Title: **Seasonal changes in sperm chromatin condensation in ram (*Ovis aries*), red deer (*Cervus elaphus*) and brown bear (*Ursus arctos*).**

Short title: Sperm DNA quality and season in wild species

Key Words: chromatin, SCSA, season, ram, Iberian red deer, brown bear

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ABSTRACT

The effect of seasonality (temperate environment; Spain) on the chromatin status of ovine (Churra breed), Iberian red deer and brown bear spermatozoa was studied. This work aims to improve genetic resource banks (GRBs) by enhancing existing knowledge of the effect of season on sperm quality. Samples were obtained by electroejaculation in Iberian red deer and brown bear and by artificial vagina in ram. We used the sperm chromatin structure assay (SCSA) to study the level of chromatin condensation of the spermatozoa in each studied period. These periods were: ram, breeding season (from September to January), non-breeding season (from February to June) and summer (July and August); red deer, breeding season (September and October), post-breeding (November) and non-breeding (from January to March); brown bear, pre-breeding (March and April), breeding (May and June), post-breeding (July and August) and non-breeding (September to February). Chromatin in ram was more decondensated in summer and no differences were observed between the breeding and non-breeding season. However, in red deer, spermatozoa obtained during the non-breeding season showed more condensed chromatin than those obtained in the rut and post-rut periods. Similarly, brown bear rendered sperm with loose chromatin in the pre-breeding and breeding seasons. Less condensed chromatin in the breeding season may be related to faster epididymal transit due to enhanced spermatogenesis.

INTRODUCTION

Genetic Resource Banks (GRBs) in combination with assisted reproductive techniques offer substantial benefits in species conservation programmes for maintaining biodiversity and helping the conservation of highly endangered species (Holt et al, 1996) and genetic recovery of autochthonous breeds in domestic animals.

Many mammals are threatened or in danger of becoming extinct within the next few decades, which implies a loss of biological diversity. This is happening not only to wild species but also to domestic breeds. Indeed, autochthonous breeds are being replaced by more productive ones, thus leading to the loss of genetic traits which may be needed in the future.

In this study, we worked with three Spanish species in different situations. The Cantabrian brown bear (*Ursus arctos*) is an endangered species in Spain, with very high symbolic value, though. It probably constitutes the last pure breed aggregate of brown bear in the world. These bears live in a fragmented habitat in the Cantabrian mountains (North of Spain) which are subjected to intense pastoral activity and crossed by many forest roads. The population of brown bear is currently split into two small nuclei (totalling around 100 individuals). The case of Iberian red deer (*Cervus elaphus hispanicus*) is different. They are subjected to controlled hunting and are considered the most valuable trophy in the Iberian peninsula. Red deer also face habitat problems because most of them are constricted to small areas separated by barriers. Consequently, inbreeding and loss of genetic variability are a recurrent hazard for both species. We have included Churra sheep as a domestic species. This breed is important in the regions were it is reared (Castilla y León) because of its good rusticity. However, it is currently

undergoing a process of absorption, with a considerable reduction in the number of pure-breed animals, which could lead to a loss of rusticity (San Primitivo and de la Fuente, 2000).

Many factors must be taken into account in the setting-up of GRBs. Seasonality affects sperm quality and must thus be considered a high impact factor. Some mammals undergo a complete reproductive arrest in their annual cycle, with the males presenting testicular quiescence and lack of sperm, followed by another period of testicular recrudescence and sexual activity (Suzuki et al, 1992; Blottner et al, 1995; Martinez-Pastor et al, 2004a). This sexual cycle is evident in wild animals from temperate areas (such as red deer and brown bear). Iberian red deer have a circannual cycle, undergoing marked variations in their behaviour, body condition and reproductive parameters (Gizejewski, 2004; Martinez-Pastor et al, 2004a; Martinez-Pastor et al, 2005). In Spain, the breeding season is between September and December, reaching its peak in September (rut). In the case of brown bear, mating in Spain takes place in May and June.

Although less pronounced in domestic species, some studies have reported seasonal influences in the reproductive activity of rams, affecting testicular size, gonadal endocrine patterns, quantitative and qualitative sperm production and sexual behavior, elicited by the variations of photoperiod and environmental cues (Gerlach and Aurich, 2000). It is well known that testicular size and spermatogenetic efficiciency in rams are maximum during the breeding season and decrease during the non-breeding season (Dacheux et al, 1981; Gerlach and Aurich, 2000). These variations change substantially

according to male and breed and demonstrate the sensitivity of each animal or breed to environmental factors. In fact, reproductive management (mating) is also carried out during the non-breeding season with acceptable results.

It is important to underline the effect of seasonal changes on the quality of sperm samples preserved in a GRB. To our knowledge, sperm DNA or chromatin quality variations due to season have not been studied in depth. Some studies show chromatin changes due to season in human (Sanchez et al, 2000; Henkel et al, 2001) and ram sperm (Rodriguez et al, 1985) but there are no reports on red deer and brown bear.

Several techniques have been developed to analyse sperm chromatin status and DNA fragmentation, such as In Situ Nick Translation (ISNT), the terminal deoxyribonucleotidyl transferase mediated dUTP nick end labelling (TUNEL), comet assay and the SCSA technique. The SCSA technique assesses chromatin status and has many major advantages over other assays, which are often labor intensive and lack statistical power for diagnosis and prognosis (Evenson et al, 2002). SCSA is a flow cytometry-based assay developed by Evenson et al (Evenson et al, 1980), and defines abnormal chromatin structure as increased susceptibility of sperm DNA to acid-induced denaturation *in situ.* The method uses flow cytometry (FCM) to measure the extent of this denaturation. Increased susceptibility to denaturation corresponds to heterogeneity in the chromatin structure, which is associated with disturbances in spermatogenesis leading to morphologically abnormal spermatozoa and reduced fertility (Evenson et al, 1980; Ballachey et al, 1987). This assay has been applied mainly in humans but also in several other species, such as ram (Martinez-Pastor et al, 2004b) .

The main objective of the present study was to investigate the influence of season on chromatin sperm quality in ram, red deer and brown bear. We chose red deer and brown bear because of the great interest in Spain in their conservation, and ram because of their financial value and also because our group has an active research line on them (Anel et al, 2003; Kaabi et al, 2003, Martinez-Pastor et al, 2004b). SCSA application in red deer and brown bear was another objective in this study as it has not been tried previously in these species.

MATERIALS AND METHODS

All the products used in this paper were obtained from Sigma (Madrid, Spain), unless otherwise specified.

Sample obtention

Ejaculated sperm samples were obtained from Iberian red deer (Cervus elaphus hispanicus), brown bear (Ursus arctos) and ram (Ovis aries).

Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD223/1998, which conforms to European Union Regulation 86/609 and adheres to guidelines established in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the American Society of Andrology.

The first decision in this study was how to define periods within the reproductive chronogram of each species. In red deer and brown bear, decisions were based on the record of rutting and mating activity reported by gamekeepers and on previous reports on the behaviour of red deer (Martinez-Pastor et al, 2004a; Martinez-Pastor et al, 2005). Thus, we determined that Iberian red deer develop their maximum rutting activity during the early autumn, and brown bear during the late spring (May and June). In the case of brown bear, we also had to take into account denning in early winter. In the case of Churra sheep, we had in-depth knowledge of their physiology and information on breeding periods in our latitude was provided by ANCHE (Churra Breeders Association).

We defined the study periods as follows:

- Iberian red deer: breeding season (September and October), post-breeding season (November and December) and non-breeding season (the rest of the year). However, we

only obtained samples in November in the post-breeding season and from January to March in the non-breeding season.

- Brown bear: pre-breeding season (March and April), breeding season (May and June), post-breeding season (July and August) and non-breeding season (September to February).

- Ram: breeding season (September to January), non-breeding season (February to June) and summer (July and August). In this species, we separated July and August (which could be included in the non-breeding season) as summer because in these months AI centers have more problematic samples and so we can study the well-known effect of temperature on spermatogenesis (Setchell, 1998). In the other two species summer was not considered separately because in brown bear it coincided with the post-breeding season and in Iberian red deer we have no samples to study.

Ram samples were obtained from 20 different Churra breed males (one collection per male in each season). The animals were 2-5 years old and belonged to ANCHE. They were maintained under natural day-length conditions at a latitude of 42º 36'N and fed with barley (1 $kg \times male^{-1} \times day^{-1}$), alfalfa (0.5 $kg \times male^{-1} \times day^{-1}$) and straw *ad libitum* supplemented with molasses. Semen samples were obtained by artificial vagina. In this center, the number of sample collections were according to season: approximately 3 times/week during the breeding season and 1-2 times/week in the non-breeding season and summer. All of these animals were used for routine service and were selected for good fertility.

Deer samples were from 14 adult stags. In the post-breeding season, only 8 males were available, because of management planning. The deer were housed in the University of Castilla la Mancha (ETSIA, Albacete, Spain), in a half-freedom regime, and maintained

under natural day-length conditions (38º 57'N). During the experiment they were in an artificial meadow of 6500 m^2, composed of *Festuca arundinacea* (52.4%), *Dactylis glomerata* (28.6%), *Medicago sativa* (14.3%) and *Trifolium repens* (4.8%). They were given barley, alfalfa and oats and had *ad libitum* access to cereal, straw and water. Prior to each electroejaculation, the animals were immovilized and treated with xylazine + ketamine (Rompun 2% ®; Bayer, Leverkusen, Germany and Imalgene 1000 ® Merial, Lyon, France) to induce general anesthesia. The prepucial area was washed with physiological saline serum and shaved. Electrical stimuli were applied using a 3-electrode probe, 250 mm length and 30 mm diameter, with a double-reading (voltage and amperage) electroejaculator (PT-Electronics®; Oregon, USA). Average parameters for provoking ejaculation were 4.5 V and 90 mA. Anaesthesia was reverted with yohimbine hydrochloride (0.9)%.

Brown bear samples from 20 different males (20 in the breeding season, 14 in the pre-breeding season, 13 in the post-breeding season and 8 in the non-breeding season) were also obtained by electroejaculation. The animal age was unknown but we estimated that all the captured males were adults, though not old. They were housed in a half-freedom regime in Cabarceno Park (Cantabria, Spain; 43º 33'N). During the experiment they were fed diets based on chicken meat, bread and fruit and feeding followed the hierarchical order, the dominant bears taking most food. General anaesthesia was by application of tiletamine + zolazepan (Zoletil100®; Virbac, Carros, France) 7 mg/kg, and ketamine (Imalgene 1000®) 2 mg/kg. Electroejaculation was with the same electroejaculator as in deer, but the transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were given until ejaculation (10 V and 250 mA, in average).

Sample preparation

Ram samples were accepted with a volume of ≥ 0.5 ml (using collector tubes graduated in milliliters), ≥ 4 in wave motion assessment (warming stage at 37ºC, ×40; score:0-5) and a concentration of ≥ 3000×10^6 spz/ml (photocolorimetric method at 540 nm, on a specific calibrated scale). These thresholds are frequently used in this ram breed in AI centers. The good-quality ejaculates were diluted (UL extender: Tes-Tris-Fructose, 10% egg yolk, 4% glycerol) (Anel et al, 2003) and frozen immediately (using biofreezer Kryo 10-16 II, Planer™). In red deer we used samples of acceptable quality (individual motility ≥60%) (Garcia-Macias et al, In press) diluted (Triladyl® Minitüb, Tiefenbach, Germany) and then cryopreserved in liquid nitrogen vapour by placing racks with straws 10 cm above the surface of the liquid nitrogen for 10 min. The brown bear samples were selected according to >50% motility and above 100×10^6 spz/ml; we rejected urine-contaminated samples (> 80 mg urea/dl, measured using Merckognot® Urea Rapid Screening test, Merck, Barcelona, Spain). The selected samples were diluted in cryopreservation extender (Tes-Tris- Fructose complemented with 8% glycerol, 20% egg yolk, 2% EDTA and 1% equex; 320 mOsm/kg).

Assessment of sperm chromatin condensation (SCSA)

We used the SCSA technique (Evenson et al, 1980; Evenson et al, 2002) to assess chromatin stability using the metachromatic staining Acridine Orange (AO: Polysciences, Inc, Warrington, PA). This dye fluoresces in the green band when combined with the intact double DNA helix, and in the red band when combined with denatured DNA (Evenson and Jost, 2000).

Once the samples were thawed (6 s, 65ºC water bath) they were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM EDTA; pH 7.4) in polypropylene tubes at a final sperm concentration of approximately 1-2×10^6 cells/ml. Samples were stored immediately in LN2 in an ultra-cold freezer (-80ºC) until needed. For analysis, samples were thawed on crushed ice and mixed with 400 µl of an acid-detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% Triton X 100; pH 1.4). Exactly 30 seconds later, 1.20 ml of acridine orange staining solution (0.037 M citric acid, 0.126 M Na2HPO4, 0.0011 M dissodium EDTA, 0.15 M NaCl; pH 6.0, 4ºC) containing 6 µg/ml electrophoretically purified AO was added. The stained samples were analyzed just 3 min after AO staining.

The samples were analyzed on a FACScalibur flow cytometer (Becton Dickinson Immunochemistry Systems; San Jose, CA, USA), equipped with standard optics and an argon-laser tuned at 488 µm and running at 200 mW. Calibration was carried out periodically using standard beads (Calibrites; Becton Dickinson). Per sample, 10,000 events were acquired with a flow rate of ~200 cells/s. Data corresponding to the red (FL3) and green fluorescence (FL1) of acquired particles were recorded.

Data processing

We used the parameters defined by Evenson et al. 2002, for SCSA interpretation, modified in order to adapt the technique to each particular species. We calculated the DFI (DNA Fragmentation Index) of each event (spermatozoa) as the relation among red and total fluorescence, by means of the formula red/(red+green)×100. Then, we classified spermatozoa into three groups, according to DFI: normal DFI (< 20% DFI),

moderate DFI (from 20% to 75% DFI) and high DFI (from 75% to 100% DFI); we calculated the percentage of spermatozoa with a moderate (DFIm) and high (DFIh) DNA fragmentation index for each sample. Total DNA fragmentation index (DFIt) was defined as DFIm+DFIh. We also calculated mean DFI and standard deviation of DFI (SD DFI) for each sample, and detected spermatozoa with high DNA stainability (HDS; FL1 channels above 600 in FL1/ FL3 flow cytometry dot plot).

FCS data were obtained using Cell Quest Version 3 (Becton Dickinson). All the parameters were obtained directly from the red/green dot plot provided by the acquisition software (Cell Quest Version 3, Becton Dickinson). FCS files were processed with WinMDI software and saved as tabulated text, and imported as Excel spreadsheets. Further analyses were performed using Excel macros programmed ad hoc.

Statistical analysis

Statistical analyses were performed with the SAS^{TM} V. 8 package (SAS Institute Inc., Cary, NC, USA). Data were not normally distributed so an arc-sine transformation was done. Comparisons between periods for the seasonality study were carried out using the Kruskal-Wallis test and the Wilcoxon rank-sum test.

We observed great dispersion in data from deer and bear (some outliers with high values). Many factors can affect the outcome of samples taken from these species because of lack of homogeneity, which was not a problem in ram. In these cases, we removed extreme values calculating the third quartile interquartil range (IQrange) and (Q3), and keeping data below (1.5x IQrange)+Q3 value.

RESULTS

Chromatin analysis of ram semen showed no statistical differences between the breeding and non-breeding season in the SCSA parameters (Table 1; figures 1 and 2). However all parameters differed ($p<0.01$) between summer and both the breeding and non-breeding season, indicating a higher chromatin decondensation and lower proportion of high DNA stainability (HDS).

In red deer, SD DFI, HDS and DFIt presented statistical differences (Table 1; figures 1 and 2). SD DFI was lower in the non-breeding season compared with the breeding and post-breeding seasons ($p<0.01$), and the same occurred with DFIt. HDS was significantly different between the three seasons showing the highest value in the breeding season. DFIt only showed differences between the breeding and the non-breeding seasons. DFIm showed a trend ($p=0.07$) towards a difference between the breeding and the non-breeding season.

Sperm chromatin was also affected by season in brown bear, as shown in Table 1 and figures 1 and 2. Mean of DFI showed the highest values in the pre-breeding and breeding seasons ($p<0.01$). SD DFI showed the highest value in the breeding season, being different ($p<0.05$) to the pre- and post-breeding seasons. HDS was higher in the post-breeding season and different to the pre-breeding season ($p<0.05$). Considering chromatin decondensation, DFIm was significantly higher ($p<0.01$) in the pre-breeding and breeding seasons, in comparison with the post-breeding season. DFIh was higher in the breeding season, showing significant differences with the post-breeding and non-

breeding season. Furthermore, DFIt was higher in the breeding and pre-breeding seasons and differed with post-breeding results ($p<0.01$).

Specific variations were observed between species. Thus, we observed only one peak in the FL-1 (green fluorescence) histogram in the brown bear samples whereas two peaks were observed in the ruminant species. The general appearance of FL-1 vs FL-3 (green vs red fluorescence) plots was similar in the three studied species (Figure 3).

DISCUSSION

In this study we found that chromatin status in ram, red deer and brown bear varied according to different periods of the year. There is ample bibliography on seasonality in red deer (Gizejewski 2004; Martinez-Pastor et al, 2004a; Martinez-Pastor et al, 2005), bear (black and polar) (Tsubota et al, 1997; Howell-Skalla et al, 2000; Howell-Skalla et al, 2002; Boone et al, 2003) and in several ram breeds (Rodriguez et al, 1985; Mandiki et al, 1998; Gerlach et al, 2000; D'Alessandro and Martemucci, 2003) regarding behavior, body condition, and hormonal and reproductive status. However, the effect of seasonality on sperm chromatin has not been studied in depth.

The results obtained in ram indicated that chromatin status was similar in both the breeding and non-breeding seasons. Instead of the well-known seasonal variations observed in routine semen parameters in ram, some reports confirm the stability of chromatin results in the breeding and non-breeding season. Rodriguez et al, 1985 studied nuclear chromatin decondensation in ram by controlled exposure to dithiotreitol (DTT) and sodium dodecyl sulphate (SDS) *in vitro*, observing a slightly higher percentage of spermatozoa with stable nuclei in the non-breeding season than in the breeding season but they did not observe any significant differences. However, the considerable increase in DFI parameters (Mean DFI, SD DFI, DFIm, DFIh and DFIt) in summer suggests that chromatin may be more susceptible to denaturation in this period. These results coinicide with those of Henkel et al, 2001 in human sperm using aniline blue stain to assess chromatin condensation status. They hypothesized that the replacement of histones by cysteine-rich protamines, which occurs during late spermatid stages might be temperature-sensitive, because the Sertoli cells that control

spermatogenesis are temperature-sensitive in specific functions. Regarding the decrease in HDS recorded in summer, some authors have determined that HDS is related to sperm chromatin immaturity (higher stainability). However, in our study the decrease in HDS in summer may be due to something other than?? chromatin immaturity. Molecular studies must be carried out to explain the difference between summer and the other seasons regarding HDS, although temperature may play a key role here.

The situation was totally different in the other ruminants studied. In red deer, not only HDS but also DFIt showed the highest values in the breeding season, thus indicating a higher level of chromatin immaturity and decondensation than in the other periods. Increased spermatogenesis may induce a shorter stay in the epididymis, where sperm chromatin undergoes important changes, including compaction. Indeed, epididymal transit implies changes in DNA-protamine interaction, regarding modification of DNA-bound proteins and the formation of disulfure bonds, which would result in more compact chromatin (Golan et al, 1996; Lewin et al, 1999). Therefore, faster epididymal transit could result in a less compact chromatin structure. A recent study (Rodriguez and Bustos, 1996) demonstrated that the chromatin of stallion sperm was more condensed in the non-breeding season. The authors proposed that sperm underwent hypermaduration because of prolonged epididymal storage, and they concluded that this highly condensated chromatin may cause faulty male pronuclear formation.

We have previously reported better results for epididymal semen from red deer in progressive motility, viability and acrosomal status in the post-breeding season (Martinez-Pastor et al, 2004a). Moreover, as demonstrated in the present work, a higher level of chromatin immaturity and decondensation were observed in the breeding and

post-breeding seasons. This fact supports the hypothesis of changes in the maturation process related to seasonality (through changes associated to the sexual chronogram). The maturation process in each season is reflected by better quality during the post-breeding season in classical parameters, but increased chromatin decondensation during the breeding and post-breeding seasons. Nevertheless, in our opinion, slight chromatin decondesation during the breeding season would not represent a handicap, since it would not be justified from an evolutive approach. Not enough information is available on the threshold of SCSA parameters for indicating subfertility in deer. However, when compared with human thresholds (subfertility with >15% HDS and >30% DFI), (Evenson et al, 2002) our data would not reflect fertility problems in any of the studied seasons. Nevertheless, it is important to know that there are species-specific variations in chromatin structure. Rybar et al (2004) observed that the threshold for subfertility in bull and boar is much lower than the 30% DFI for humans, being approximately 8% DFI and 10-20% range, respectively. This aspect would not be critical in sperm collection in different seasons for GRBs. Related to species-specific differences is the appearance in FL-1 histograms. This distribution is an artifact due to the geometry and high index of refraction of the flat sperm head, and this occurred in brown bear due to the smaller and shorter size when compared with the two ruminants as observed in previous studies comparing dog with ram and red deer (Garcia-Macias et al, In press).

The study of brown bear indicated a similar trend to that of red deer. Sperm with decondensated chromatin was more frequent in the pre-breeding and breeding seasons. However, we found a higher variation between samples than in the ram and red deer. In this species there were a lot of factors we were unable to evaluate and, due to the

characteristics of the studied population (very heterogeneous, different origins of the animals, unknown ages) data presented a very wide dispersion. Thus, we found males with high proportions of spermatozoa with damaged chromatin (>20 % in DFIt), but with normal chromatin in other collections. We must take into account that, for ram, we worked with an homogeneous population (2-5 year old males selected for good fertility and with genetic uniformity). In the case of red deer, although there was no previous selection process as in ram, the studied population was very homogeneous. We also have to consider physiological differences when comparing carnivores with ruminants, which may also affect sperm chromatin stability and heterogeneity. Knowledge of the sources of the variations recorded in this work may greatly help the creation of GRBs for brown bear. It is possible that animal age, stress due to captivity or competition, and hierarchy, amongst others, alter sperm quality.

In summary, our results show that chromatin status, assessed by SCSA, varies according to season in ram, Iberian red deer and brown bear. In brown bear, differences between samples within each season were more marked than for the other species, possibly due to other factors we could not control in this study. In general, regarding chromatin status, it is possible to obtain spermatozoa of acceptable quality in any season (except in summer for ram), for any of the studied species. Because of the importance of this parameter in fertility, we think that sperm chromatin assessment should be a key step for GRBs management. Furthermore, the SCSA technique appears to be suitable assay for the studied species, considering the lack of previous studies in red deer and brown bear.

Table 1. Results for each species and season[1] (means ??).

Param.[2]	Ram			Red Deer			Brown Bear			
	BS	NBS	Sm	BS	PostBS	NBS	PreBS	BS	PostBS	NBS
MeanDFI	16.27^{a}	15.76^{a}	20.12^{b}	18.02	17.42	17.32	18.49^{a}	18.76^{ab}	16.59^{c}	17.13^{bc}
SD DFI	3.11^{a}	3.44^{a}	6.76^{b}	3.66^{a}	4.96^{a}	1.81^{b}	5.34^{a}	7.38^{b}	5.88^{a}	2.89^{ab}
HDS	0.97^{a}	0.78^{a}	0.24^{b}	2.41^{a}	0.34^{b}	0.78^{c}	0.52^{a}	0.60^{ab}	0.98^{b}	0.49^{ab}
DFIm	0.98^{a}	0.58^{a}	2.24^{b}	1.69	1.06	0.99	3.80^{a}	3.55^{a}	0.99^{b}	0.96^{ab}
DFIh	0.17^{a}	0.26^{a}	1.22^{b}	0.17	0.20	0.07	0.75^{ab}	1.42^{b}	0.57^{ac}	0.14^{c}
DFIt	1.42^{a}	0.97^{a}	3.11^{b}	2.37^{a}	1.43^{ab}	1.06^{b}	4.43^{a}	5.92^{a}	1.70^{b}	1.09^{ab}
n	20	20	20	14	8	14	14	20	13	8

[1]Ram: Breeding season (BS), non-breeding season (NBS) and summer (Sum**);** red deer: breeding season (BS), post-breeding (PostBS) and non-breeding season (NBS); bear: pre-breeding season (PreBS), breeding season (BS), post-breeding season (PostBS) and non-breeding season (NBS)

[2]Mean DFI: mean of DFI (red/red+green fluorescence); SD DFI: standard deviation of DFI; HDS: high DNA stainability; DFIm: moderate DNA fragmentation index; DFIh: high DNA fragmentation index and DFItn: total DNA fragmentation index (DFIm+DFIh).

[a,b,c] Different letters between seasons in the same row indicate significant differences within species ($p<0.05$).

FIGURE LEGENDS

-Figure 1. Sperm chromatin parameters. Mean DFI (DNA fragmentation index), SD DFI (standard deviation of DFI) and HDS, depending on season in ram (BS: breeding season; NBS: non-breeding season; Sum: summer), Iberian red deer (BS: breeding season; PostBS: post-breeding season and NBS: non-breeding season) and brown bear (PreBS: pre-breeding season; BS: breeding season; PostBS: post-breeding season and NBS: non-breeding season). ^{a,b,c} Different letters between seasons indicate significant differences within species ($p<0.05$).

- Figure 2. DNA fragmentation indexes DFIm, DFIh and DFIt, depending on season in ram (BS: breeding season; NBS: non-breeding season; Sum: summer), Iberian red deer (BS: breeding season; PostBS: post-breeding season and NBS: non-breeding season) and brown bear (PreBS: pre-breeding season; BS: breeding season; PostBS: post-breeding season and NBS: non-breeding season). ^{a,b,c} Different letters between seasons indicate significant differences within species ($p<0.05$).

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