Available online at www.sciencedirect.com
SCIENCE DIRECT®
Theriogenology
ELSEVIER
Theriogenology xxx (2004) xxx–xxx
www.elsevier.com/locate/theriogenology

Season effect on genitalia and epididymal sperm from Iberian red deer, roe deer and Cantabrian chamois

Felipe Martinez-Pastor[a], Camino Guerra[b], Mohammed Kaabi[b], Vanesa Garcia-Macias[b], Paulino de Paz[a], M. Alvarez[b], Paz Herraez[a], Luis Anel[a,*]

[a]*Department of Cell Biology and Anatomy, Faculty of Biology, University of León, 24071 León, Spain*
[b]*Reproduction and Obstetrics, Veterinary Clinic Hospital, University of León, 24071 León, Spain*

Received 23 April 2004; received in revised form 18 August 2004; accepted 19 August 2004

Abstract

Seasonality deeply affects the physiology and behavior of many species, and must be taken into account when biological resource banks (BRBs) are established. We have studied the effect of seasonality on many reproductive parameters of free-ranging Iberian red deer, roe deer and Cantabrian chamois, living in Spain. Testicles from hunted animals were collected and sent to our laboratory at different times during the year. We recorded the weight and volume of testis, the weight of the epididymis and its separate parts (caput, corpus, and cauda), the weight of the sperm sample collected from the cauda epididymis, and several sperm parameters (sperm concentration, spermatozoa recovered, motility, HOS test reactivity, acrosomal status, and viability). We studied the data according to several periods, defined accordingly to each species. For red deer, we defined rut (mid-September to mid-October), post-rut (mid-October to mid-December), and non-breeding season (February). For roe deer, they were pre-rut (June), rut (July), post-rut (first fortnight of August), and non-breeding season (September). For chamois: non-breeding season (June to mid-September) and breeding season (October–November). The rut/breeding season yielded significantly higher numbers for almost all parameters. However, in the case of red deer, sperm quality was higher in the post-rut. For roe deer, testicular weight was similar in the pre-rut and in the rut, and sperm quality did not differ significantly between these two periods, although we noticed higher values in the rut. In the case of chamois, sperm quality did not differ significantly from the breeding season, but

* Corresponding author. Tel.: +34 987 2913 20; fax: +34 987 2013 22.
E-mail address: dsalar@unileon.es (L. Anel).

0093-691X/$ – see front matter
doi:10.1016/j.theriogenology.2004.08.006

data distribution suggested that in the non-breeding season there are less males with sperm of good quality. On the whole, we find these results of interest for BRB planning. The best season to collect sperm in this species would be the breeding season. However, post-rut in red deer, pre-rut in roe deer, and non-breeding season in chamois could be used too, because of the acceptable sperm quality, despite the lower quantity salvaged. More in-depth research needs to be carried out on the quality of sperm salvaged at different times of the year in order to confirm these findings.

Keywords: Iberian red deer; Roe deer; Chamois; Post-mortem recovery; Epididymal spermatozoa; Seasonality

1. Introduction

Biological resource banks (BRBs) are important tools for the conservation of species and valuable breeds, and have been strongly developed during the last decade. The term BRB comprises many techniques and protocols, the purpose of which is to collect, preserve and utilize tissues and germplasm of selected individuals in order to ensure the continuity and the genetic variability of breeds, populations and species [1–3]. One simple method to achieve the objectives of BRB is the collection of sperm from males, followed by its cryopreservation and its use in AI when needed. This way, the genetic variability of a population can be maintained in an easy and inexpensive manner [4]. However, there are many drawbacks, some of them related to the collection of the sperm sample, which is a difficult task in wild species. Indeed, one of the most attractive uses of BRBs is the preservation of wild species, since they can be of use in management programs dedicated to protecting not only endangered species but also those that could be at risk in the future [2].

In this context, post-mortem sperm recovery appears as an attractive strategy for sperm collection in order to provide germplasm banks. Sperm from the epididymes of males killed in hunts or by accident can be salvaged and cryopreserved. Since spermatozoa from the cauda epididymis have a high degree of maturity and functionality [5], they can be stored in liquid nitrogen for later use in AI programs [6]. However, to assure good status for the collected sperm, one has to consider many variables such as animal condition, pre-freezing handling (post-mortem time and storage), and season, which can heavily affect the quality of the sample [7–9].

The influence of season on sperm production and quality has been largely considered, as a factor of high impact. Most species, at least in non-tropical latitudes, present a circannual cycle, undergoing more or less marked variation in their behavior, body condition and reproductive parameters. There are great differences between species, even between those closely related [10]. In fact, some mammals undergo a complete reproductive arrest in their annual cycle, in which the males present testicular quiescence and lack of sperm, followed by another period of testicular recrudescence and sexual activity (such as the members of the Cervidae) [11,12]. On the other hand, other species maintain some level of spermatogenic activity throughout the whole year, however it is much more intense during their breeding season [13–15]. Seasonality is less pronounced between males of domestic species, but there are still differences in behavior and sperm characteristics depending on the time of the year [13,16–20]. Photoperiod, mediated through the hormone melatonin, is the main factor triggering events related with season [21,22]. There is abundant literature in

this respect, and numerous studies have been carried out on circannual variations of many hormones and its importance on body, gonadal and gametogenesis changes [8,13,23–25]. In cervids (and in other mammals too), there are also very pronounced changes in body condition and morphology throughout the year, which are strongly related to sexual activity. In fact, the same hormones control both kinds of changes, with testosterone being an important regulator both of antler growth and spermatogenesis [26–28].

In this study, we approached the setting up of BRBs by considering the influence of season on some parameters of testis, epididymis and epididymal sperm from hunted wild ruminants. We have chose Iberian red deer (*Cervus elaphus hispanicus*), roe deer (*Capreolus capreolus*) and Cantabrian chamois (*Rupicapra pyrenaica parva*), because of their value in Spain as hunting trophies and their importance in the environments they inhabit. Furthermore, they have different breeding seasons, namely the beginning of summer, for roe deer, early autumn for red deer, and mid-autumn for chamois. Our aim was to assess the differences in testicular and epididymal morphology, sperm production, and sperm quality in different periods of the year, hence providing data that may be of use in the creation of germplasm banks for these and similar species.

2. Material and methods

All chemicals were acquired from Sigma (The Netherlands). Media were not bought as such, but prepared in our laboratory as described.

2.1. Genitalia collection

Genitalia were collected from shot Iberian red deer (*Cervus elaphus hispanicus*), roe deer *(Capreolus capreolus*) and Cantabrian chamois (*R. pyrenaica parva*), in the hunting reserves of Ancares, Mampodre and Picos de Europa (Cantabrian mountains in León, Spain), and in several private hunting reserves of Cáceres, Burgos, Ciudad Real, Toledo and Jaén (Spain; only Iberian red deer). All the animals lived in a free-ranging regime. Ages ranged 3–8 years for red deer, 2–6 years for roe deer and 3–10 years for chamois. Sample collection was limited by hunting activity, which is regulated and higher around the breeding season (September–October for red deer, October–November for chamois, and July for roe deer), but not constrained exclusively to this period. Thus, samples could be collected in February and from mid-September to mid-December for Iberian red deer, from June to September for roe deer, and from June to November for chamois. The reproductive calendar for these species was defined accordingly to the observations and experience of the wardens of the game reserves. For red deer, rut starts by the end of August, and lasts until mid-October. The rest of the Autumn was considered post-rut, in which animals show sexual activity, but with much less frequency that in the rut. For roe deer, we considered a pre-rut (comprising the end of May and June), without sexual display, the rut (July), which is very short for this species (sexual display and competition, and mating last only few weeks), and a post-rut, with very rare sexual activity. And for chamois, we considered the October and November as the breeding season. For the three species, samples obtained in a period different to the described ones were considered as out of the breeding season.

Harvest plans followed Spanish Harvest Regulation, Law 4/96 of Castilla y León and Law 19/01 of Extremadura, which conform to European Union Regulation. Furthermore, species and number of individuals that can be hunted, as well as the exact periods of the year when hunting can take place, are reviewed each year in the Annual Hunting Regulation of the respective regions. Animal handling was performed in accordance with the 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.

Scrotum, including testicles and epididymes, was removed from the carcass and refrigerated down to 5 °C as soon as possible. Date and time of death, collection and refrigeration were noted and attached to the corresponding sample. Refrigerated genitalia were sent to our laboratory at the Veterinary Clinic Hospital of the University of León (Spain). A total of 291 Iberian red deer samples, 148 roe deer samples, and 99 chamois samples were processed in this study.

2.2. Measurements of testicles, epididymis and sperm sample

Sample manipulation was carried out in a walk-in fridge (5 °C). Testicles with epididymes and vas deferens attached were isolated from scrotum and other tissues. Epididymes were dissected free from the testicles, cleaned of connective tissue, and weighed, after removing the vas deferens. After that, caput, corpus and cauda were separated, and weighed. Testicles were weighted, and their volumes were estimated by sinking them in a glass tube half-filled with water, and observing the volume of displaced liquid. We recorded the mean values of both left and right side.

Sperm sample was obtained by multiple incisions and gently squeezing of cauda. To avoid blood contamination, superficial blood vessels were previously cut, wiping their content and drying thoroughly the surface of the cauda. Sperm samples obtained from both cauda epididymis of the same animal were mixed and weighed.

The concentration of each sample (spermatozoa/mL) was calculated using a Bürker counting chamber, after diluting the sample in a glutaraldehyde solution (5 μL of sample in 500 μL of 2% glutaraldehyde in an aqueous solution made of 29 g/L glucose monohydrate, 10 g/L sodium citrate tribasic dihydrate and 2 g/L sodium bicarbonate). We determined the number of spermatozoa by g/mL of sperm sample, by multiplying the weight of salvaged sperm by the sperm concentration. In a preliminary experience we found that 1 mL of epididymal sperm sample weighted 0.94 g, therefore spermatozoa × g/mL would roughly represent the total number of spermatozoa in the sample. Finally, we divided this parameter by the cauda epididymis weight, obtaining the relative sperm content of the cauda epididymis.

2.3. Sperm quality assessment

Only those samples with a post-mortem time of 24–48 h were analyzed for quality. The reason for this limitation is that post-mortem time has a deleterious effect in the quality of the samples, therefore, which we tried to reduce. We chose this period because there were

an adequate number of samples in it, for most seasons and species, and the variation of sperm quality during this period seemed to be small and steady, according to the bibliography [29], whereas choosing longer post-mortem intervals would had been an undesirable source of variation in the study.

For motility assessment, 5 μL of sample were diluted in 500 μL of Hepes medium (20 mmol/L Hepes, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose; pH 7, 400 mOsm/kg). Diluted samples were put on a warming plate at 37 °C for 20 min. A Makler chamber warmed up to 37 °C, was filled with 5 μL of sample and examined with a phase-contrast microscope (Nikon Labophot-2) on a warming stage at the same temperature. At least five fields were observed at 200×. Total motility (percentage of cells exhibiting any kind of movement) and progressive motility (percentage of cells with straight movement) were estimated subjectively.

The functional integrity of the sperm plasma membrane was evaluated using the hypoosmotic swelling test (HOS test). Five microliters of sample were diluted in 500 μL of a hypoosmotic sodium citrate solution (100 mOsm/kg). After 18 min at room temperature, samples were fixed with a drop of glutaraldehyde solution. Response to the test was determined by counting 100 cells with a phase-contrast microscope (400×). The percentage of positive cells (those with a swollen flagelle) was recorded for each sample [30].

Aliquots of the samples were fixated in a glutaraldehyde solution (5 μL in 500 μL; 2% glutaraldehyde in an aqueous solution of 146 mmol/L glucose, 34 mmol/L sodium citrate tribasic dihydrate and 24 mmol/L sodium bicarbonate). Five microliters were put on a microscope slide, covered with a coverslip and observed with a phase-contrast microscope (400×) [31]. Acrosomal status (% of cells with an intact acrosome) was evaluated counting at least 100 cells.

The fluorescent dye propidium iodide (PI) was used to assess sample viability. Five microliters of sample were diluted in 500 μL of PI solution (25 μg/L PI in Hepes solution, see above). Samples were kept in the dark at room temperature for 10 min before being analyzed with an epifluorescence microscope (Nikon; 400×). At least 100 cells were counted and the percentage of non-stained cells (viable cells) was noted [32].

2.4. Statistical analysis

Statistical analysis was carried out using the SAS™ package v. 8 (SAS Institute, Cary, NC), and $P < 0.05$ was used in all tests for statistical significance. Data were distributed between seasons (pre-rut, rut/breeding, post-rut, and non-breeding), depending on the species. These groups were defined according to the observations of the wardens of the hunting reserves on the behavior of the animals. As described above, for red deer, the groups were non-breeding season (February), rut (mid-September to mid-October), and post-rut (mid-October to mid-December); for roe deer: pre-rut (June), rut (July), post-rut (first fortnight of August), and non-breeding season (September); for chamois: non-breeding season (June to mid-September), and breeding season (October and November). It must be noted that, because of the hunting calendar, samples were constrained to defined dates, and we could not cover the whole reproductive calendar (for instance, red deer rut begins by the end of August, but we started to receive samples in mid-September). For this

same reason, we could study a pre-rut period in roe deer, but not in red deer. Comparisons between seasons were carried out using the Kruskal–Wallis test and the Wilcoxon rank-sum test.

Sperm quality was studied similarly. However, due to the post-mortem time limitation described above, a lower number of samples were available. Furthermore, in the case of roe deer, we could not carry out a complete statistical analysis, because of the different distribution of the samples between seasons and post-mortem time. Only one sample from the post-rut and the non-breeding season fell within the 24–48 h interval. Thus, in this case, the comparison was carried out only between the pre-rut and rut seasons.

3. Results

3.1. Testicular and epididymal characteristics and sperm recovery between seasons

Fig. 1 displays the distribution of individual testicular weights throughout the year, showing how its distribution varied between the chosen periods. The comparison of different periods of the years showed very clear differences regarding to the quantitative parameters. Figs. 2–5 summarize the measurements of the testis, epididymis and the quantitative data for salvaged sperm (weight of collected sperm, concentration, and spermatozoa × g/mL), for Iberian red deer, roe deer and chamois. Median values are detailed in Table 1.

We found significant differences between the compared time periods for all parameters, with higher values in the breeding season, and lower ones in the non-breeding season. For red deer and chamois, all seasons differed between them. In the case of roe deer, comparison rendered a more complex result. In this case, testicular weight was similar in the pre-rut and rut periods, and sperm sample weight and sperm concentration were similar in the pre-rut, post-rut and non-breeding season. Variation in testis weight and volume between breeding and non-breeding seasons were higher in the case of red deer and roe deer. For instance, testicular weight was 3.5 and 2.8 times higher in the breeding season, for red deer and roe deer, respectively, whereas it was only 1.7 times higher for chamois. In the case of testicular volume, it was 3.4 times higher for red deer, 3.7 for roe deer, and 1.5 for chamois. However, considering other parameters, the variations were more similar between roe deer and chamois, and higher in the case of red deer, possibly because of body size. For instance, epididymis weight variation was 1.5 for roe deer and chamois, but 2.6 for red deer, and, in the case of the sperm sample weight, the difference was more pronounced: 8.5 times higher for red deer, whereas it was only 2.6 for roe deer and 3 for chamois.

3.2. Sperm quality between seasons

Figs. 5 and 6 show the variation in sperm quality parameters between the corresponding seasons for each species. Median values are detailed in Table 1. Red deer sperm yielded the highest quality values in the post-rut period. Comparing with the rut period, acrosomal integrity and viability where significantly higher. In the non-breeding season, motility

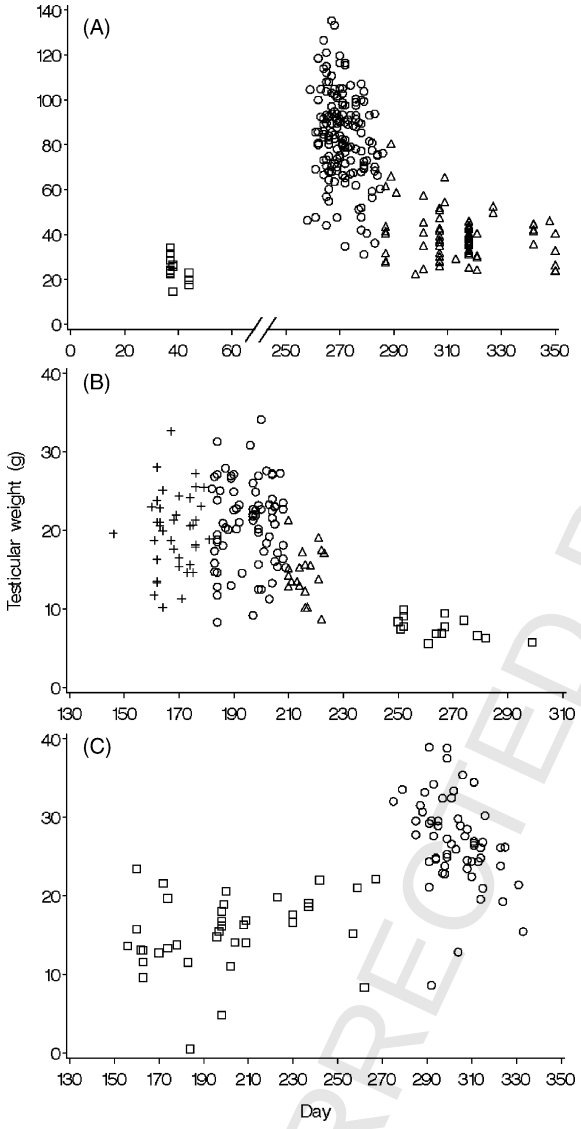

Fig. 1. Distribution of testicular weights along the year, for Iberian red deer (A), roe deer (B), and chamois (C). Each mark corresponds to one sample (mean value of the two testicles). Figures identify each season (+: pre-rut; ○: rut/breeding season; △: post-rut; □: non-breeding season).

parameters dropped, and, in fact, most samples had very poor or no motility at all, but acrosomal integrity and HOS test results were similar to those recorded during the rut.

In the case of Chamois, we found no significant differences, although median values seemed lower in the non-breeding season. In this sense, dispersion of data was higher during the non-breeding season, which suggested the existence of different groups within

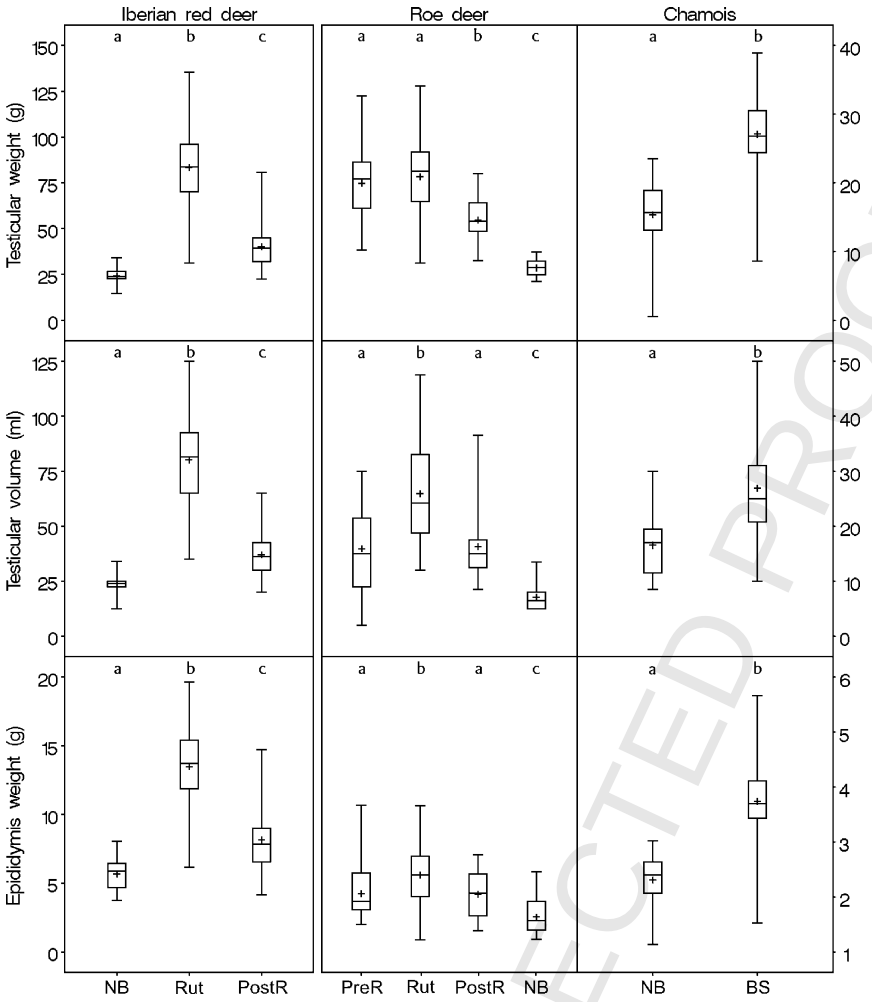

Fig. 2. Evolution of testicular weight, testicular volume, and epididymal weight depending on season (NB: non-breeding; PreR: pre-rut; PostR: post-rut; BS: breeding season). Left scale refers to red deer, whereas right scale refers to roe deer and chamois. Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside indicates the median score. The whiskers reach the maximum and minimum values of the range. The mean is shown with a cross. Different letters on the top of each plot indicate significant differences between seasons ($P < 0.05$).

241 the class. Indeed, considering individual samples, all those studied during the breeding
242 season rendered good motility results (TM > 60%) together with good acrosomal integrity,
243 HOS test and viability values. On the other hand, almost half of the samples collected
244 during the non-breeding season had almost no motility, and also low acrosomal integrity,
245 HOS test and viability results. Interestingly, the rest of the samples in the non-breeding
246 season were comparable to those of the breeding season.

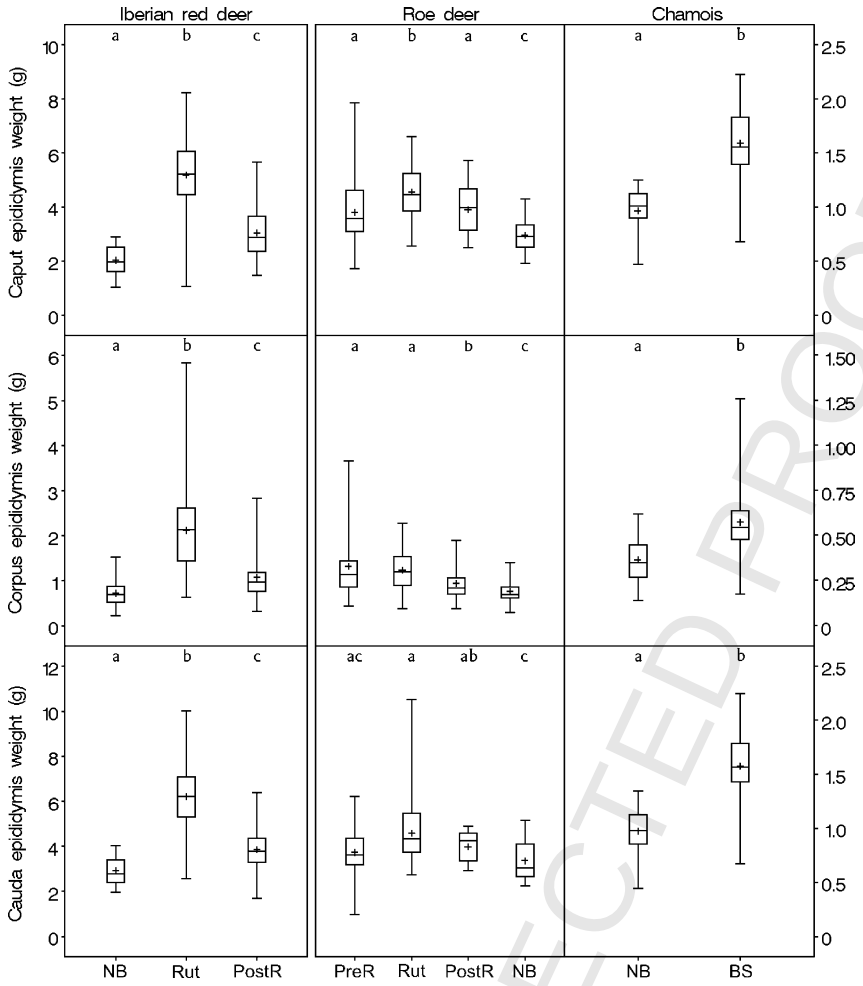

Fig. 3. Evolution of caput, corpus and cauda epididymis weights depending on season (NB: non-breeding; PreR: pre-rut; PostR: post-rut; BS: breeding season). Left scale refers to red deer, whereas right scale refers to roe deer and chamois. Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside indicates the median score. The whiskers reach the maximum and minimum values of the range. The mean is shown with a cross. Different letters on the top of each plot indicate significant differences between seasons ($P < 0.05$).

For roe deer, there were no significant differences between the pre-rut and rut period, albeit results were lower in the pre-rut period. In the case of post-rut and non-breeding seasons, we could not include them in the statistical analysis, because only one sample in each period fell within the 24–48 h post-mortem interval. However, taking the 60–120 h post-mortem interval, we noticed that the percentage of samples with TM < 10% was 29% for pre-rut, 27% for rut, 63% for post-rut, and 100% for non-breeding season, but with similar values for the other parameters.

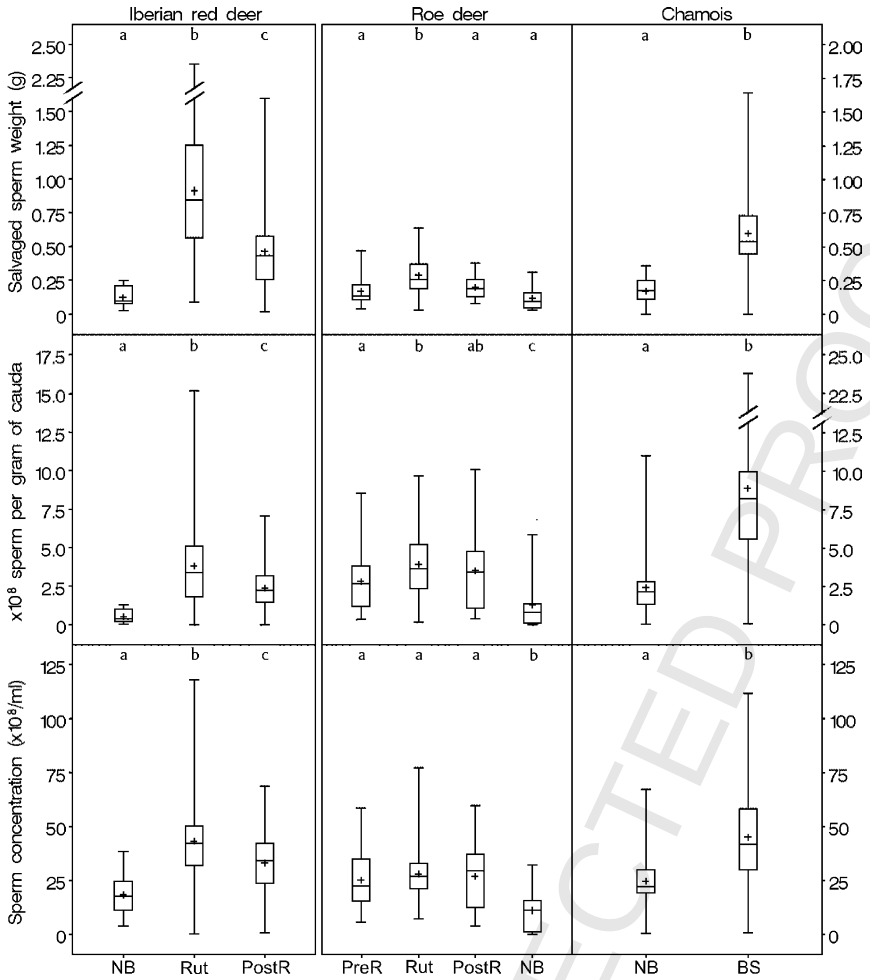

Fig. 4. Evolution of sperm sample weight, number of sperm per gram of cauda, and sperm concentration depending on season (NB: non-breeding; PreR: pre-rut; PostR: post-rut; BS: breeding season). Left scale refers to red deer, whereas right scale refers to roe deer and chamois. Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside indicates the median score. The whiskers reach the maximum and minimum values of the range. The mean is shown with a cross. Different letters on the top of each plot indicate significant differences between seasons ($P < 0.05$).

4. Discussion

In this study we have shown that many reproductive parameters of Iberian red deer, roe deer and chamois vary between different periods of the year. The morphology of testicles and epididymis, and the quantity and quality of the salvaged sperm underwent important variations between these periods, as expected in seasonal species. There is ample bibliography about this subject on the red deer and roe deer, containing much information on behavior, body condition, and hormonal and reproductive status in different periods of

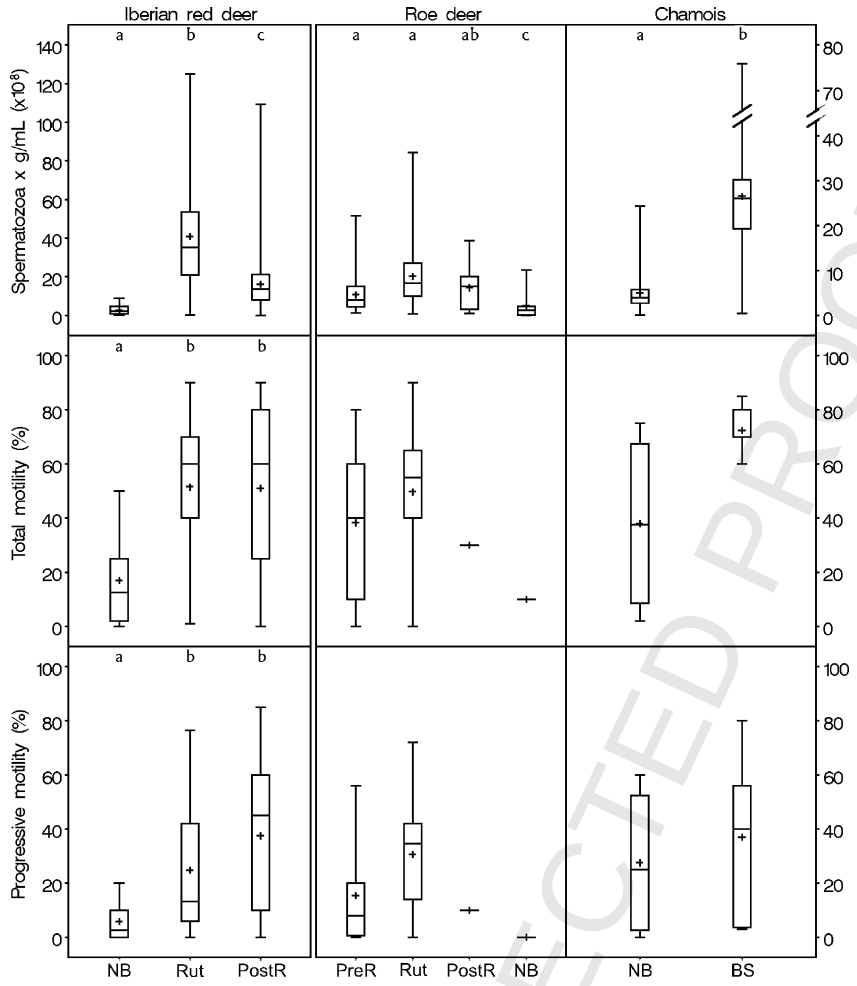

Fig. 5. Evolution of spermatozoa × g/mL (approximately, total spermatozoa in the sample), total motility, and progressive motility depending on season (NB: non-breeding; PreR: pre-rut; PostR: post-rut; BS: breeding season). Left scale refers to red deer, whereas right scale refers to roe deer and chamois. Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside indicates the median score. The whiskers reach the maximum and minimum values of the range. The mean is shown with a cross. Different letters on the top of each plot indicate significant differences between seasons ($P < 0.05$).

the year. Besides, this subject has been studied in other cervids. On the other hand, to our knowledge, there are no studies on the reproductive cycle of male chamois, apart from a recent report by our group [33]. However, this study was limited by the extent of the hunting seasons for each species, which determined the availability of samples.

Seasonality is controlled by fluctuations in the hormone melatonin, which is produced by the pineal gland during the night. Hence, depending on the photoperiod, the concentration of melatonin in the body rises or lowers, and regulates the production of GnRH. One of its final effects is the control of testosterone levels, through LH pulses,

Table 1
Results for each species and season (medians)

Param.[a]	Red deer			Roe deer				Chamois	
	NB	Rut	PostR	PreR	Rut	PostR	NB	NB	BS
TESW	23.9	83.8	39.3	20.6	21.7	14.4	7.6	15.5	26.8
TESV	24	81.5	37.5	15	24.3	15	6.5	17	25
EPIW	5.9	13.7	7.7	1.9	2.4	2.1	1.6	2.4	3.7
CAPW	2.0	5.2	2.8	0.9	1.1	1.0	0.7	1.0	1.6
CORW	0.7	2.1	1.0	0.3	0.3	0.2	0.2	0.4	0.6
CAUW	2.8	6.2	3.8	0.8	0.9	0.9	0.6	1.0	1.6
SPEW	0.1	0.9	0.4	0.1	0.3	0.2	0.1	0.2	0.5
SPZC	0.3	2.9	1.9	2.7	3.6	3.4	0.8	2.2	8.2
CONC	17.6	42.2	34.8	22.5	27.0	29.4	11.3	22.0	41.7
SPZ	2.1	35.1	13.2	3.4	7.2	6.4	1.2	3.9	25.9
TM	12.5	60	60	40	55	30[b]	10[b]	37.5	70
PM	2.5	13.2	40	8	34.6	10[b]	0[b]	25	40
ACR	60.5	67	88.5	49	56.5	64[b]	79[b]	36.5	74
HOST	72.5	76	78.5	62	70.5	80[b]	88[b]	55	69
VIAB	–	73.5	82	51	67	4.5[b]	–	46.5	67

[a] TESW: testicular weight (g); TESV: testicular volume (mL); EPIW: epididymis weight (g); CAPW: caput weight (g); CORW: corpus weight (g); CAUW: cauda weight (g); EFW: sperm sample weight (g); SPZC: $\times 10^6$ spermatozoa/g cauda; CONC: sperm concentration ($\times 10^8$ sperm/mL); SPZ: spermatozoa$\times 10^8$ $\times$ g/mL (approximately, total spermatozoa in the sample); TM: total motility (%); PM: progressive motility (%); HOST: HOS test reactivity (%); ACR: acrosomal status (%); VIAB: sperm viability (%).
[b] Only one sample.

which rise before the breeding season and peak during that period, dropping afterwards [13]. Since testosterone is needed for testicular recrudescence and adequate spermatogenesis, this hormonal cycle synchronizes the sexual activity of the males. Consequently, sperm is produced only in the right period of the year.

The first matter in this study was to define periods or ''seasons'' for each species, so we could group the samples in these periods and compare them. These periods should make sense in the reproductive cycle of the respective species, thus we based our choices on the record of rutting and mating activity reported by the wardens of the hunting reserves. According to this, in the Cantabrian mountains (north of Spain), Iberian red deers develop their maximum rutting activity during the early autumn. Besides, many of the red deer samples came from Cáceres, a more southern and warmer area. In this zone, the rut is considered to be longer and some rutting behavior is often observed even by the end of autumn. Nevertheless, we did not observe a divergence between the data of the two zones, so they were pooled and analyzed together. Finally, the samples collected in February were considered as belonging to the non-breeding season, due to the absence of rutting activity in that period and the proximity of antler casting (around April).

In the case of roe deer, the rut and mating period is much shorter. According to Blottner et al. [12,23], spermatogenesis and other reproductive parameters peak just before the breeding season of roe deer, and fall quickly afterwards. These authors worked in a higher latitude than us (Germany), and reported that the breeding season of the roe deer comprises from mid-July to mid-August. In the north of Spain, rut occurs in July, but it seems to have a similar length. Thus, we can consider that there is a shift in the sexual cycle of the roe deer

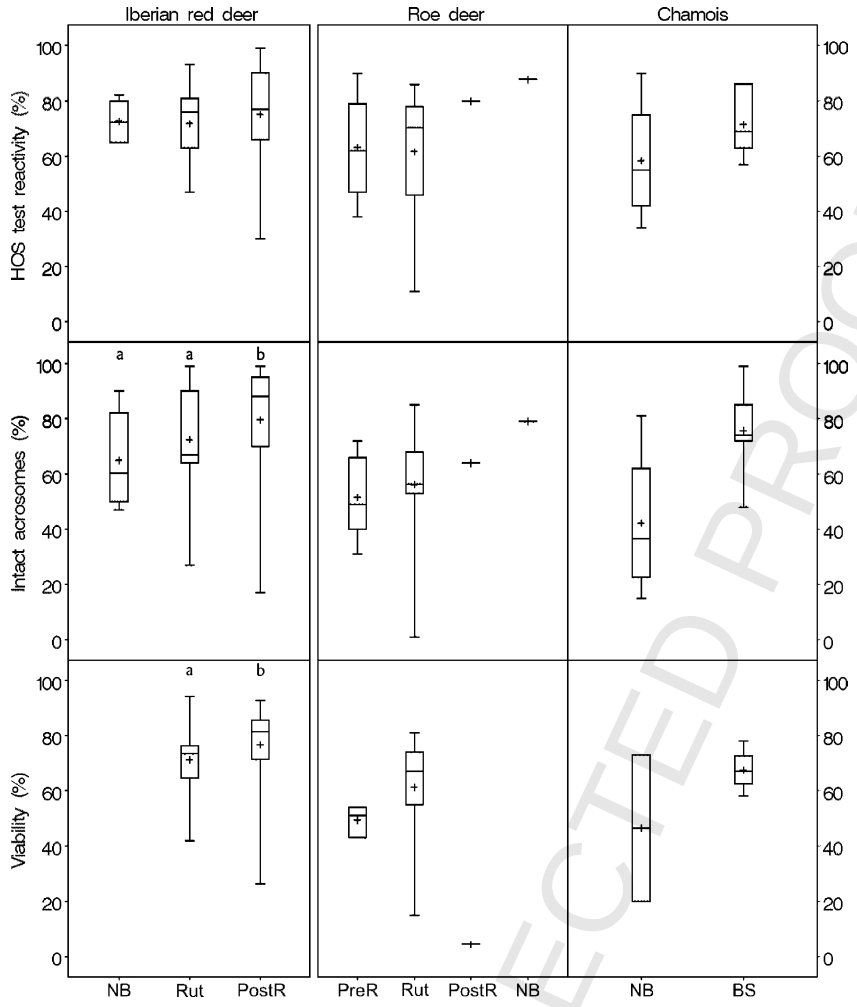

Fig. 6. Evolution of HOS test reactivity, acrosomal status, and sperm viability depending on season (NB: non-breeding; PreR: pre-rut; PostR: post-rut; BS: breeding season). Left scale refers to red deer, whereas right scale refers to roe deer and chamois. Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside indicates the median score. The whiskers reach the maximum and minimum values of the range. The mean is shown with a cross. Different letters on the top of each plot indicate significant differences between seasons ($P < 0.05$).

because of the different environments (latitude, climate), which could be of use for populational studies. The samples that fell out of the breeding season were included either in a pre-rut or post-rut period, or in a non-breeding season, if they were collected much later (early autumn). The classification was much simpler with the chamois, as we only distinguished two periods, breeding and non-breeding.

The differences we found between seasons, regarding morphological measurements and quantitative sperm parameters, were expected. Many studies have shown that seasonal

mammals undergo a cycle of involution and growth that affects not only testicles, but also other components of the genital tract [8], and secondary sexual characteristics, very evident in cervids [34]. In the case of Iberian red deer, our results suggests a very rapid involution of spermatogenic tissues after rut, possibly due to the decrease in the plasma testosterone levels, which has been reported to occur after the rut in similar species. According to these studies [10,11,34,35], plasma testosterone concentration peaks just before the moment of most intense mating activity in the rut, and falls quickly afterwards, so it presents low values only 1 month after the end of the rut. Seminiferous tubules, which had reached their maximum development, regress, following testosterone drop. Our data collected in February (non-breeding season) showed lower values for all parameters, corresponding with a situation of long-term low testosterone levels. Other authors have obtained similar results studying red deer and other cervid species. For instance, Hochereau-de Reviers and Lincoln [36], working with red deer, found a mean weight of the left testis of 24.4 g in the non-breeding season (February–April), and of 70.7 g in the breeding season (August–October). Comparing with our results, the lower weight obtained by these authors in the breeding season could be due, apart from other factors, to the longer period considered as breeding season, which may include the pre-rut period, when testes have not reached their maximum weight yet. Furthermore, Suzuki et al. [11], studying Sika deer, determined a testicular size coefficient by means of multiplying the three testicular axes and calculating the cubic root of the resulting number. In this way, the relation of the mean values obtained in the rut and non-breeding season was 1.46, and between the rut and the post-rut was 1.13. Treating our numbers in the same way, we have 1.5 and 1.3. Other studies in Eld's deer showed similar results [10]. Moreover, our coefficients for roe deer are 1.55 and 1.17. Therefore, different species of cervids possibly follow the same trend.

Roe deer reproductive seasonality has been thoroughly described in many articles. Blottner et al. [23], and Blottner and Roelants [37,38] indicated that spermatogenesis in roe deer increases considerably immediately prior to the rut, decreasing quickly thereafter. These authors found that sperm production depends not only on the changes in testis mass, but also on mitotic and meiotic activity. Spermatogenic activity during the non-breeding season is exclusively limited to spermatogonia proliferation, whereas sperm production is activated due to a testosterone increase in the pre-rut period. Testosterone reaches its maximum during the rut and falls abruptly afterwards [39]. In this sense, we had noted a clear differentiation between the rut and the pre-and post-rut periods, considering morphometric parameters. Testicular weight did not change between the pre-rut and rut, but testicular volume increased. This could indicate that, during the pre-rut, testicular tissues reach their full growth, but other changes, related to their full activation, may occur in these tissues only during the rut (testosterone peak). Blottner et al. [23] described the evolution of testis mass throughout the year, with a trend similar to the one shown here. These authors also studied the number of spermatozoa per gram of testis, which marked a peak in the rut, reaching almost 100×10^6 sperm/g testis, whereas it was around 50×10^6 sperm/g testis just before or after the rut, and nearly 0 sperm/g testis during the winter. These data are in accordance with the number of sperm we salvaged from the cauda epididymis in each season (twice during the rut than during the pre-rut, and six times lower in the non-breeding season). The similarity of our results in the rut and post-rut could be due to a delay between the lowering of testicular spermatozoa and the depletion of sperm

reserves in the cauda epididymis. In fact, the stability of the relative sperm content of the cauda (millions of spermatozoa per gram of cauda) between these two seasons does support this idea. We have to keep in mind that all the other studies considered here [23,37–39] used animals living in a higher latitude than ours (around 52°N versus 42°N in the north of Spain), where rut takes place between July and August, instead of July, so there is about a 15-day shift we have to take into account when comparing results. Goeritz et al. [8] carried out an ultrasonographic study in roe deer and found that testicular volume and size varied very little from May to September, contrarily to our findings (comparable to a period between April and August, in our work). Another difference is that corpus epididymis reached its maximum diameter between September and October (between the post-rut and non-breeding season), whereas we have found its maximum weight in the pre-rut and the rut, being lower in the post-rut. These differences are possibly due to the different methodology (ultrasounds versus direct measuring of the testis and epididymis), and the different kind of animals used (captive versus free-ranging; different subpopulations). Nevertheless, cauda epididymis volume and number of spermatozoa collected, by electroejaculation, agreed with our results.

Chamois reproductive biology has been little studied. In a preliminary study [33], we compared some morphometric and seminal parameters between breeding and non-breeding periods, finding results for testicular and cauda epididymis weights, and salvaged sperm similar to the ones shown here. However, sperm concentration between the two periods was not different, but in this study, using more extensive data, we have found that it was significantly higher in the breeding season than in the non-breeding season. Comparing with red deer and roe deer, it can easily be seen that, even though results for the breeding season were higher, differences between both periods were much smaller than in the case of cervids. In fact, seasonality affects less dramatically to members of the Bovidae family. Nevertheless, its effect on genitalia and sperm production is important, even in domestic species [13,20,40]. According to our results, it is evident that spermatozoa production is clearly depressed during the non-breeding season, although a certain amount of sperm can be collected from the cauda epididymis. Lincoln [24], working with mouflon (*Ovis musimon*), described rapid testicular development preceding the breeding season. This process could be similar in the case of the chamois, with a rapid increase of testicular weight and size prior to the rut. As we did not find differences between the samples collected in different periods within the non-breeding season, we treated this entire interval as homogeneous. However, we could expect some variation in the samples collected just before the breeding season, in September. Unfortunately, we only had four samples collected in September, and during the first fortnight, which did not allow us to carry out such a study. The analysis of samples collected in September would be interesting in order to complement the present study.

These results are of great interest when considering strategies for setting up BRBs of these and similar species. Most reports regarding seasonality and sperm collection in cervids either omit data on the quantity of sperm salvaged from the cauda epididymis, or deal with semen obtained by electroejaculation. In the case of chamois, information is almost nonexistent. Our study shows that, for red deer, samples collected during the post-rut can yield an appreciable amount of sperm, much less than during the rut, though. Indeed, after the rut, spermatogenesis lowers rapidly, but there is still enough activity to maintain spermatogenesis for many weeks [11]. Even though the total number of

spermatozoa salvaged in this period was around one-third of the value obtained during the rut, these samples could have being processed into many seminal doses for conservation in BRBs. On the other hand, sperm collected in February would have meant that only very few doses could be produced. In the case of roe deer, the most favorable seasons were the rut and the post-rut. There were acceptable numbers, at least in many samples, during the pre-rut, and again very low quantity in the non-breeding season. Similarly, the breeding season of chamois is much more favorable for sperm collection, although an appreciable amount of sperm can be salvaged in the non-breeding season.

Furthermore, the study of sperm quality in each season is compulsory in order to establish a good collection strategy. In the case of red deer, quality is clearly poor in the non-breeding season. Monfort et al. [10], working with Eld's deer, indicated that motility and acrosomal status were better around the rut (winter solstice and spring equinox), and the number of abnormal forms was higher in the non-breeding period (summer solstice and autumn equinox). Interestingly, in our study, progressive motility, HOS test reactivity and acrosomal status yielded better results in the post-rut season than in the rut. In this sense, increased number of motile sperm in the post-rut have been noted by other authors [40], and would be a consequence of spermatogenetic changes at the end of the rut, with lower number of sperm but of better quality. Recently, Gizejewski [41] studied the characteristics of red deer semen collected with artificial vagina along the year and found some traits that could relate with our results. First, the author studied the different fractions of the ejaculate (''grey'', ''white'' and ''yellow'') during the pre-mating (August), mating (September–December), transition (December–February) and post-mating (February–April) periods. He found that, during the mating season (rut and post-rut seasons in our study), the fractions were yellow and white, the latter rich in spermatozoa. However, during the post-mating period (including February) he could obtain only grey fraction (very poor in spermatozoa). Furthermore, he obtained higher sperm concentration in the first part of the mating period, corresponding to the moment of higher libido (corresponding to the rut season in this study). However, sperm motility was better in that period than in the rest of the mating period, contrarily to our findings in epididymal sperm. This issue deserves more attention, and may be related to the interaction of seminal plasma with spermatozoa after leaving the epididymis. In fact, Strzezek et al. [42] reported important changes in seminal plasma composition, even during the mating season.

In the case of roe deer, our study is necessarily incomplete, due to the lack of appropriate samples in the post-rut and non-breeding periods. However, our study suggests that sperm quality might be inferior in the post-rut, and worse in the non-breeding season, at least regarding motility. In this sense, other authors [8,23] showed that motility quickly improves before the rut and drops just after the rut, reaching very low values in the non-breeding season, and that the proportion of abnormal sperm varied almost inversely to motility values. Blottner et al. [23] found also that sperm from the caput epididymis gave better results in a denaturation-resistant chromatin assay in the rut than in the pre-rut or the post-rut, but differences were not significant when studying cauda epididymis sperm. In a preliminary report [14], comparing only pre-rut and rut periods, we found that progressive motility was significantly lower in the pre-rut. In this work, we have found a high individual variability within seasons regarding quality parameters, which could have masked some differences between pre-rut and rut.

We faced the same problem with chamois, since medians are lower in the non-breeding season, but we did not found significant differences with the breeding season. However, Anel et al. [33] found differences studying motility and acrosomal status. Again, the problem seems to be due to individual differences. Due to the low number of samples analyzed (because of the restriction of 24–48 h post-mortem), we cannot reach definite conclusions, but probably some animals can yield acceptably good sperm during the non-breeding season, whereas other undergo higher variation between both seasons. We have to highlight the presence of samples of good quality in the non-breeding season, contrarily to the case of cervids, where no samples gave acceptable quality results in that period.

In conclusion, we have found important differences between the breeding season and other periods of the year, regarding both testicular and epididymal morphometry and sperm parameters. Our results mostly agree with other studies in cervids, and contribute with novel data for chamois, which has received little attention regarding its reproductive biology. We want to highlight the consequences for BRBs funding based on post-mortem sperm recovery (for these or similar species), since season modified sperm availability and quality. Considering quality and quantity, breeding season was the better period to collect samples. For the other periods, post-rut for Iberian red deer, pre-rut for roe deer, and the non-breeding season for chamois (at least the intervals studied here) seemed to offer good opportunities to collect samples, although at the cost of less quantity and possibly lower quality (excepting red deer, with very good quality in the post-rut). The post-rut of the roe deer may be discarded because of the low quality of the samples. In the same sense, the non-breeding season is not a good period to collect samples from cervids, considering the very low sperm numbers and quality of the samples. Nevertheless, in the case of endangered species or valuable individuals, it may be worthwhile to collect sperm even in the worst season, since techniques such as sperm selection, IVF or ICSI can overcome the low numbers or the lack of functionality of sperm. However, the quality of the sperm collected at different times during the year should be tested in depth, especially its fertility, in order to confirm our findings.

Acknowledgements

This study has been supported by the Junta de Castilla y León (AB29). The authors thank Juan José Martínez, César Gómez, Juan Carlos Peral, the Territorial Service of Environmental Affairs of León, and the gamekeepers of the hunting reserves of Picos de Europa, Mampodre and Ancares (León, Spain), and Carlos Bernabé (Cáceres, Spain) for their collaboration in the collection of the samples used in this work; and Ana Rocío Díaz, Enrique Anel, Juan Daniel Muro, and Patri Martínez for their help in the processing of the samples. F. Martinez-Pastor received a grant from The Ministry of Education, Culture and Sports of Spain (AP99 12776847).

References

[1] Holt W, Bennett P, Volobouev V, Watson P. Genetic resource banks in wildlife conservation. J Zool 1996;238:531–44.

[2] Holt W, Pickard A. Role of reproductive technologies and genetic resource banks in animal conservation. Rev Reprod 1999;4:143–50.
[3] Gilmore J, McGann L, Ashworth E, Acker J, Raath J, Bush M, et al. Fundamental cryobiology of selected African mammalian spermatozoa and its role in biodiversity preservation through the development of genome resource banking. Anim Reprod Sci 1998;53:277–97.
[4] Johnston L, Lacy R. Genome resource banking for species conservation: selection of sperm donors. Cryobiology 1995;32:68–77.
[5] Turner T. On the epididymis and its role in the development of the fertile ejaculate. J Androl 1995;16:292–8.
[6] Foote R. Fertilizing ability of epididymal sperm from dead animals. J Androl 2000;21:355.
[7] Garde J, Ortiz N, Garcia A, Gallego L, Landete CT, Lopez A. Postmortem assessment of sperm characteristics of the red deer during the breeding season. Arch Androl 1998;41:195–202.
[8] Goeritz F, Quest M, Wagener A, Fassbender M, Broich A, Hildebrandt T, et al. Seasonal timing of sperm production in roe deer: interrelationship among changes in ejaculate parameters, morphology and function of testis and accessory glands. Theriogenology 2003;59:1478–502.
[9] Kaabi M, de Paz P, Alvarez M, Anel E, Boixo J, Rouissic H, et al. Effect of epididymis handling conditions on the quality of ram spermatozoa recovered post-mortem. Theriogenology 2003;56:1–2.
[10] Monfort S, Brown J, Bush M, Wood T, Wemmer C, Vargas A, et al. Circannual interrelationships among reproductive hormones, gross morphometry, behaviour, ejaculate characteristics and testicular histology in Eld's deer stags (*Cervus eldi thamin*). J Reprod Fertil 1993;98:471–80.
[11] Suzuki M, Kaji K, Nigi H. Annual changes of testis size, seminiferous tubules and plasma testosterone concentration of wild Sika deer (*Cervus nippon yesoensis* Heude, 1884) in Hokkaido. J Vet Med Sci 1992;54:551–6.
[12] Blottner S, Hingst O, Meyer H. Inverse relationship between testicular proliferation and apoptosis in mammalian seasonal breeders. Theriogenology 1995;44:321–7.
[13] Gerlach T, Aurich J. Regulation of seasonal reproductive activity in the stallion, ram and hamster. Anim Reprod Sci 2000;58:197–213.
[14] Anel L, Guerra C, Alvarez M, Anel E, Martinez A, Rodriguez C, et al. Post-mortem spermatozoa recovery in roe deer (*Capreolus capreolus*): differences between pre-rutting and rutting season. Theriogenology 2001;55:380.
[15] Morai RN, Mucciolo RG, Gomes ML, Lacerda O, Moraes W, Moreira N, et al. Seasonal analysis of semen characteristics, serum testosterone and fecal androgens in the ocelot (*Leopardus pardalis*), margay (*L. wiedii*) and tigrina (*L. tigrinus*). Theriogenology 2002;57:2027–41.
[16] Ciereszko A, Ottobre J, Glogowski J. Effects of season and breed on sperm acrosin activity and semen quality of boars. Anim Reprod Sci 2000;64:89–96.
[17] Karagiannidis A, Varsakeli S, Karatzas G. Characteristics and seasonal variations in the semen of alpine, saanen and damascus goat bucks born and raised in Greece. Theriogenology 2000;53:1285–93.
[18] Alm K, Taponen J, Dahlbom M, Tuunainen E, Koskinen E, Andersson M. A novel automated fluorometric assay to evaluate sperm viability and fertility in dairy bulls. Theriogenology 2001;56:677–84.
[19] Blottner S, Warnke C, Tuchscherer A, Heinen VV, Torner H. Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. Anim Reprod Sci 2001;65:75–88.
[20] Chacon J, Perez E, Rodriguez-Martinez H. Seasonal variations in testicular consistency, scrotal circumference and spermiogramme parameters of extensively reared brahman (*Bos indicus*) bulls in the tropics. Theriogenology 2002;58:41–50.
[21] Webster J, Suttie J, Corson I. Effects of melatonin implants on reproductive seasonality of male red deer (*Cervus elaphus*). J Reprod Fertil 1991;92:1–11.
[22] Lincoln G. Photoperiod-melatonin relay in deer. Acta Vet Hung 1998;46:341–56.
[23] Blottner S, Hingst O, Meyer H. Seasonal spermatogenesis and testosterone production in roe deer (*Capreolus capreolus*). J Reprod Fertil 1996;108:299–305.
[24] Lincoln G. Reproductive seasonality and maturation throughout the complete life-cycle in the mouflon ram (*Ovis musimon*). Anim Reprod Sci 1998;53:87–105.
[25] Hoffmann B, Landeck A. Testicular endocrine function, seasonality and semen quality of the stallion. Anim Reprod Sci 1999;57:89–98.

[26] Lincoln G, Kay R. Effect of season of LH and testosterone in intact and castrated red deer stag (*Cervus elaphus*). J Reprod Fertil 1979;55:75–80.
[27] Gosch B, Fischer K. Seasonal changes of testis volume and sperm quality in adult fallow deer (*Dama dama*) and their relationship to the antler cycle. J Reprod Fertil 1989;85:7–17.
[28] Brown J, Wildt D, Raath J, de Vos V, Howard J, Janssen D, et al. Impact of season on seminal characteristics and endocrine status of adult free-ranging African buffalo (*Syncerus caffer*). J Reprod Fertil 1991;92:47–57.
[29] Soler A, Pérez-Guzmán M, Garde J. Storage of red deer epididymides for four days at 5 °C: effects on sperm motility, viability, and morphological integrity. J Exp Zool 2003;295A:188–99.
[30] Jeyendran R, Van der Ven H, Perez-Pelaez M, Crabo B, Zaneveld L. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil 1984;70:219–28.
[31] Saacke R, White J. Semen quality test and their relationship to fertility. In: Proceedings of the Fourth Technical Conference of Artificial Insemination Reproduction NAAB, p. 22.
[32] Brito LF, Barth AD, Bilodeau-Goeseels S, Panich PL, Kastelic JP. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. Theriogenology 2003;60:1539–51.
[33] Anel L, Guerra C, Alvarez M, Kaabi M, Anel E, Boixo J, et al. Basic parameters in spermatozoa recovered post-mortem from the Spanish Cantabrian chamois (*Rupicapra pyrenaica parva*). Theriogenology 2000;53:323.
[34] Ditchkoff S, Spicer L, Masters R, Lochmiller R. Concentrations of insulin-like growth factor-I in adult male white-tailed deer (*Odocoileus virginianus*): associations with serum testosterone, morphometrics and age during and after the breeding season. Comp Biochem Physiol A 2001;129:887–95.
[35] Rolf H, Fischer K. Serum testosterone, 5-alpha-dihydrotestosterone and different sex characteristics in male fallow deer (*Cervus dama*): a long-term experiment with accelerated photoperiods. Comp Biochem Physiol A Physiol 1996;115:207–21.
[36] Hochereau-de Reviers M, Lincoln G. Seasonal variation in the histology of the testis of the red deer, *Cervus elaphus*. J Reprod Fertil 1978;54:209–13.
[37] Blottner S, Roelants H. Calculation of spermatogenic transformations based on dual-flow cytometric analysis of testicular tissue in seasonal breeders. Andrologia 1998;30:331–7.
[38] Blottner S, Roelants H. Quantification of somatic and spermatogenic cell proliferation in the testes of ruminants, using a proliferation marker and flow cytometry analysis. Theriogenology 1998;49:1275–87.
[39] Roelants H, Schneider F, Göritz F, Streich J, Blottner S. Seasonal changes of spermatogonial proliferation in roe deer, demonstrated by flow cytometric analysis of c-kit receptor, in relation to follicle-stimulating hormone, luteinizing hormone, and testosterone. Biol Reprod 2002;66:305–12.
[40] McMillan W, Shackell G, Vishwanath R, Fielden E, Smith J. Comparative reproductive performance. Reprod Manage Grazing Rumin NZ 1998;12:43–64.
[41] Gizejewski Z. Effect of season on characteristics of red deer (*Cervus elaphus* L.) semen collected using modified artificial vagina. Reprod Biol 2004;4:51–66.
[42] Strzezek J, Krzywinski A, Swidowicz K. Seasonal changes in the chemical composition of red deer (*Cervus elaphus*) semen. Anim Reprod Sci 1985;9:195–204.