velocity, as indicated by VCL, VSL and VAP, high linearity, as indicated by LIN and STR, and high values of oscillatory movement, as indicated by WOB, mean ALH and BCF. The percentage of spermatozoa included in this subpopulation was 29.4% ± 3.5% of all motile sperm (Table 2, Fig. 1A).

Subpopulation 3: This subpopulation had high values of VCL (69.2 ± 2.6 μm/s). Subpopulation 3 was made up of sperm with high velocity and relatively high linearity, as indicated by VCL, VSL, VAP, LIN and STR. Moreover, sperm included in this subpopulation also had a relatively high oscillatory movement, as indicated by values of WOB, mean ALH and BCF. The cells included in this subpopula-

Table 2
Effects of freezing/thawing on motility parameters of the motile-sperm subpopulations determined in boar samples

	Mean values		Subpopulation 1		Subpopulation 2	
	Before freezing	After freezing	Before freezing	After freezing	Before freezing	After freezing
Total motility (%)	75.9 ± 1.2^{a}	46.9 ± 0.9^{b}				
VCL (μm/s)	38.8 ± 0.7^{a}	85.2 ± 2.8^{b}	25.5 ± 2.6^{a}	34.4 ± 5.3^{b}	43.9 ± 2.1^{a}	68.6 ± 7.3^{b}
VSL(μm/s)	21.9 ± 0.5^{a}	49.6 ± 2.2^{b}	9.8 ± 1.8^{a}	10.1 ± 3.3^{a}	28.0 ± 3.1^{a}	42.1 ± 6.8^{b}
VAP (μm/s)	28.7 ± 0.7^{a}	62.6 ± 2.7^{b}	15.2 ± 2.0^{a}	15.3 ± 3.4^{a}	35.1 ± 2.8^{a}	57.0 ± 4.9^{b}
LIN (%)	52.6 ± 0.5^{a}	53.0 ± 2.2^{a}	38.2 ± 5.7^{a}	27.6 ± 5.3^{b}	67.5 ± 5.3^{a}	65.4 ± 5.0^{a}
STR (%)	70.8 ± 0.5^{a}	73.8 ± 1.9^{a}	63.9 ± 5.2^{a}	62.8 ± 5.3^{a}	82.0 ± 4.4^{a}	76.9 ± 7.0^{a}
WOB (%)	70.8 ± 0.4^{a}	68.6 ± 1.8^{a}	61.4 ± 5.3^{a}	44.2 ± 4.3^{b}	83.9 ± 3.9^{a}	87.6 ± 6.4^{a}
Mean ALH(μm)	1.65 ± 0.02^{a}	2.98 ± 0.08^{b}	1.40 ± 0.03^{a}	1.62 ± 0.15^{a}	1.79 ± 0.06^{a}	2.44 ± 0.15^{b}
BCF (Hz)	5.8 ± 0.1^{a}	7.0 ± 0.3^{b}	4.5 ± 1.5^{a}	3.4 ± 1.3^{a}	7.0 ± 0.6^{a}	7.9 ± 0.9^{a}

	Subpopulation 3		Subpopulation 4	
	Before freezing	After freezing	Before freezing	After freezing
VCL (μm/s)	69.2 ± 2.6^{a}	80.2 ± 4.3^{b}	102.3 ± 6.6^{a}	122.6 ± 4.3^{b}
VSL(μm/s)	47.2 ± 2.3^{a}	51.1 ± 4.5^{a}	78.0 ± 4.9^{a}	104.5 ± 8.6^{b}
VAP (μm/s)	58.6 ± 3.2^{a}	70.2 ± 4.2^{b}	92.4 ± 4.0^{a}	115.8 ± 4.8^{b}
LIN (%)	73.0 ± 6.1^{a}	66.5 ± 3.8^{a}	79.3 ± 4.2^{a}	88.4 ± 4.8^{a}
STR (%)	84.3 ± 5.7^{a}	79.5 ± 6.1^{a}	87.6 ± 4.5^{a}	93.6 ± 3.5^{a}
WOB (%)	88.1 ± 3.0^{a}	91.1 ± 4.2^{a}	91.7 ± 4.3^{a}	95.9 ± 3.7^{a}
Mean ALH(μm)	2.31 ± 0.08^{a}	3.08 ± 0.14^{b}	2.71 ± 0.09^{a}	3.14 ± 0.16^{a}
BCF (Hz)	7.7 ± 1.6^{a}	8.7 ± 2.3^{a}	8.2 ± 1.6^{a}	8.6 ± 2.2^{a}

The motility parameters shown have been determined as explained in Section 2. Results are expressed as means ± S.E.M. of 15 different experiments, which implies the total number of analysed sperm for motility characteristics were of 1981 (before freezing) and 1763 (after freezing). Different superscript letters between rows in the same subpopulation or between the mean values indicates significant ($P < 0.05$) differences.

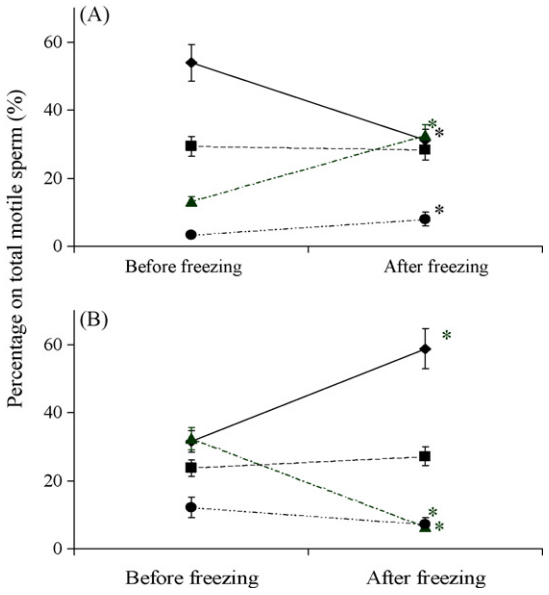

Fig. 1. Changes in the proportion of each motile sperm subpopulation in boar and donkey samples before and after freezing. The proportion of sperm in each subpopulation has been determined as described in Section 2. Results are means ± S.E.M. for 15 (boar) and 8 (donkey) different experiments. Asterisks indicate significant ($P < 0.05$) differences between samples before and after freezing. (A) Boar spermatozoa. (B) Donkey spermatozoa. Rhombuses: Subpopulation 1. Squares: Subpopulation 2. Triangles: Subpopulation 3. Circles: Subpopulation 4.

tion accounted for 13.3% ± 2.5% of all motile sperm (Table 2, Fig. 1A).

Subpopulation 4: Finally, this subpopulation included that sperm with the highest VCL (102.3 ± 6.6 μm/s). Cells included in Subpopulation 4 showed the highest velocity and linearity characteristics, as indicated by values of VCL, VSL, VAP, LIN and STR. Furthermore, the overall oscillatory movement of these spermatozoa was also very high, as indicated by WOB, mean ALH and BCF values. The percentage of motile sperm included in this subpopulation was the lowest, including only 3.4% ± 0.9% of the total motile-sperm subpopulation (Table 2, Fig. 1A).

When the motile sperm subpopulations structure of donkey samples was analysed, they also showed a structure that was composed of four separate subpopulations, with very specific motility characteristics (Table 3, Fig. 1B), namely:

Subpopulation 1: This subpopulation was composed of motile sperm with overall, relatively low velocity characteristics, high linear movement and both low BCF and mean ALH values. These spermatozoa comprised 31.5% ± 4.3% of the total motile-sperm subpopulation.

Table 3
Effects of freezing/thawing on motility parameters of the motile-sperm subpopulations determined in donkey samples

	Mean values		Subpopulation 1		Subpopulation 2	
	Before freezing	After freezing	Before freezing	After freezing	Before freezing	After freezing
Total motility (%)	83.6 ± 1.3[a]	48.4 ± 1.0[b]				
VCL (μm/s)	93.9 ± 1.0[a]	91.7 ± 1.7[a]	31.7 ± 1.7[a]	27.4 ± 1.5[a]	67.5 ± 2.0[a]	62.3 ± 2.2[a]
VSL(μm/s)	40.3 ± 0.6[a]	35.2 ± 1.1[b]	19.9 ± 1.1[a]	16.6 ± 0.9[a]	15.3 ± 1.2[a]	12.2 ± 1.4[a]
VAP (μm/s)	58.5 ± 0.7[a]	50.2 ± 1.7[b]	22.4 ± 1.2[a]	18.6 ± 1.0[a]	33.9 ± 1.3[a]	25.8 ± 1.5[b]
LIN (%)	47.4 ± 0.6[a]	46.2 ± 1.1[a]	63.2 ± 1.1[a]	61.6 ± 1.0[a]	22.8 ± 1.2[a]	19.5 ± 1.4[a]
STR (%)	68.1 ± 0.7[a]	68.7 ± 1.1[a]	87.7 ± 1.1[a]	87.5 ± 1.0[a]	46.2 ± 1.3[a]	45.5 ± 1.4[a]
WOB (%)	64.4 ± 0.6[a]	60.3 ± 0.9[a]	71.2 ± 0.9[a]	69.4 ± 0.8[a]	50.0 ± 1.1[a]	45.5 ± 1.4[b]
Mean ALH(μm)	3.65 ± 0.06[a]	3.72 ± 0.10[a]	1.37 ± 0.10[a]	1.17 ± 0.09[a]	3.59 ± 0.12[a]	3.11 ± 0.14[b]
BCF (Hz)	12.2 ± 0.2[a]	14.4 ± 0.4[b]	11.3 ± 0.4[a]	11.9 ± 0.3[a]	11.2 ± 0.4[a]	12.0 ± 0.5[a]

	Subpopulation 3		Subpopulation 4	
	Before freezing	After freezing	Before freezing	After freezing
VCL (μm/s)	111.8 ± 1.7[a]	92.3 ± 4.5[b]	164.6 ± 2.7[a]	184.7 ± 4.3[b]
VSL(μm/s)	90.9 ± 1.0[a]	87.3 ± 3.1[a]	34.9 ± 1.7[a]	36.7 ± 2.7[a]
VAP (μm/s)	95.4 ± 2.0[a]	87.3 ± 3.1[b]	82.4 ± 1.9[a]	78.9 ± 3.0[a]
LIN (%)	81.8 ± 1.0[a]	94.4 ± 3.1[b]	21.6 ± 1.7[a]	20.0 ± 2.7[a]
STR (%)	85.8 ± 0.9[a]	85.4 ± 2.5[a]	43.5 ± 1.8[a]	44.2 ± 2.8[a]
WOB (%)	85.8 ± 0.9[a]	96.4 ± 2.5[b]	50.8 ± 1.5[a]	44.2 ± 2.4[a]
mean ALH(μm)	2.14 ± 0.10[a]	1.94 ± 0.28[a]	7.43 ± 0.17[a]	8.67 ± 0.27[b]
BCF (Hz)	13.1 ± 0.6[a]	17.0 ± 1.0[b]	13.4 ± 0.4[a]	12.7 ± 1.0[a]

The motility parameters shown have been determined as explained in Section 2. Results are expressed as means ± S.E.M. of 8 different experiments, which implies the total number of analysed sperm for motility characteristics were of 770 (before freezing) and 903 (after freezing). Different superscript letters between rows in the same subpopulation or between the mean values indicates significant ($P < 0.05$) differences.

Subpopulation 2: This subpopulation was composed of motile sperm with overall, relatively low velocity characteristics, but with much lower linear movement characteristics than that showed by Subpopulation 1.Furthermore, BCF was similar to Subpopulation 1, whereas mean ALH was relatively high. These spermatozoa comprised 23.8% ± 2.9% of the total motile sperm subpopulation.

Subpopulation 3: Motile sperm with high velocity characteristics combined with a very high linearity. This was combined with high values of BCF and mild mean ALH characteristics. This subpopulation contained 32.4% ± 3.2% of the total motile-sperm subpopulation.

Subpopulation 4: Spermatozoa with high velocity and low linearity. Moreover, both BCF and specially mean ALH were very high. Spermatozoa included in this subpopulation were 12.2% ± 2.5% of the total motile-sperm subpopulation.

3.3. Effects of freezing/thawing on the mean motile sperm subpopulations structure of boar and donkey semen samples

Freezing/thawing of boar sperm from fresh ejaculates induced important changes in the mean motion parameters of motile sperm included in each specific subpopulation. However, it is worth noting that the effects of freezing/thawing were different on each subpopulation. Furthermore, the combined, observed changes in each subpopulation by themselves did not entirely explain the observed changes in the mean overall values of motility parameters after thawing. Thus, whereas VCL significantly ($P < 0.05$) increased in all subpopulations after thawing, only VSL from Subpopulation 2 (from 28.0 ± 3.1 to 42.1 ± 6.8 μm/s after thawing) and Subpopulation 4 (from 78.0 ± 4.9 to 104.5 ± 8.6 μm/s after thawing, see Table 2) experienced a similar increase. Thawing induced an increase in VAP parameters in only Subpopulation 2 (from 35.1 ± 2.8 to 57.0 ± 4.9 μm/s after thawing), Subpopulation 3 (from 58.6 ± 3.2 to 70.2 ± 4.2 μm/s after thawing) and Subpopulation 4 (from 92.4 ± 4.0 to 115.8 ± 4.8 μm/s after thawing, see Table 2). A similar increase was also observed in mean ALH after thawing in Subpopulation 2 (from 1.79 ± 0.06 to 2.44 ± 0.15 μm after thawing) and Subpopulation 3 (from 2.31 ± 0.08 to 3.08 ± 0.14 μm after thawing). On the other hand, freezing/thawing induced a significant ($P < 0.05$) decrease in the LIN and WOB of Subpopulation 1 (from 61.4% ± 5.3% to 44.2% ± 4.3% after thawing, see Table 2).

As in boar, the effects of freezing/thawing on motility characteristics of each motile-sperm subpopulation in fresh donkey samples was different and specific for each subpopulation and, on the whole, these changes did not entirely explain the observed changes in the mean overall values of motility parameters after thawing. However, the effects of freezing/thawing of motile descriptors of donkey-sperm motile subpopulations were different from those observed in boar samples. Thus, as shown in Table 3, freezing/thawing did not significantly change any motility parameter of Subpopulation 1, whereas Subpopulation 2 underwent a significant ($P < 0.05$) decrease of VAP, WOB and mean ALH, with Subpopulation 3 having a significant ($P < 0.05$) decrease of VCL and VAP that was concomitant to a significant ($P < 0.05$) increase of LIN, WOB and BCF. Finally, Subpopulation 4 had a significant ($P < 0.05$) increase of VCL (from 164.6 ± 2.7 to 184.7 ± 4.3 μm/s after thawing) and mean ALH (from 7.43 ± 0.17 to 8.67 ± 0.27 μm after thawing, see Table 3).

On the other hand, the percentage of motile sperm included in each subpopulation experienced important changes after freezing/thawing in both boar and donkey samples. Thus, as shown in Fig. 1A, freezing/thawing induced a significant ($P < 0.05$) decrease in the percentage of motile sperm included in Subpopulation 1 in boar samples (from $53.9\% \pm 4.7\%$ to $31.2\% \pm 3.9\%$ after thawing). This decrease was compensated for by concomitant, significant ($P < 0.05$) increases in the percentages of sperm included in Subpopulations 3 (from $13.3\% \pm 2.5\%$ to $32.6\% \pm 3.9\%$ after thawing) and 4 (from $3.4\% \pm 0.9\%$ to $8.0\% \pm 1.1\%$ after thawing). Strikingly, an inverse effect was observed in donkey semen. In these samples, freezing/thawing induced a significant ($P < 0.05$) increase in sperm included in Subpopulation 1 (from $31.5\% \pm 4.3\%$ to $58.8\% \pm 4.9\%$ after thawing) that was concomitant with significant ($P < 0.05$) decreases in the percentages of both Subpopulation 3 (from $32.4\% \pm 3.2\%$ to $6.6\% \pm 1.8\%$ after thawing) and Subpopulation 4 (from $12.2\% \pm 2.5\%$ to $7.3\% \pm 1.9\%$ after thawing, see Fig. 1B).

4. Discussion

Our results clearly establish that motility changes induced by the freezing/thawing protocol are linked to concomitant changes in both the specific parameters and the specific percentage of each of the motile sperm subpopulations present in both boar and donkey ejaculates. Furthermore, it is noteworthy that these changes did not affect the general motile-sperm structure present in both boar and donkey. This indicates that in both species the maintenance of an overall subpopulation structure could be important in order to maintain the general function of the ejaculate, regardless of the specific functional status. These results pointed to a specific, important role for the maintenance of a specific subpopulational structure in mammalian ejaculates, regardless of the species in which the studies were carried out. In this way, the existence of an specific subpopulational structure has been described in very different mammals like boar [8,15,24,25], horse, [10], donkey [12], dog [9], red deer [11], gazelle [8], golden hamster [7] and rabbit [13], indicating that it is a common feature of all mammalian ejaculates. More importantly, recent studies from our laboratory indicate that motility changes associated with processes like ''in vitro'' capacitation in boar semen [26] do not modify the overall subpopulational structure of these samples, the mean motility changes linked to these processes being induced mainly to concomitant changes in the percentage of motile sperm included in each specific subpopulation. Thus, the maintenance of this specific subpopulational structure would be important in the maintenance of the overall semen function in mammals.

It is noteworthy that, whereas freezing/thawing causes a significant increase in the motility characteristics of boar sperm, the same process did not exert a similar increase on donkey cells. In fact, this effect has already been described in both species [5,20], and it could be linked to two attendant causes. The first, the great differences observed in the physiological and metabolic characteristics of boar and donkey spermatozoa. Thus, boar sperm from fresh ejaculates shows very low mitochondrial activity together with a very high glycolytic rate [27]. These metabolic characteristics are linked to a mean, low motility pattern when compared with other species, such as dog and equine [9,10]. On the other hand, donkey sperm from fresh samples shows a glycolytic rate that was lower than that observed in boar sperm [12]. This would be linked to high mitochondrial activity in these cells that, in turn, could be at the basis of the very high velocity characteristics observed in fresh donkey semen [12,20]. These differences in the physiological and metabolic characteristics of boar and donkey sperm could lead to different responses in each species when faced with a very stressing procedure such as freezing/thawing. In this way, whereas boar sperm would respond to freezing/thawing by inducing the already-described cryocapacitation phenomenon [1–4,6], donkey cells would respond to a relative impairment of their

motion characteristics related to a different sensitivity in response to the stress linked to freezing/thawing.

Nevertheless, although the specific characteristics are important in explaining the different behaviours of boar and donkey sperm in front of freezing/thawing, there are surely other factors implied in these differences. One of these factors would be the initial extender utilised in the first stages of the freezing/thawing process. Thus, in our procedure, whereas donkey semen was initially utilised without any previous storage step, boar samples were previously diluted in a commercial extender for storage at 16–17 °C for a time lapse of never less than 4 h. This can be important, since practically all commercial extenders for boar semen have, as a constitutive element, some substances intended for decreasing sperm motility, such as ethylenediaminotetraacetic acid (EDTA) salts. EDTA is a strong calcium chelant, thus interfering with the biochemical processes linked to the maintenance of sperm flagellar movement [28]. In this way, boar sperm diluted in these media has motion characteristics that are lower than those observed in undiluted samples. However, in our working conditions, the utilisation of undiluted boar-semen samples was impossible without producing considerable stress on cells, thus compelling the utilisation of previously diluted ejaculates. All of these points explain why the motility of boar samples prior to freezing/thawing was low. The first steps of the freezing/thawing protocol include a centrifugation/washing phase, in which boar sperm is separated from its diluting medium. During this phase, EDTA is eliminated, in this way allowing boar sperm to recover a fully functional flagellar movement. Thus, the separation of boar sperm from the initial commercial extender could have an activating effect on sperm movement, which could be one of the reasons for the increase in overall motility in frozen/thawed boar sperm.

The maintenance of the overall, four-subpopulations structure in both boar and donkey ejaculates after freezing/thawing, despite the very different response to the process in both species, was linked to symmetrical changes in the specific percentage of each subpopulation. Hence, the overall increase in mean motility of frozen/thawed boar sperm was linked to a decrease in the percentage of sperm in Subpopulation 1 and a concomitant increase of sperm from Subpopulations 3 and 4. On the contrary, changes in mean motility of frozen/thawed donkey sperm were linked to an increase in the percentage of sperm in Subpopulation 1 and a concomitant decrease of sperm from Subpopulations 3 and 4. This suggests that the specific response to freezing/thawing of an ejaculate could depend on the concrete relationship among the percentages of sperm included in Subpopulations 1, 3 and 4. However, we have to stress that subpopulations with the same numerical identification would not necessarily have the same functional and/or physiological meaning in boar and in donkey semen (i.e., Subpopulation 1 of boar semen is not necessarily equivalent to Subpopulation 1 in donkey samples). Concomitantly, a subpopulation designed with the same number does not necessarily to be equivalent between fresh and frozen/thawed samples from a concrete species (i.e., Subpopulation 1 of boar fresh semen does not necessarily coincide with Subpopulation 1 in frozen/thawed samples). Taking into account all of these precautions, at this moment we cannot specify what the precise mechanism/s is/are related with this assert. In this way, further experiments regarding ejaculates with different sensitivities to freezing/thawing are in progress in order to evaluate this assumption.

In conclusion, our results seem to establish that the motility changes induced by the freezing/thawing protocol are linked to concomitant changes in both the specific parameters and the specific percentage of each of the motile sperm subpopulations present in mammalian species with very different sperm motility characteristics, such as boar and donkey. Moreover, these changes did not affect the general motile-sperm structure present in both boar and donkey, which is conserved despite the detrimental effect caused by freezing/thawing in both species. All of these results suggest, then, that a specific, motile sperm subpopulations structure is a common feature in mammalian ejaculates, which seems to play some role in the maintenance of the overall function of the whole mammalian ejaculates.

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