Animal Reproduction Science xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

The percentage of spermatozoa lost during the centrifugation of brown bear (*Ursus arctos*) ejaculates is associated with some spermatozoa quality and seminal plasma characteristics

Q1 M. Alvarez[a,b], M. Nicolas[a,b], S. Borragán[c], E. Lopez-Urueña[a,b], L. Anel-López[a,b], F. Martinez-Pastor[a,d], J. Tamayo-Canul[a,b], L. Anel[a,b], P. de Paz[a,d,*]

[a] ITRA-ULE, INDEGSAL, University of León, 24071 León, Spain
[b] Animal Reproduction and Obstetrics, University of León, 24071 León, Spain
[c] Cabárceno Park, Cantabria, Spain
[d] Cell Biology, University of León, 24071 León, Spain

ARTICLE INFO

Article history:
Received 25 June 2012
Received in revised form
10 September 2012
Accepted 12 September 2012
Available online xxx

Keywords:
Centrifugation
Brown bear
Semen quality
Seminal plasma
Sperm recovery

ABSTRACT

Cryopreservation of brown bear (*Ursus arctos*) semen requires centrifugation to increase concentration and/or remove urine contamination. However, a percentage of the spermatozoa are lost in the process. This percentage varies considerably between males and ejaculates, and we have studied the effect of sperm quality and seminal plasma characteristics on the spermatozoa recovery rate after centrifugation. One hundred and thirty one sperm samples obtained from fifteen brown bear males by electroejaculation under general anaesthesia were used. The ejaculates were centrifuged 600 × *g* for 6 min. Motility was assessed by CASA, and acrosomal status (PNA-FITC) and viability (SYBR-14/propidium iodide) were determined by flow cytometry. Seminal plasma characteristics (albumin, alkaline phosphatase, calcium, cholesterol, creatine, glucose, glutamic oxaloacetic transaminase (GOT), lactate, lipase, magnesium, phosphate and total protein) were determined by a biochemical and gas analysis. Total motility ($r = 0.26$; $P = 0.005$) and cell viability ($r = 0.20$; $P = 0.033$) were positively correlated with the percentage of recovered spermatozoa. Sperm recovery was correlated with the concentration of several components of seminal plasma: negatively with glucose concentration ($r = -0.47$; $P = 0.005$) and positively with the enzymes GOT ($r = 0.36$; $P = 0.040$) and lactate dehydrogenase ($r = 0.36$; $P = 0.041$). After sorting the data into classes according to sperm recovery (Low: 0–39, Medium: 40–69, High: 70–100), we observed that the samples with a lower recovery rate derived from ejaculates with lower values for TM, VAP and viability ($P < 0.05$). Multiple regression analysis rendered two models to define the post-centrifugation spermatozoa recovery which included total motility and damaged acrosome or glucose, GOT and lactate dehydrogenase. We discuss these relationships and their implications in the electroejaculation procedure and the handling of the sample during centrifugation.

1. Introduction

The Cantabrian brown bear (*Ursus arctos*) is the last autochthonous bear population in the Iberian Peninsula, and may constitute the last pure breed of brown bear in the world (García et al., 2007). This population is considered at risk of extinction by Spanish law (Real Decreto

* Corresponding author at: Department of Molecular Biology, University of León, 24071 León, Spain. Tel.: +34 9872911320; fax: +34 9872911322.
E-mail address: ppazc@unileon.es (P. de Paz).

0378-4320/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.anireprosci.2012.09.009

439/1990, regulation of the National Catalogue of Endangered Species). Conservation plans for this population include developing artificial reproductive techniques for the brown bear (Anel et al., 2008). Our group has conducted several studies to obtain a specific protocol for freezing bear semen, including: (1) evaluating the effects of cryopreservation on the morphometry of spermatozoa (Álvarez et al., 2008), (2) developing specific semen extenders (Anel et al., 2010; Álvarez-Rodríguez et al., 2011; de Paz et al., 2012), (3) validating commercial extenders in epididymal sperm freezing (Anel et al., 2011), (4) testing different extenders and dilution ratios during centrifugation (Nicolás et al., 2011), (5) analysing different g forces and times in centrifugation methods (Nicolás et al., 2012a), and (6) selecting spermatozoa by gradient centrifugation (Nicolás et al., 2012b).

In semen cryopreservation in many species, centrifugation of ejaculates is necessary to remove seminal fluids, increase sperm concentration and recover urine-contaminated samples. In some species, it is known that electroejaculation yields semen with a low spermatozoa concentration, and this is a great problem in carnivores (Ishikawa et al., 2002). Our previous experience has shown that a large percentage of brown bear ejaculates do not achieve a suitable concentration to be cryopreserved ($423.0 \pm 53.7 \times 10^6$ spermatozoa/mL (Anel et al., 2010); $256.3 \pm 98.3 \times 10^6$ spermatozoa/mL (Nicolás et al., 2011); $186.7 \pm 79.4 \times 10^6$ spermatozoa/mL (Nicolás et al., 2012a)). Physiological contamination of ejaculates by urine occurs frequently in many species such as horse (Althouse et al., 1989). In bears, ejaculates collected by electroejaculation frequently show contamination by urine (Anel et al., 2008; Kojima et al., 2001).

Damage caused by sperm handling procedures is accumulative, and small successive injuries may result in a reduction in sperm quality that could negatively impact fertility. Centrifugation is an inefficient process because a percentage of spermatozoa are lost when the supernatant is discarded. We observed high variability between males and ejaculates in the percentage of spermatozoa recovery.

The aim of this work was to evaluate the relationship between some factors of semen sampling (ejaculate characteristics, sampling period, spermatozoa quality and seminal plasma characteristics) and the percentage of spermatozoa recovered after centrifugation, evaluating its ability to predict the efficiency of centrifugation and enable optimized handling of seminal samples.

2. Materials and methods

Chemicals were acquired from Sigma (Madrid, Spain), unless otherwise specified. Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Directive 2003/65/EC.

2.1. Animals and sample collection

131 sperm samples from 15 sexually mature male brown bears, obtained by electroejaculation during the period March to July and October (from 2003 to 2010), were analysed. From these samples, 30 samples of seminal plasma from 13 male brown bears (2 or 3 samples per male), obtained in the breeding season (April–June), were used to analyse seminal plasma. The animals were housed in a half-freedom regime in Cabarceno Park (Cantabria, Spain; 43°21′N, 3°50′W; altitude: 143 m), and fed with a diet based on chicken meat, bread and fruits.

The animals were immobilized by teleanaesthesia with 750 mg of zolazepam HCl + tiletamine HCl (Zoletil 100®; Virbac, Carros, France, 100 mg/mL) and 6 mg of medetomidine (Zalopin®, Orion Pharma Animal Health, Finland, 10 mg/mL) and monitored during the time of anaesthesia (pulse, saturation of peripheral oxygen and respiration). The pubic region was cleaned, the prepuce and penis were washed with sterile saline and the rectum emptied of faeces. Electroejaculation was carried out using a PT Electronics electroejaculator (Boring, OR, USA). The transrectal probe was 320 mm long, with a diameter of 26 mm. Electric stimuli were given at intervals of 3 s of shock and three of rest, until ejaculation (10 V and 250 mA), in average, following to (García-Macías et al., 2006). The bladder was catheterized during semen collection to prevent urine contamination. The ejaculates were collected in graduated glass tubes. A second electroejaculation was performed 20 min after the first. All electroejaculation and sample collection procedures were performed by the same experienced operators, in order to minimize any effects on the composition of the collected samples.

To prevent urine contamination, the ejaculates were collected as isolated fractions in 15 mL graduated glass tubes. Immediately after collection, the volume of each fraction was recorded, osmolality was measured using a cryoscopic osmometer Osmomat-030 (Gonotec™, Berlin, Germany) and the pH value was determined by a CG 837 pH meter (Schott Instruments, Main, Germany). Sperm concentration (spz $\times 10^6$/mL) was assessed using a Bürker haemocytometer (Marienfeld GmbH, Marienfeld, Germany) and CASA (ISAS, Integrated Semen Analyser System; Proiser, Valencia, Spain). For each fraction, urospermia was evaluated using a Merckgnost Urea Rapid Screening test (Merck, Barcelona, Spain). Urine-contaminated fractions (>80 mg urea/dL) were rejected. All valid fractions of the same electroejaculation were mixed and constituted one ejaculate.

2.2. Semen handling and evaluation

Fresh semen samples were centrifuged at $600 \times g$ for 6 min. After centrifugation, the supernatant was removed and the pellet processed for experimental design or banking. Spermatozoa quality was assessed before and after the centrifugation procedure.

The recovery rate (recovered spermatozoa in the pellet/spermatozoa in the pre-centrifugation sample, %) was calculated. These rates were classified as follows: Low (0–39%), Medium (40–69%) and High (70–100%).

The motility and kinematic parameters were evaluated using the CASA system. The samples were diluted ($10–20 \times 10^6$ cells/mL) in a buffer (HEPES 20 mmol/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose; pH 7; 300 mOsm/kg) with 1% egg yolk, and warmed on a

Please cite this article in press as: Alvarez, M., et al., The percentage of spermatozoa lost during the centrifugation of brown bear (*Ursus arctos*) ejaculates is associated with some spermatozoa quality and seminal plasma characteristics. Anim. Reprod. Sci. (2012), http://dx.doi.org/10.1016/j.anireprosci.2012.09.009

Table 1
Parameters of semen, spermatozoa and seminal plasma of brown bear ejaculates (mean, SEM, minimum and maximum).

	Mean	SEM	Minimum	Maximum
Semen				
Volume (mL)	2.1	0.1	0.7	5.8
Concentration (spz × 10^6/mL)	228.9	24.9	4.6	1275.6
pH	8.2	0.3	7.3	8.8
Osmolality (mOsm/kg)	294.4	2.6	210.0	376.0
Urea (mg/dL)	53.6	2.2	36.1	77.8
Spermatozoa				
Recovery rate	58.3	24.4	6.6	99.3
TM (%)	74.1	1.7	18.9	97.2
PM (%)	42.7	1.8	2.8	82.1
VAP (μm/s)	99.8	2.7	39.9	154.6
dACRO(%)	3.7	0.5	0.0	24.8
VIAB (%)	74.0	13.7	22.3	93.1
Seminal plasma				
Albumin (g/dL)	0.1	0.1	0.0	0.4
Alkaline phosphatase (U/L)	1093.7	169.6	145.0	2931.5
Calcium (mmol/L)	0.4	0.2	0.1	0.9
Cholesterol (mmol/L)	0.1	0.1	0.0	0.3
Creatine (mg/dL)	3.3	6.1	0.3	29.4
Glucose (mg/dL)	15.1	19.8	0.2	88.0
GOT (U/L)	93.7	64.4	22.8	266.7
Lactate dehydrogenase (mmol/L)	5.0	1.6	2.1	8.1
Lipase (U/L)	6.1	2.9	1.2	11.9
Magnesium (mg/dL)	2.0	1.9	0.3	8.3
Potassium (mg/dL)	3.6	2.4	0.8	12.1
Total protein (g/dL)	0.2	0.2	0.0	0.7

TM, total motility; PM, progressive motility; dACRO, spermatozoa with damaged acrosomes (PNA+); VIAB, viable spermatozoa (SYBR+/PI−); GOT, glutamic oxaloacetic transaminase.

37.5 °C plate for 5 min. A 5-μL sperm sample was then placed in a Makler cell counting chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel) and examined using a negative phase contrast microscope (10×) with a warmed stage (38 °C). The standard settings of the CASA used correspond to the values of dog spermatozoa defined by ISAS. The settings used to define progressive motility are specific to bears and are defined by our previous experience (Anel et al., 2010). The settings were as follows: 25 frames/s; 5–80 μm^2 for head area; curvilinear velocity >10 μm/s to classify a spermatozoon as motile. At least 5 fields or 200 spermatozoa were saved and analysed afterwards. Reported parameters were total motility (TM, %), progressive motility (PM, %; spermatozoa were considered progressive if VCL > 25 and STR > 80) and average path velocity (VAP, μm/s).

Sperm viability was evaluated using the double stain SYBR-14 with propidium iodide (PI) (LIVE/DEAD Sperm Viability Kit; Invitrogen, Barcelona, Spain). The sperm samples were diluted with PBS down to 5 × 10^6 sperms/mL, and 300 μL were transferred to a polypropylene tube to which we added 3 μL PI (3 mg/mL in water) and 1.5 μL SYBR-14 (1 mM in DMSO). The tubes were kept at 37 °C for 20 min in the dark. We detected three populations corresponding to viable spermatozoa (green), moribund spermatozoa (red + green) and dead spermatozoa (red). We recorded the percentage of viable spermatozoa (VIAB).

To evaluate the sperm acrosomes, we used the double stain PI/PNA-FITC. The sperm samples were diluted in PBS (5 × 10^6 sperms/mL), and 300 μL were transferred to a polypropylene tube, adding 2.5 μL PI (1 mg/mL in water) and 2.5 μL PNA-FITC (0.2 mg/mL in water). We obtained the percentage of spermatozoa with damaged acrosomes (dACRO) as those green-stained.

For flow cytometry evaluation (viability and acrosomal status) we used a FACScalibur flow cytometer (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA), equipped with an argon ion laser (488 nm). We used the FL3 photodetector channel to read the red emission light of PI (650 long pass filter), and the FL1 photodetector channel to read the green emission light of FITC (530/30 band pass filter). In all cases, we assessed 10,000 events per sample with a flow rate of 200 cells/s.

2.3. Seminal plasma

Seminal plasma was obtained from the supernatant of the centrifuged samples, and was centrifuged again at 12,000 × *g* for 6 min to remove the remaining cells and debris. The seminal plasma sample was stored at −80 °C until analysis.

Biochemical analysis of plasma was performed using Cobas Integra 400 (Roche Diagnostic, Mannheim, Germany) with Precinorm U (176136-05) and Precipath U (150412-06) controls. The components assessed were albumin (g/dL), alkaline phosphatase (U/L), calcium (mmol/L), cholesterol (mmol/L), creatine (mg/dL), glucose (mg/dL), glutamic oxaloacetic transaminase (GOT, U/L), lactate dehydrogenase (mmol/L), lipase (U/L), magnesium (mg/dL), potassium (mg/dL) and total protein (mg/dL).

2.4. Statistical analysis

The results are shown as means and standard errors (SEM). Statistical analyses were performed with the

Please cite this article in press as: Alvarez, M., et al., The percentage of spermatozoa lost during the centrifugation of brown bear (*Ursus arctos*) ejaculates is associated with some spermatozoa quality and seminal plasma characteristics. Anim. Reprod. Sci. (2012), http://dx.doi.org/10.1016/j.anireprosci.2012.09.009

SAS/STAT™ package, Version 9.1 (SAS Institute Inc., Cary, NC, USA). Variables measured as percentages (spermatozoa quality) were normalized by transformation to angles corresponding to arc sine of the square root of percentage. The log 10 transformation was used for some plasma seminal parameters not normally distributed.

Differences between classes of sperm recovery rate (Low, Medium, and High) and differences between treatments (spermatozoa concentration in ejaculate, volume of ejaculate, month) were analysed using linear mixed-effects models (MIXED procedure) considering male and ejaculate as random effects. The Tukey test was used to separate main-effect means when treatment of F ratios were significant ($P < 0.05$).

The relationship between the sperm recovery rate and the other qualitative variables of semen (spermatozoa quality and seminal plasma characteristics) was assessed using the CORR procedure (Pearson correlation coefficients).

A linear regression model (REG procedure) was used to evaluate the degree of importance of each variable in explaining variations in the recovery rate, and the use of these variables to obtain models to predict the spermatozoa recovery rate after centrifugation. The recovery rate was assumed to follow the normal distribution and multiple linear regression models were then fit to evaluate the efficacy of combinations of variables in explaining variations in the recovery rate.

3. Results

Mean (±SEM), minimum and maximum values of the parameters analysed are shown in Table 1. There was a high variability between males regarding the sperm recovery rate after centrifugation, and this variability was also detected in each seminal collection from each bear (Fig. 1). The mean sperm recovery rate was 58.3 ± 24.4%, distributed heterogeneously.

There were no significant differences among the sperm recovery rate of consecutive electroejaculations (first: 61.5 ± 2.8% vs. second: 55.1 ± 3.2%). The recovery rate was not affected by ejaculate volume (52.3 ± 7.0%, 59.0 ± 3.1% and 59.1 ± 3.2% for samples with volume <1 mL, between 1 and 2 mL or >2 mL, respectively), sperm concentration (Fig. 2), or month of sampling (Fig. 3).

The Low recovery rate group (RR) showed lower TM values than the Medium ($P = 0.021$) and High ones ($P = 0.017$) (Table 2). Also, the Low RR group showed lower viability (Medium: $P = 0.023$; High: $P = 0.023$) and a higher percentage of damaged acrosome cells (Medium: $P < 0.001$; High: $P < 0.001$).

The sperm recovery rate was positively correlated with total motility ($r = 0.26$; $P = 0.005$) and sperm viability ($r = 0.20$; $P = 0.033$) and negatively correlated with the percentage of damaged acrosome cells ($r = -0.28$; $P = 0.001$). Moreover, several components of seminal plasma were significantly correlated with the percentage of sperm recovery. Glucose concentration was negatively correlated with the sperm recovery rate ($r = -0.47$; $P = 0.005$), whereas the activity of glutamic oxaloacetic transaminase ($r = 0.36$; $P = 0.040$) and lactate dehydrogenase ($r = 0.36$; $P = 0.041$) were positively correlated with the sperm recovery rate.

Multiple linear regressions showed the prediction equations of the spermatozoa recovery rate for both the spermatozoa quality parameters and the seminal plasma parameters (Table 3). A diagnostic of the fitting of linear models is illustrated in Fig. 4 which represents a plot of residual values against predicted value and allows a diagnostic graphic for the presence of graphic outliers and other influential values. The equation for the seminal plasma parameters best explains the spermatozoa recovery rate observed (R-square: 0.39).

4. Discussion

Brow bear ejaculates often yield an acceptable volume but with low spermatozoa concentrations (Nicolás et al., 2012a,b). This concentration is a problem during semen freezing in order to achieve a final sperm concentration of 100×10^6 spermatozoa/mL after two 1:1 dilutions; to obtain this result, the fresh sample should provide a minimum concentration of 400×10^6 spermatozoa/mL. Moreover, the need to remove the seminal plasma or solve the problem of urospermia requires the centrifugation of ejaculates in order to recover spermatozoa.

In Cantabric brown bear, an endangered population, "poor" or "bad" ejaculates (low concentration, urine-contaminated, etc.) cannot be discarded, and maximum recovery should be achieved after centrifugation.

The centrifugation yield depends on the characteristics of each species ejaculate. Centrifugation of viscous semen (primates and camelids) yielded a low quantity of spermatozoa, requiring a previous treatment to liquefy the sample (Giuliano et al., 2008). Higher sperm recovery (90–95%) has been reported in species with less viscous semen (boar: Carvajal et al., 2004; Matás et al., 2007; dog: Rijsselaere et al., 2002; stallion: Hoogewijs et al., 2010). These results contrast with recovery rates obtained in the present study. Although some samples had a 90% recovery rate after centrifugation, at most there is a remarkable reduction in these rates of up to 60%.

In previous studies (Nicolás et al., 2011, 2012a), we noticed that the post-centrifugation yield could be influenced by many factors, such as male or semen quality. Rijsselaere et al. (2002), studying the centrifugation of fresh diluted canine spermatozoa at $720 \times g$, reported a high variation in sperm concentration in the supernatant, as shown by the standard deviation. These authors observed that the percentage of lost spermatozoa upon supernatant removal declines with increasing initial sperm concentration. The effect of individual factors that strongly influence the post-centrifugation yield (Rijsselaere et al., 2002) could be the principal cause of the high variation in sperm recovery rate in our study but other factors related to the procedure used may also influence. In fact, we may consider not only between-male variability but also within-male variability. The ultimate cause of this high variability might be the interaction among individual characteristics, season and management, possibly through seminal plasma composition and sperm quality.

Please cite this article in press as: Alvarez, M., et al., The percentage of spermatozoa lost during the centrifugation of brown bear (*Ursus arctos*) ejaculates is associated with some spermatozoa quality and seminal plasma characteristics. Anim. Reprod. Sci. (2012), http://dx.doi.org/10.1016/j.anireprosci.2012.09.009

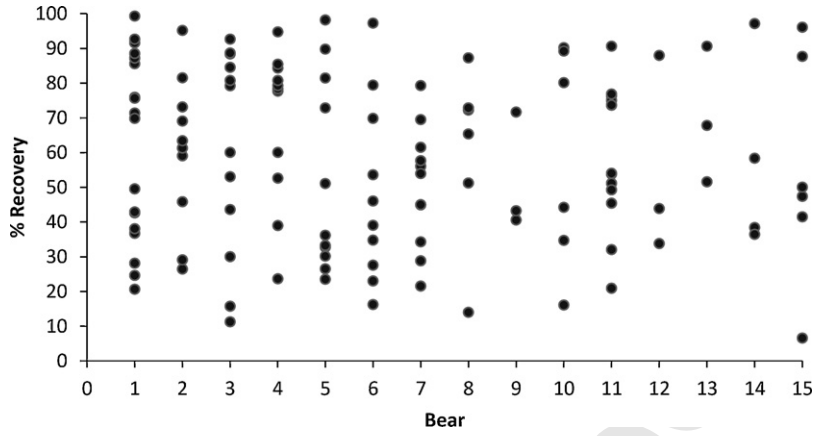

Fig. 1. Sperm recovery rate after centrifugation (recovered spermatozoa in the pellet/spermatozoa in the pre-centrifugation sample, %) of each electroejaculation from each brown bear.

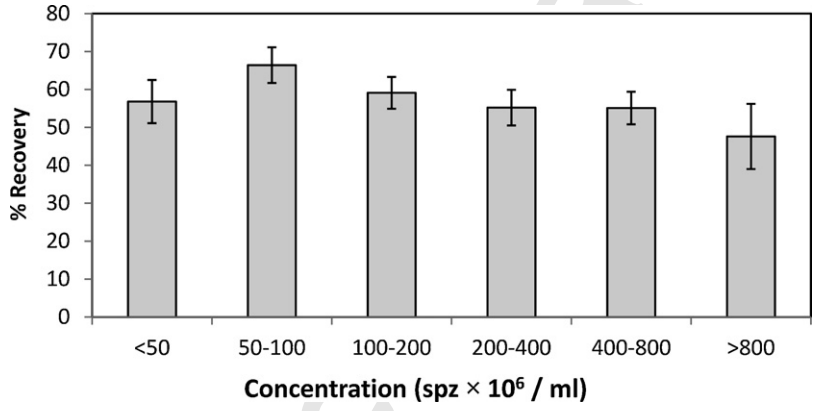

Fig. 2. Effect of spermatozoa concentration in brown bear ejaculate on percentage of sperm recovery rate (recovered spermatozoa in the pellet/spermatozoa in pre-centrifugation sample, %) (mean ± SEM).

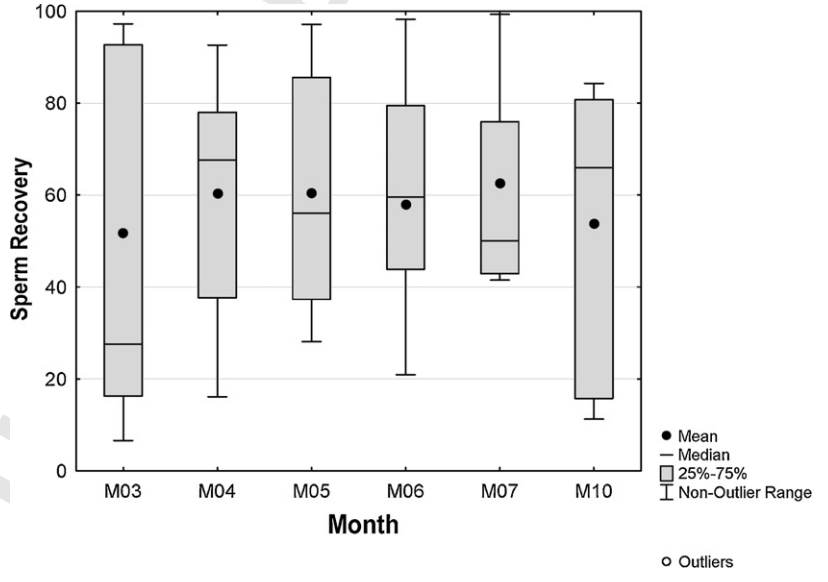

Fig. 3. Boxplot of the sperm recovery rate (recovered spermatozoa in the pellet/spermatozoa in pre-centrifugation sample, %) after centrifugation of brown bear ejaculate for each month of sampling (M03: March, M04: April, M05: May, M06: June, M07: July, M10: October).

Please cite this article in press as: Alvarez, M., et al., The percentage of spermatozoa lost during the centrifugation of brown bear (*Ursus arctos*) ejaculates is associated with some spermatozoa quality and seminal plasma characteristics. Anim. Reprod. Sci. (2012), http://dx.doi.org/10.1016/j.anireprosci.2012.09.009

Table 2
Values (mean ± SEM) of motility, acrosome status and viability of brown bear spermatozoa in three classes of recovery rate (Low, Medium and High) observed before and after centrifugation.

Class	TM	PM	VAP	LIN	dACRO	VIAB	*n*
Pre-centrifugation							
Low	66.8 ± 3.9[a]	38.5 ± 3.3	95.1 ± 5.2[a]	50.7 ± 1.4	6.9 ± 1.4[a]	71.2 ± 2.4[a]	36
Medium	76.8 ± 2.7[b]	44.1 ± 2.9	106.7 ± 4.3[b]	51.1 ± 1.6	2.6 ± 0.4[b]	77.4 ± 1.1[b]	42
High	76.5 ± 2.2[b]	45.3 ± 2.5	97.6 ± 3.7[ab]	52.1 ± 1.2	2.5 ± 0.3[b]	77.5 ± 1.3[b]	53
Post-centrifugation							
Low	62.4 ± 4.6[a]	29.6 ± 3.3[a]	67.6 ± 4.4[a]	39.6 ± 1.3	5.5 ± 0.9[a]	60.8 ± 3.1[a]	31
Medium	78.8 ± 2.4[b]	40.3 ± 2.5[b]	79.1 ± 2.7[b]	45.3 ± 1.4	3.6 ± 0.5[ab]	70.5 ± 2.3[b]	39
High	76.5 ± 2.6[b]	40.1 ± 2.3[b]	72 ± 2.7[ab]	40.2 ± 1.8	3.3 ± 0.3[b]	69.2 ± 2[b]	46

Low recovery class (%): 0–39; Medium recovery class: 40–69; High recovery class: 70–100; TM, total motility (%); PM, progressive motility (%); VAP, average patch velocity (μm/s); dACRO, percentage of cells with damaged acrosome (PNA+); VIAB, percentage of viable cells (SYBR+/PI−). Different letters indicate significant differences ($P < 0.05$) between three classes of recovery rate observed before or after centrifugation.

Table 3
Q2 Results of linear regressions used to determine, after centrifugation of brown bear ejaculates, the relationships between the spermatozoa recovery rate (dependent variable) and some parameters of sperm quality (predictor variables). (A) Relationships vs. parameters of spermatozoa quality (total motility and damaged acrosomes). (B) Relationships vs. parameters of seminal plasma (glucose, glutamic oxaloacetic transaminase and lactate dehydrogenase).

	Parameter estimate	Standard error	*t* value	*P*-value
(A) Spermatozoa quality				
Intercept	0.76	0.22	3.38	0.001
Total motility (TM)	0.25	0.13	1.94	0.052
Damaged acrosomes (dACRO)	−0.58	0.23	−2.55	0.012
R-square: 0.12				
The fitted equation: 0.76 + 0.25 TM − 0.58 dACRO				
(B) Seminal plasma				
Intercept	−0.16	0.35	−0.44	0.663
Glucose	−0.80	0.03	−2.61	0.015
Glutamic oxaloacetic transaminase[a]	0.24	0.10	2.03	0.045
Lactate dehydrogenase (LDH)	1.12	0.14	0.85	0.041
R-square: 0.39				
The fitted equation: −0.16 − 0.8 Glucose + 0.24 GOT + 1.12 LDH				

[a] Glutamic oxaloacetic transaminase: GOT.

Based on the present study, we inferred that spermatozoa concentration in ejaculate does not affect the sperm recovery rate after centrifugation. However, Edmond et al. (2012) reported that the equine sperm number for centrifugal fractionation did have an impact on the sperm recovery rate, so centrifuging 1 mL of semen containing 250–500 × 10^6 sperm produced the highest sperm recovery rate from what was observed centrifuging 4 mL of semen containing 1–2 × 10^6 sperm. The presence of a conditioned medium in this study (EquiPure) could explain the differences when compared with our results.

Brown bear reproduction is markedly seasonal with variations among different species. In the Cabarceno Nature Park, the mating season for brown bear spans from late April to late June (Anel et al., 2008). It is well known that the composition of seminal plasma is influenced by season in several species (ram: Cardozo et al., 2006; pig: Murase et al., 2007). Seasonal changes in semen volume and sperm concentration have been described (Smith et al., 1997), as well as monthly changes in the ram seminal plasma protein profile (Cardozo et al., 2006). Murase et al. (2007) have observed clear seasonal changes in the concentrations of total protein and albumin in boar semen, with minimum values being observed in summer. These seasonal changes in seminal plasma could affect semen viscosity and sperm quality, and both factors could possibly influence post-centrifugation recovery rates. We did not find evidence that post-centrifugation sperm recovery changes significantly between the months of sampling, but Fig. 4 shows that these recovery rates have a very clear heterogeneity in March and October (non-breeding season). These observations could be related to changes in seminal plasma associated with the season although we do not have sufficient data to assess changes in plasma quality. We have some preliminary data on the total amount of protein in the seminal plasma of bear semen associated with three periods of the year (pre-breeding: 0.44 ± 0.9 g/dL, breeding season: 0.22 ± 0.04 g/dL and post-breeding: 0.07 ± 0.01 g/dL; unpublished data). These data indicate quantitative changes that we suppose are associated with qualitative changes, which must undergo analysis by electrophoresis.

An inverse relationship between recovery rate of spermatozoa after sperm filtration and quality of semen or spermatozoa has been found (Anzar and Graham, 1993). However, Januskauskas et al. (2005) did not observe any significant effect of filtration of bovine spermatozoa on sperm motion characteristics or on the recovery of spermatozoa with intact acrosome. In the present study, we observed a weak relationship between semen

Please cite this article in press as: Alvarez, M., et al., The percentage of spermatozoa lost during the centrifugation of brown bear (*Ursus arctos*) ejaculates is associated with some spermatozoa quality and seminal plasma characteristics. Anim. Reprod. Sci. (2012), http://dx.doi.org/10.1016/j.anireprosci.2012.09.009

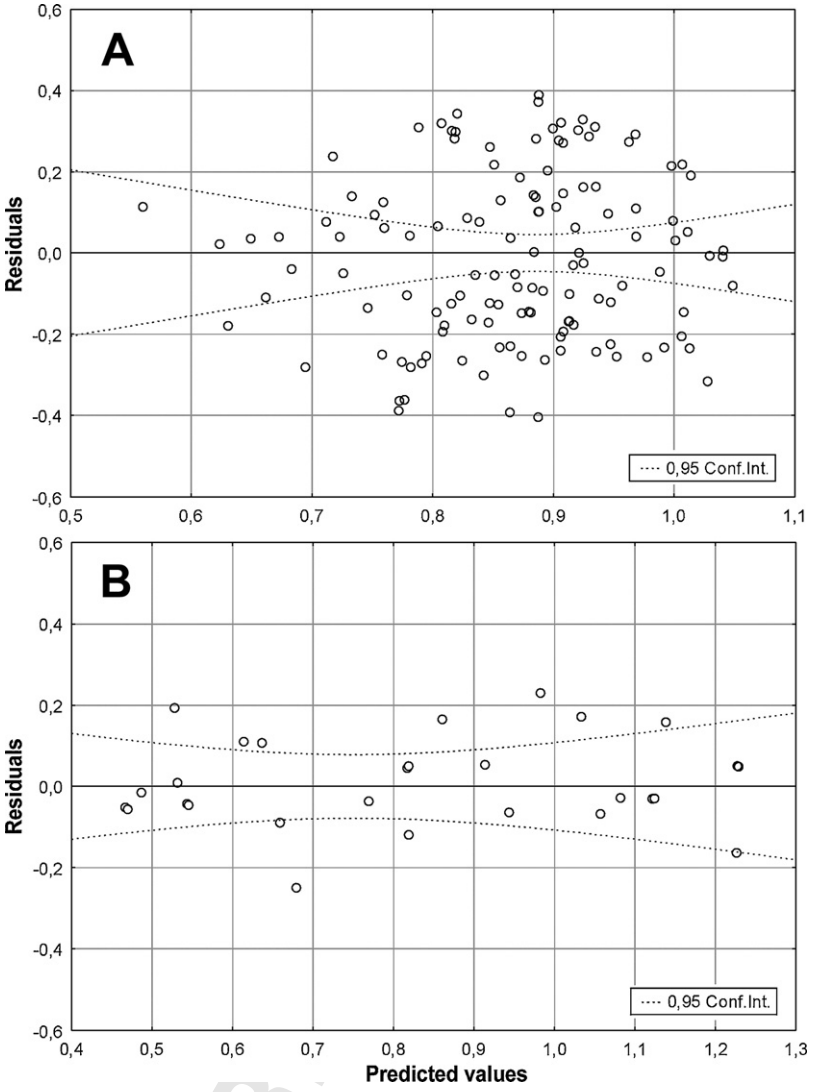

Fig. 4. Plots of residual values against the predicted value of two models of multiple linear regressions for the spermatozoa recovery rate in brown bear ejaculates, to graphically diagnose the presence of outliers and other influential values. (A) Plot of residuals from the analysis of the spermatozoa recovery rate against total motility and damaged acrosome (variables arcsin transformed). (B) Plot of residuals from the analysis of the spermatozoa recovery rate against glucose, glutamic oxaloacetic transaminase (GOT) and lactate dehydrogenase (variables log 10 transformed).

quality (total motility and acrosome status) and recovery rate after centrifugation of bear semen as suggested by plotting residual values against predicted values (Fig. 4A). Also, some seminal plasma parameters have been seen to act as acceptable predictors of this recovery rate (Fig. 4B). Glutamic oxaloacetic transaminase and lactate dehydrogenase correlated positively with sperm recovery, whereas glucose correlated negatively. Rigau et al. (2001) observed that canine sperm treated with glucose in the incubation medium showed motility similar to that described for hyperactivated canine spermatozoa, these authors thus concluding that glucose induced an oscillatory motility pattern in dog spermatozoa. This oscillatory movement of spermatozoa would explain the negative correlation between the glucose level in seminal plasma and sperm recovery. A positive correlation was seen between sperm count and glucose in human semen (Patel et al., 1988).

Cardozo et al. (2006) suggest that proteins in the seminal plasma play an important role in sperm membrane stability, and subsequent viability, motility and concentration. This mechanism could explain the data observed in our study for the above enzymes. Most of the enzymes, the activities of which have been estimated in seminal plasma, are also to be found in spermatozoa and there is a considerable possibility of 'leakage' of some of the intracellular enzymes, e.g. LDH and GOT (Chauhan and Srivastava, 1973). Most of the GOT is found on or in the sperm cell initially and not in the seminal plasma, and the measurement of cellular injury can be monitored using GOT release (Pace and Graham, 1970).

Spermatozoa meet their energy demands primarily through use of glucose in mammals. After ejaculation, fructose represents an important glycolytic substrate for spermatozoa in seminal plasma (Pellicer-Rubio et al.,

Please cite this article in press as: Alvarez, M., et al., The percentage of spermatozoa lost during the centrifugation of brown bear (*Ursus arctos*) ejaculates is associated with some spermatozoa quality and seminal plasma characteristics. Anim. Reprod. Sci. (2012), http://dx.doi.org/10.1016/j.anireprosci.2012.09.009

1997). Lactate and pyruvate are also used by spermatozoa of several species as a suitable substrate for energy production, and hence, the lactate dehydrogenase enzyme present in seminal plasma may play a role in energy metabolism of spermatozoa (Singh et al., 1990). According to these authors, the variation in LDH values in seminal plasma might be attributed to individual variations, differences in sperm concentration in ejaculates and seasonal variations. LDH and GOT levels in stallion seminal plasma were positively correlated with semen volume and sperm concentration (Pesch et al., 2006). In this study, LDH seems to be the most predictive enzyme for semen quality. These data confirm our results as we have observed that these two plasma proteins are the best predictors of the recovery rate after centrifugation of semen bear.

Kaya et al. (2002) detected a decrease in LDH activity and an elevated AST (GOT) level in the seminal plasma of ram in response to increased semen collection intensity that may partly result from a reduced proportion of accessory gland fluids in the ejaculate. Since we were performing electroejaculation, which is different to physiological ejaculation, this suggests that varying the electroejaculation protocol could increase the chances of obtaining higher sperm recovery after centrifugation, by a lower stimulation of seminal glands. To develop a suitable protocol, it must be taken into account that the electroejaculation procedure is influenced by a large number of variables, such as: anaesthesia (cat, Zambelli et al., 2007) or treatment with hormones (bull, Palmer et al., 2004).

In conclusion, sperm recovery after centrifugation of brown bear spermatozoa depends on many factors. Between and within-male variation is very large, but recovery can be associated to some sperm quality and seminal plasma characteristics, allowing for some predictive modelling. The relationship between recovery and seminal plasma composition suggests that post-centrifugation recovery could be improved by acting upon the electroejaculation procedure.

Acknowledgements

This work was supported in part by CICYT (CGL 2010-19213/BOS) and CANTUR S.A. Felipe Martínez-Pastor was supported by the Ramón y Cajal Program (Spanish Ministry of Science and Innovation). The authors thank Miguel Ángel Marañón and Miguel Angel Prieto, the game keepers of the Cabárceno Nature Park, Susana Gomes Alves, María Mata Campuzano, Manuel Alvarez Rodríguez, and Patricia Manrique Revuelta.

References

Althouse, G.C., Seager, S.W., Varner, D.D., Webb, G.W., 1989. Diagnostic aids for the detection of urine in the equine ejaculate. Theriogenology 31, 1141–1148.

Álvarez, M., García-Macías, V., Martínez-Pastor, F., Martínez, F., Borragán, S., Mata, M., Garde, J., Anel, L., de Paz, P., 2008. Effects of cryopreservation on head morphometry and its relation with chromatin status in brown bear (*Ursus arctos*) spermatozoa. Theriogenology 70, 1498–1506.

Álvarez-Rodríguez, M., Álvarez, M., Gomes-Alves, S., Borragán, S., Martínez-Pastor, F., de Paz, P., Anel, L., 2011. Quality of frozen-thawed semen in brown bear is not affected by timing of glycerol addition. Theriogenology 75, 1561–1565.

Anel, L., Álvarez, M., Anel, E., Martínez-Pastor, F., Martínez, F., Chamorro, C., de Paz, P., 2011. Evaluation of three different extenders for use in emergency salvaging of epididymal spermatozoa from a Cantabric brown bear. Reprod. Domest. Anim. 46, e85–e90.

Anel, L., Álvarez, M., Martínez-Pastor, F., Gomes, S., Nicolás, M., Mata, M., Martínez, A.F., Borragán, S., Anel, E., de Paz, P., 2008. Sperm cryopreservation in brown bear (*Ursus arctos*): preliminary aspects. Reprod. Domest. Anim. 43 (Suppl. 4), 9–17.

Anel, L., Gomes-Alves, S., Álvarez, M., Borragán, S., Anel, E., Nicolás, M., Martínez-Pastor, F., de Paz, P., 2010. Effect of basic factors of extender composition on post-thawing quality of brown bear electroejaculated spermatozoa. Theriogenology 74, 643–651.

Anzar, M., Graham, E.F., 1993. Filtration of bovine semen. II: factors affecting the recovery rate of spermatozoa. Anim. Reprod. Sci. 31, 197–204.

Cardozo, J.A., Fernandez-Juan, M., Forcada, F., Abecia, A., Muino-Blanco, T., Cebrian-Perez, J.A., 2006. Monthly variations in ovine seminal plasma proteins analyzed by two-dimensional polyacrylamide gel electrophoresis. Theriogenology 66, 841–850.

Carvajal, G., Cuello, C., Ruíz, M., Vázquez, J.M., Martínez, E.A., Roca, J., 2004. Effects of centrifugation before freezing on boar sperm cryosurvival. J. Androl. 25, 389–396.

Chauhan, R.A., Srivastava, R.K., 1973. Enzyme composition of buffalo seminal plasma. J. Reprod. Fertil. 34, 165–166.

de Paz, P., Álvarez-Rodríguez, M., Nicolás, M., Álvarez, M., Chamorro, C., Borragán, S., Martínez-Pastor, F., Anel, L., 2012. Optimization of glycerol concentration and freezing rate in the cryopreservation of ejaculate from brown bear (*Ursus arctos*). Reprod. Domest. Anim. 47, 105–112.

Edmond, A.J., Brinsko, S.P., Love, C.C., Blanchard, T.L., Teague, S.R., Varner, D.D., 2012. Effect of centrifugal fractionation protocols on quality and recovery rate of equine sperm. Theriogenology 77, 959–966.

García, P., Lastra, J., Marquinez, J., Nores, C., 2007. Detailed model of shelter areas for the Cantabrian brown bear. Ecol. Inform. 2, 207–307.

García-Macías, V., Martínez-Pastor, F., Álvarez, M., Borragán, S., Chamorro, C.A., Soler, A.J., Anel, L., de Paz, P., 2006. Seasonal changes in sperm chromatin condensation in ram (*Ovis aries*), Iberian red deer (*Cervus elaphus hispanicus*), and brown bear (*Ursus arctos*). J. Androl. 27, 837–846.

Giuliano, S., Director, A., Gambarotta, M., Trasorras, V., Miragaya, M., 2008. Collection method, season and individual variation on seminal characteristics in the llama (*Lama glama*). Anim. Reprod. Sci. 104, 359–369.

Hoogewijs, M., Rijsselaere, T., Vliegher, S.D., Vanhaesebrouck, E., Schawuer, C.S., Govaere, J., Thys, M., Hoflack, G., Van Soom, A., de Kruif, A., 2010. Influence of different centrifugation protocols on equine semen preservation. Theriogenology 74, 118–126.

Ishikawa, A., Matsu, M., Sakamoto, H., Katagiri, S., Takahashi, Y., 2002. Cryopreservation of the semen collected by electroejaculation from the Hokkaido brown bear (*Ursus arctos yesoensis*). J. Vet. Med. Sci. 64, 373–376.

Januskauskas, A., Lukoseviciute, K., Nagy, S., Johannisson, A., Rodriguez-Martinez, H., 2005. Assessment of the efficacy of Sephadex G-15 filtration of bovine spermatozoa for cryopreservation. Theriogenology 63, 160–178.

Kaya, A., Aksoy, M., Tekeli, T., 2002. Influence of ejaculation frequency on sperm characteristics, ionic composition and enzymatic activity of seminal plasma in rams. Small Rumin. Res. 44, 153–158.

Kojima, E., Tsuruga, H., Komatsu, T., Murase, T., Tsubota, T., Kita, I., 2001. Characterization of semen collected from beagles and captive Japanese black bears (*Ursus thibetanus japonicus*). Theriogenology 55, 717–731.

Matás, C., Decuadro, G., Martínez-Miro, S., Gadea, J., 2007. Evaluation of a cushioned method for centrifugation and processing for freezing boar semen. Theriogenology 67, 1087–1091.

Murase, T., Imaeda, N., Yamada, H., Miyazawa, K., 2007. Seasonal changes in semen characteristics, composition of seminal plasma and frequency of acrosome reaction induced by calcium and calcium ionophore A23187 in Large White boars. J. Reprod. Dev. 53, 853–865.

Nicolás, M., Álvarez, M., Gomes-Alves, S., Anel, E., Martínez, F., Borragán, S., Martínez-Pastor, F., de Paz, P., Anel, L., 2012a. Spermatozoa recovery and post-thawing quality of brown bear ejaculates is affected for centrifugation regimes. Eur. J. Wildl. Res. 58, 77–84.

Nicolás, M., Álvarez, M., Borragán, S., Martínez-Pastor, F., Chamorro, C.A., Álvarez-Rodríguez, M., de Paz, P., Anel, L., 2012b. Evaluation of the qualitative and quantitative effectiveness of three media of centrifugation (Maxifreeze, Cushion Fluid Equine, and PureSperm 100) in preparation of fresh or frozen-thawed brown bear spermatozoa. Theriogenology 77, 1119–1128.

Please cite this article in press as: Alvarez, M., et al., The percentage of spermatozoa lost during the centrifugation of brown bear (*Ursus arctos*) ejaculates is associated with some spermatozoa quality and seminal plasma characteristics. Anim. Reprod. Sci. (2012), http://dx.doi.org/10.1016/j.anireprosci.2012.09.009

Nicolás, M., Álvarez, M., Gomes-Alves, S., Mata-Campuzano, M., Borragán, S., Martínez-Pastor, F., Anel, L., de Paz, P., 2011. Effects on brown bear (*Ursus arctos*) spermatozoa freezability of different extender and dilution ratios used for pre-freezing centrifugation. Eur. J. Wildl. Res. 57, 259–266.

Pace, M.M., Graham, E.F., 1970. The release of glutamic oxaloacetic transaminase from bovine spermatozoa as a test method of assessing semen quality and fertility. Biol. Reprod. 3, 140–146.

Palmer, C.W., Amundson, S.D., Brito, L.F., Waldner, C.L., Barth, A.D., 2004. Use of oxytocin and cloprostenol to facilitate semen collection by electroejaculation or transrectal massage in bulls. Anim. Reprod. Sci. 80, 213–223.

Patel, S.M., Skandhan, K.P., Mehta, Y.B., 1988. Seminal plasma fructose and glucose in normal and pathological conditions. Acta Eur. Fertil. 19, 329–332.

Pellicer-Rubio, M.T., Magallon, T., Combarnous, T., 1997. Deterioration of goat sperm viability in milk extenders is due to a bulbourethral 60-kDa glycoprotein with triglyceride lipase activity. Biol. Reprod. 57, 1023–1031.

Pesch, S., Bergmann, M., Bostedt, H., 2006. Determination of some enzymes and macro- and microelements in stallion seminal plasma and their correlations to semen quality. Theriogenology 66, 307–313.

Rigau, T., Farré, M., Ballester, J., Mogas, T., Peña, A., Rodríguez-Gil, J.E., 2001. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology 56, 801–815.

Rijsselaere, T., Van Soom, A., Maes, D., de Kruif, A., 2002. Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. Theriogenology 57, 1669–1681.

Singh, G.B., Pangawkar, G.R., Biswas, R.K., Srivastava, R.K., Sharma, R.D., 1990. Studies on lactic dehydrogenase and sorbitol dehydrogenase release in relation to deep freezing of buffalo semen in certain extenders. Theriogenology 34, 371–378.

Smith, J.F., Parr, J., Smith, J.K., Briggs, R.M., Duganzich, D.M., 1997. Seasonal patterns in production and quality of semen of rams from flocks selected for or against an early breeding season. Proc. N. Z. Soc. Anim. Reprod. 57, 259.

Zambelli, D., Cunto, M., Prati, F., Merlo, B., 2007. Effects of ketamine or medetomidine administration on quality of electroejaculated sperm and on sperm flow in the domestic cat. Theriogenology 68, 796–803.

Please cite this article in press as: Alvarez, M., et al., The percentage of spermatozoa lost during the centrifugation of brown bear (*Ursus arctos*) ejaculates is associated with some spermatozoa quality and seminal plasma characteristics. Anim. Reprod. Sci. (2012), http://dx.doi.org/10.1016/j.anireprosci.2012.09.009