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Post mortem time and season alter subpopulation characteristics of Iberian red deer epididymal sperm

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Abstract

We have studied the effect of post mortem time and season on sperm subpopulation pattern and characteristics. We used epididymal samples from free-ranging Iberian red deers harvested during the hunting season. We studied samples at different moments of the year (rut, transition period and post-rut), and at different times post mortem (up to 4 days). Sperm were extracted from the cauda epididymis and their motility was evaluated by means of a CASA system. A principal component and clustering analysis were carried out to identify subpopulations. Post mortem time caused a significant decrease in motility quality, and a general deterioration in subpopulation characteristics. We found three subpopulations the first day, and the one indicating good sperm quality decreased with post mortem time until it disappeared on the fourth day. This may indicate considerable impairment of the samples after 72 h post mortem, which could compromise their use in AI programs. With regard to season, subpopulation pattern and characteristics were better in the transition and post-rut periods. Moreover, we found one subpopulation formed by mature spermatozoa, which increased from rut to post-rut. This might be a negative fact, because samples collected after the rut may undergo hypermaturation, which possibly impairs fertility. Our results are of interest for the management of wildlife germplasm banks based on post mortem sperm recovery.

Keywords: Post mortem; Seasonality; Epididymis; Sperm subpopulations; Red deer

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1. Introduction

The use of germplasm banks has enabled the genetic wealth of many domestic and wild species to be preserved, and nowadays there are both economical and conservational reasons for creating and developing them [1]. These banks must be set up with great care, especially when the aim in to keep material from wild species, as there are many factors complicating the acquisition and use of the germplasm. In this case, sperm recovered post mortem from the cauda epididymis has become a widely used source [2], since collection by other means is often very difficult or unaffordable. Also, if the species are hunted, a considerable number of samples may be available each year, which can eventually be used to restore populations challenged by inbreeding or genetic drift.

However, since animals usually die (hunted or accidentally) far away from laboratories, post mortem time is a factor to be taken into account. Many studies have considered this issue, and conclude that epididymal sperm is clearly affected by the time elapsed between the death of the animal and its cryopreservation (for instance, [3–6]). It is generally agreed that only a few hours are enough to impair epididymal sperm, although its quality can be acceptable for several days. Thus, post mortem time can be an important challenge if we intend to use these sperm samples in AI programs.

Seasonality is another factor of special relevance when dealing with wild species, although it also affects domestic species. This phenomenon is due to complex hormonal interactions, ultimately based on daylength, which differ amongst breeding and non-breeding periods in the year [7–9]. Moreover, its importance depends highly on species and location (mainly the latitude) [10], and it induces changes in behavior, morphology and reproductive ability [11–13]. Thus, the effect of season on the characteristics of epididymal sperm must be assessed in order to determine whether samples of a concrete season are adequate for storage and future use.

At the time of the writing of this paper, we have carried out two studies on epididymal sperm from red deer, roe deer and chamois [14,15], to determine the effect of post mortem time and seasonality, respectively. The present study was aimed at complementing these, since the motility analysis carried out in these studies was limited. We have analyzed many samples from Iberian red deer with a modern CASA system that allowed us to identify individual spermatozoa. Then, we used multivariate statistical techniques in order to differentiate sperm subpopulations in our samples, depending on sperm motility.

It is widely accepted that sperm samples are not homogeneous, and that sperm subpopulations can be identified using different parameters [16–20]. The use of motility descriptors for performing such studies has rendered interesting information, which would not be evident if only the mean values of the whole motile population were used [21–24]. In fact, motility subpopulations may act as markers for good or bad sample quality [25], and some authors have found relationships between the presence of determined subpopulations and sample fertility [26]. The study of sperm subpopulations is relatively recent and still little explored, but it represents an important source of novel information on sperm biology not available from other kinds of analyses.

Another reason for carrying out this work is the importance of the studied species. Iberian red deer are a valuable trophy in Spain, and there are plans to establish germplasm banks for this and other wild ruminant species. They are highly seasonal (including a

period of complete reproductive arrest), and the period of time between hunting and sample processing (post mortem time) can be highly variable. We believe that this type of study would provide useful information for the management of any future germplasm banks from these or related species.

2. Materials and methods

All chemicals were acquired from Sigma (Madrid, Spain). Media were not bought as such, but prepared in our laboratory as referred.

2.1. Genitalia collection and sperm recovery

Genitalia were collected from 71 Iberian red deer (*Cervus elaphus hispanicus*, Helzheimer 1909) harvested in the game reserves of Ancares, Mampodre and Picos de Europa (León, Spain) and in several private hunting reserves of the region of Cáceres (Spain). All the animals were adults and lived in a free-ranging regime. Sample collection was carried out from the second fortnight of September to the first fortnight of December.

Harvest plans followed Spanish Harvest Regulation, Law 4/96 of Castilla y León and Law 19/01 of Extremadura, which confirms 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 by 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), arriving at different times post mortem.

Sample manipulation was carried out in a walk-in fridge (5 °C). Testicles with epididymes and vas deferens attached were isolated from the scrotum and other tissues. Epididymes were dissected free from the testicles, and cleaned of connective tissue. To avoid blood contamination, superficial blood vessels were previously cut and their contents wiped out. Season and post mortem time were attached to each sample. Spermatozoa were collected making several incisions on the cauda epididymis with a surgical blade, and taking the liquid emerging from the cut tubules with the aid of the blade.

2.2. Computer assisted sperm analysis

Sperm were diluted down to 10–20 $\times$ 10^6 spermatozoa/mL in a buffered solution (20 mmol/L Hepes, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose; pH 7, 400 mOsm/kg), and warmed on a 37 °C plate for 20 min. Then, a pre-warmed Makler

113 counting chamber (10 μm depth) was loaded with 5 μL of sample. The CASA system
114 consisted on an optical phase contrast microscope (Nikon Labophot-2, equipped with
115 negative phase contrast objectives and a warming stage at 37 °C), a Sony XC-75CE camera
116 and a PC with the Sperm Class Analyzer software (SCA2002, Microptic, Barcelona,
117 Spain). The magnification was 10×. All samples were analyzed at least twice, in order to
118 discard errors due to incorrect sampling. At least five fields per sample were acquired,
119 giving at least 100 motile sperm (whenever possible, more than 200 motile sperm were
120 acquired). Image sequences were saved and analyzed afterwards. CASA acquisition
121 parameters were: 25 images acquired, at an acquisition rate of 25 images per second. For
122 each sperm analyzed, the SCA2002 rendered the following data: velocity according to the
123 actual path (VCL; μm/s), velocity according to the straight path (VSL; μm/s), velocity
124 according to the average – smoothed – path (VAP; μm/s), linearity (LIN; %), straightness
125 (STR; %), wobble (WOB; %), amplitude of the lateral displacement of the sperm head
126 (ALH; μm) and frequency of the flagellar beat (BCF; Hz). Detailed explanation of these
127 descriptors of sperm movement is provided elsewhere [25,27–29].

128 *2.3. Data preparation and statistical analysis*

129 Firstly, CASA data were pooled in a common database. Each observation (spermatozoa)
130 was identified by three fields (male, post mortem time and season). After determining total
131 sample motility (TM: percentage of spermatozoa with VCL $>$ 10 μm/s), only samples
132 with TM $>$ 10% were used in the rest of the analyses. This was decided in order to avoid
133 bias due to the presence of a lot of samples from different males, which contributed with
134 very few motile sperm to the database. The database was duplicated, and each copy was
135 utilized either for post mortem time analysis or for season analysis, and modified
136 accordingly.
137 In order to study both factors, we had to decide previously the periods we were to divide
138 post mortem time and season. Since we knew that both post mortem time and seasonality
139 affected sperm quality, we had to filter each database, in order to prevent interferences.
140 First, we determined the seasonality periods. As we could only obtain samples during the
141 hunting season, which in our case was limited to the autumn, we divided this season in
142 several periods, depending on the breeding activity of Iberian red deer. According to
143 wardens' records, rut comprised from the end of summer to mid-autumn, although mating
144 could still be observed until winter, but with decreasing frequency. Thus, and also taking
145 into account other previous observations [15], we decided to consider the second fortnight
146 in September and the first one in October as the rut season, the second fortnight in October
147 and November as the transition period, and the first fortnight in December as the post-rut
148 period.
149 Taking this division into account, we used data from the rut period only to study the
150 effect of post mortem time. These data were divided into 1-day intervals (we could not get
151 any samples below 19 h post mortem, so the first interval included only 19–24 h post
152 mortem), resulting in four intervals of 24 h each. Samples with higher post mortem times
153 (>96 h) were discarded because of very bad quality (generally almost no motility).
154 Only samples with post mortem times from 19 to 30 h were accepted for the seasonality
155 study. We decided that this period would be adequate because it included a fairly large

number of samples, and variation due to post mortem time would be acceptable, according to a previous study [14]. Samples were then assigned to the three periods defined as rut, transition and post-rut.

All statistical analyses were carried out using the SAS/STAT™ package V. 8 (SAS Institute, Cary, NC) [30]. Where applicable, $P < 0.05$ was considered as statistically significant, unless otherwise stated. As the clustering analysis we followed has been extensively described in a previous study [31], we will simply summarize it here (SAS™ procedure names are given just for reproducibility). As a first step, we used the PRINCOMP procedure in order to perform a principal component analysis (PCA) of the motility data. Principal components with eigenvalues higher than 1 (Kaiser criterion) were used in the subsequent analysis instead of motility descriptors. Then, we carried out a non-hierarchical cluster analysis using the FASTCLUS procedure, which performs a disjoint cluster analysis using euclidean distances (*k*-means model) to calculate cluster centers. We chose 15 clusters to be produced by this procedure, which were passed to the CLUSTER procedure as initial clusters, in order to perform a hierarchical clustering on them (average linkage method, UPGMA). The TREE procedure was used to draw a dendrogram of the hierarchical clustering process and an output with the final number of clusters. The final number of clusters was decided according to the pseudo t^2, the pseudo F and the cubic clustering criterion (CCC) statistics, produced in each step of the hierarchical clustering (good numbers of clusters are indicated by local peaks of the CCC and pseudo F statistics combined with a small value of pseudo t^2 and a larger pseudo t^2 for the next cluster fusion). Fig. 1 shows an example of dendrogram and the corresponding plot used for choosing the final number of clusters.

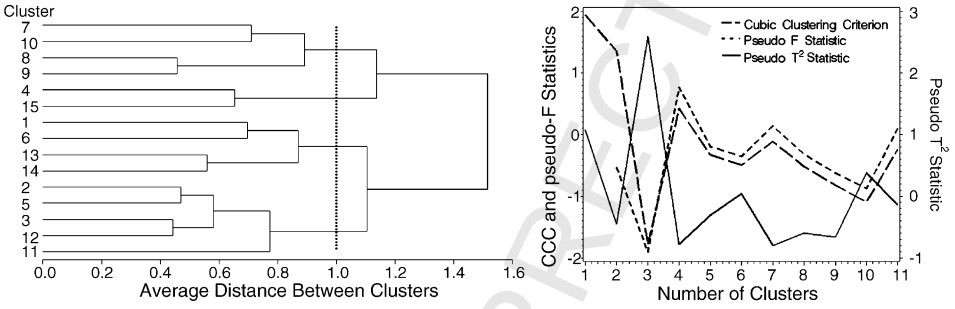

Fig. 1. Example of a dendrogram derived from the hierarchical clustering analysis, and of the plot used for the determination of an adequate final number of clusters. The plot on the left shows the dendrogram resulting from the hierarchical clustering of the 15 clusters derived of the PCA and subsequent non-hierarchical clustering of motility data (this example corresponds to the 48–72-h interval of the post mortem time study). The plot on the right shows the line plot of CCC, pseudo F and pseudo t^2 statistics produced by the hierarchical clustering procedure, which helped to find possible cut places in the dendrogram (the three variables were standardized in order to show all of them in the same plot). Each step in the hierarchical clustering joins two clusters in a new one, and generates new values for these three statistics. Suitable number of clusters are indicated by local high values of the CCC and pseudo F statistics and low values of pseudo t^2 statistic, followed with decreasing CCC and pseudo F and increasing pseudo t^2 in the next cluster fusion. In this case, the plot clearly suggests a final number of four clusters (indicated in the dendrogram), although two or seven would be also eligible.

A descriptive statistic of each cluster in each post mortem period or season, and the analysis of PCA allowed us to carry out a preliminary characterization. Therefore, clusters were compared between post mortem periods or seasons. For this comparison, we used the general linear models procedures (GLM). In the case of post mortem time, the factors included in the model were the post mortem period (days 1–7) and male nested within the post mortem period. In the case of season, factors were the season (rut, transition and post-rut) and male nested within the season. When the model was significant for the post mortem period or the season, the respective classes were compared using adjusted least-squares means (LSMEANS statement). Previously, variables were transformed for normality (log: VCL, VSL, VAP, ALH, BCF; arc sine: LIN, STR, WOB). Comparisons of cluster proportions between post mortem intervals or seasons were carried out by means of the χ^2 test. When assumptions for χ^2 test were violated, the exact Fisher's test was used instead.

On the other hand, motility was also studied in a ''classical'' way (not considering subpopulations), determining the mean values of the motility descriptors for each sample and carrying out a comparison using the general linear models as explained above.

3. Results

3.1. Sperm motility (general results)

Twenty-one samples complied with the condition for entering the post mortem time study (collected during the rut season and no more than 96 h post mortem), and 39 samples (19–30 h post mortem) were used for the season study. As Table 1 and Fig. 2 show, there were important differences between post mortem intervals and between seasons. The percentage of motile spermatozoa decreased clearly with post mortem time, the fourth day being only half the value of the ?rst. The motility descriptors underwent a similar evolution, although they seemed to drop more quickly just after 48 h post mortem and remain more stable afterwards. As for seasonality, transition and, in particular, post-rut periods rendered higher results than rut, either considering the proportion of motile sperm or the motility descriptors.

Table 1
Values of some motility descriptors depending on post mortem time and season

	Period	TM (%)[a]	VAP (μm/s)	LIN (%)	ALH (μm)	BCF (Hz)
Post mortem time (h)	0–24	70.63 a	30.33 ± 0.72 a	50.48 ± 1.05 a	2.03 ± 0.04 a	8.00 ± 0.17 a
	24–48	60.32 b	29.26 ± 0.65 b	45.68 ± 0.96 b	1.88 ± 0.04 b	6.30 ± 0.16 b
	48–72	45.06 c	13.00 ± 1.02 c	35.26 ± 1.49 c	1.22 ± 0.06 c	2.87 ± 0.25 c
	72–96	33.8 d	12.30 ± 1.14 c	38.97 ± 1.69 c	1.08 ± 0.07 d	4.09 ± 0.28 d
Season	Rut	61.98 a	29.63 ± 0.83 a	50.56 ± 0.89 a	1.92 ± 0.04 a	7.59 ± 0.15 a
	Transition	82.29 b	36.47 ± 0.62 b	49.02 ± 0.67 a	2.33 ± 0.03 b	7.09 ± 0.11 a
	Post-rut	86.22 c	43.31 ± 0.74 c	58.09 ± 0.79 b	2.17 ± 0.04 c	7.88 ± 0.14 b

Data are expressed as adjusted least-squares mean ± S.E.M. Rows (within experiment, post mortem or season) with different letters (a–d) differ $P < 0.05$.

[a] Proportion of motile sperm (VCL > 10 μm/s).

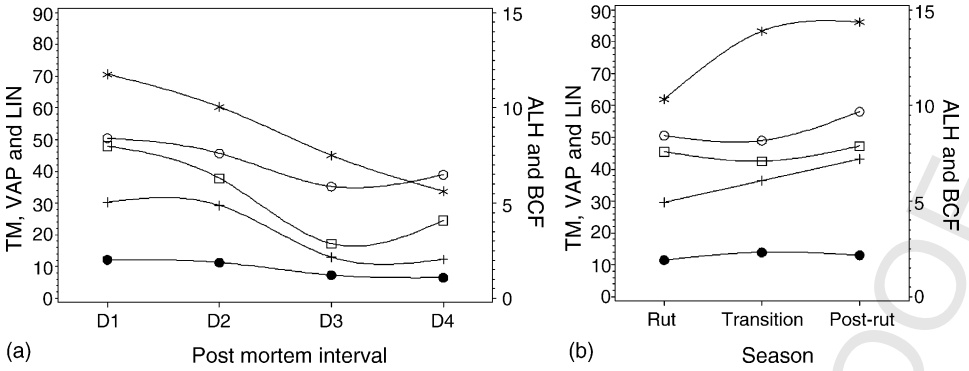

Fig. 2. Evolution of several motility parameters depending on post mortem time and season. For post mortem intervals, D1: 0–24 h; D2: 24–48 h; D3: 48–72 h; D4: 72–96 h. Note that the scales for TM (%), VAP (μm/s) and LIN (%), and ALH (μm) and BCF (Hz) are different. TM: (*); VAP: (+); LIN: (○); ALH: (●); BCF: (□).

206 *3.2. Effect of post mortem time on sperm subpopulations*

207 Cluster analysis rendered three clusters per post mortem interval, excepting for the
208 fourth day, with four clusters. Considering their motility descriptors and the results of
209 PCA, we concluded that there were four different kinds of clusters, which were called
210 CL1p, CL2p, CL3p and CL4p. In general, cluster distribution varied considerably
211 between post mortem intervals (Fig. 3(a)). Statistics of the clusters are shown in Table 2
212 and Fig. 4.

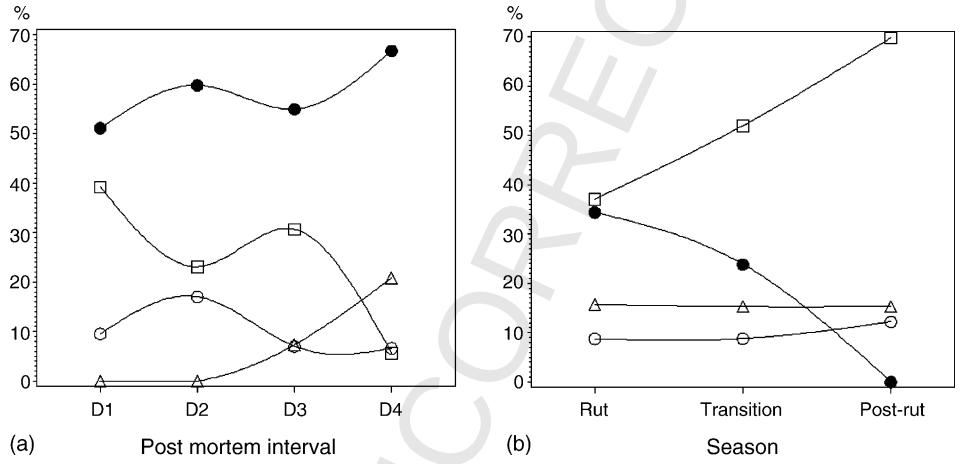

Fig. 3. Changes of the proportions of motility subpopulations depending on post mortem interval or season. For post mortem intervals, D1: 0–24 h; D2: 24–48 h; D3: 48–72 h; D4: 72–96 h. CL1: (●); CL2: (□); CL3: (○); CL4: (△).

Table 2
Values of some motility descriptors corresponding to the clusters obtained from the post mortem time analysis (CL1p–CL4p)

Cluster	Post mortem time (h)	(%)[a]	VAP (μm/s)	LIN (%)	ALH (μm)	BCF (Hz)
CL1p	0–24	51.1 a	23.22 ± 0.47 a	39.54 ± 0.87 a	1.93 ± 0.03 a	6.50 ± 0.18 a
	24–48	59.8 b	15.65 ± 0.52 b	35.78 ± 0.97 b	1.40 ± 0.04 b	4.55 ± 0.20 b
	48–72	55.0 a	8.03 ± 0.55 c	24.99 ± 1.01 c	1.08 ± 0.04 c	1.93 ± 0.21 c
	72–96	66.8 c	7.81 ± 0.76 c	27.07 ± 1.40 c	0.98 ± 0.05 c	2.99 ± 0.30 d
CL2p	0–24	39.3 a	37.45 ± 1.63 a	70.54 ± 1.77 a	1.73 ± 0.07 a	9.40 ± 0.41 a
	24–48	23.1 b	40.26 ± 1.45 b	74.33 ± 1.57 b	1.66 ± 0.07 b	7.68 ± 0.36 b
	48–72	30.7 c	28.22 ± 2.14 c	59.52 ± 2.32 c	1.68 ± 0.10 a,b	8.32 ± 0.53 a,b
	72–96	5.7 d	30.55 ± 5.59 a,b,c	71.18 ± 5.96 a,b,c	1.66 ± 0.25 a,b	7.82 ± 1.38 a,b
CL3p	0–24	9.6 a	43.68 ± 2.36 a	44.83 ± 1.95 a	3.32 ± 0.14 a	10.94 ± 0.52 a
	24–48	17.1 b	45.00 ± 1.88 a	34.39 ± 1.56 b	3.47 ± 0.11 a	8.31 ± 0.42 b
	48–72	7 c	53.68 ± 5.08 a	26.72 ± 4.20 c	3.91 ± 0.30 a	6.14 ± 1.12 b
	72–96	6.6 d	30.99 ± 6.02 b	47.66 ± 5.02 a	2.22 ± 0.35 b	9.72 ± 1.34 a,b
CL4p	48–72	7.4 a	11.91 ± 0.96 a	78.09 ± 2.36 a	0.84 ± 0.05 a	1.39 ± 0.32
	72–96	20.9 b	7.05 ± 0.68 b	62.23 ± 1.94 b	0.60 ± 0.04 b	1.89 ± 0.27

Post mortem time is given in hours. Data are expressed as adjusted least-squares means ± S.E.M. Data are expressed as percentages, and comparisons were performed using the χ^2 test on raw data. Rows (post mortem intervals within clusters) with different letters (a–d) differ $P < 0.05$.

[a] Proportion of each subpopulation respect to the total number of motile sperm (VCL > 10 μm/s).

213 *3.2.1. Subpopulation 1 (CL1p)*

214 CL1p comprised slow and non-linear sperm, with low ALH and BCF values.
215 All the motility descriptors were affected by post mortem time, although they stabilized
216 with low values after 48 h post mortem (except for the increase in BCF). Considering its
217 relative proportion, it increased from 51% in the first interval to almost 67% on the last
218 day.

219 *3.2.2. Subpopulation 2 (CL2p)*

220 CL2p was characterized by high linearity and BCF. Its proportion showed a decreasing
221 trend with increasing post mortem time. Although its motility descriptors varied between
222 post mortem intervals, there was not a trend indicating progressive changes.

223 *3.2.3. Subpopulation 3 (CL3p)*

224 CL3p showed the highest velocity, ALH and BCF values, but it was not as linear as
225 CL2p. VAP and ALH were little affected by post mortem time (decreasing only in the last
226 interval), but LIN and BCF decreased greatly in the 48–72-h interval, although they
227 recovered their initial values in the last interval.

228 *3.2.4. Subpopulation 4 (CL4p)*

229 CL4p was found only in the last two intervals, comprising more than 20% of motile
230 sperm in the 72–96-h interval. It was characterized by motility descriptors with very low
231 values, but with high linearity.

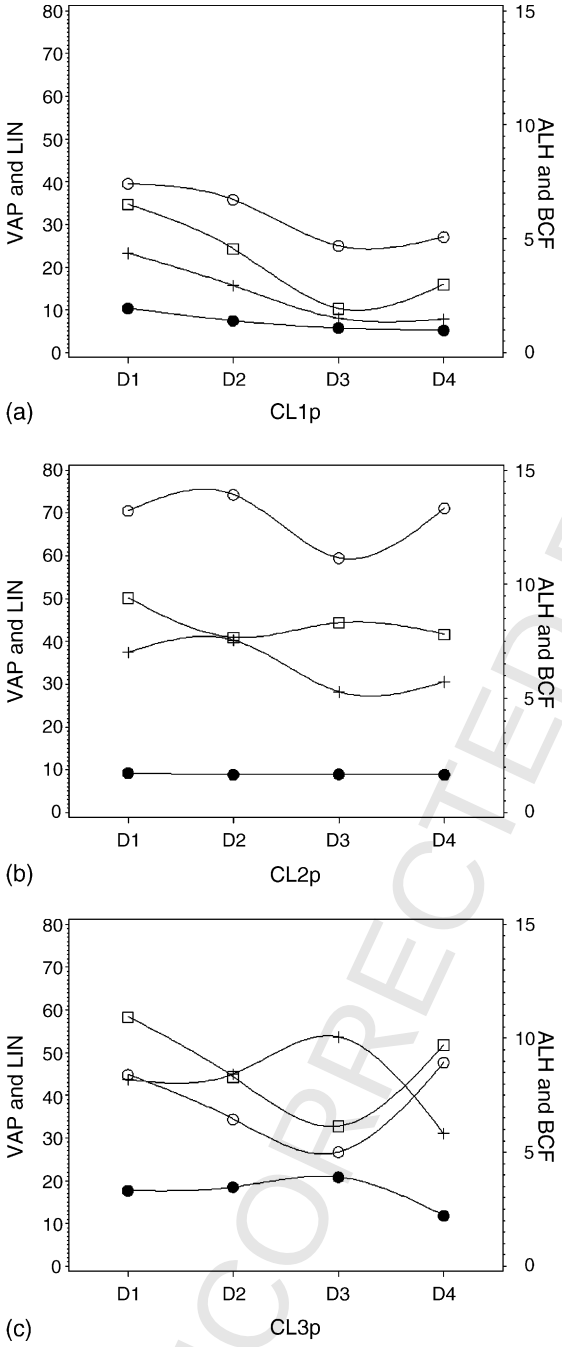

Fig. 4. Motility parameters of CL1p, CL2p and CL3p, depending on post mortem time. For post mortem intervals, D1: 0–24 h; D2: 24–48 h; D3: 48–72 h; D4: 72–96 h. Note that the scales for VAP (μm/s) and LIN (%), and ALH (μm) and BCF (Hz) are different. VAP: (+); LIN: (○); ALH: (●); BCF: (□).

Table 3
Values of some motility descriptors corresponding to the clusters obtained from the season analysis (CL1s–CL4s)

Cluster	Season	(%)[a]	VAP (μm/s)	LIN (%)	ALH (μm)	BCF (Hz)
CL1s	Rut	34.4 a	28.08 ± 0.75 a	39.14 ± 1.10 a	2.27 ± 0.04 a	8.31 ± 0.30 a
	Transition	23.9 b	43.55 ± 2.22 b	28.97 ± 1.39 b	3.93 ± 0.15 b	7.07 ± 0.36 b
CL2s	Rut	37.1 a	33.64 ± 1.32	74.42 ± 1.49 a	1.60 ± 0.06 a	7.97 ± 0.34 a
	Transition	52.0 b	38.25 ± 0.78	64.46 ± 0.88 b	1.98 ± 0.04 b	8.74 ± 0.20 b
	Post-rut	69.9 c	38.59 ± 0.83	66.88 ± 0.94 b	1.83 ± 0.04 c	8.95 ± 0.21 b
CL3s	Rut	8.8 a	53.81 ± 1.88 a	57.73 ± 1.57 a	2.95 ± 0.12	13.29 ± 0.40 a
	Transition	8.8 a	89.14 ± 3.39 b	74.98 ± 2.83 b	3.15 ± 0.21	10.43 ± 0.72 b
	Post-rut	12.2 b	77.95 ± 1.94 c	77.90 ± 1.62 b	2.83 ± 0.12	10.56 ± 0.41 b
CL4s	Rut	15.7	10.42 ± 1.12 a	33.37 ± 1.30 a	1.13 ± 0.09 a	2.63 ± 0.32 a
	Transition	15.3	13.44 ± 0.52 b	27.72 ± 0.60 b	1.57 ± 0.04 b	3.46 ± 0.15 b
	Post-rut	15.3	21.99 ± 0.64 c	25.32 ± 0.74 c	2.25 ± 0.05 c	4.80 ± 0.18 c

Data are expressed as adjusted least-squares mean ± S.E.M. Data are expressed as percentages, and comparisons were performed using the χ^2 test on raw data. Rows (seasons within clusters) with different letters (a–c) differ $P < 0.05$.

[a] Proportion of each subpopulation respect to the total number of motile sperm (VCL > 10 μm/s).

3.3. Effect of season on sperm subpopulations

In this case, we found four different kinds of clusters (CL1s, CL2s, CL3s and CL4s), unevenly distributed between seasons (Fig. 3(b)). We obtained six clusters in the rut and post-rut periods, but two of them were removed because we considered them residual (see below). Statistics of the clusters are shown in Table 3 and Fig. 5. The variation of the proportions of the different clusters between seasons can be appreciated in Fig. 3(b).

3.3.1. Subpopulation 1 (CL1s)

CL1s showed low velocity and linearity. It comprised one-third of the spermatozoa during rut, decreased to less than 25% in the transition period, and it did not appear in the post-rut. In this case, VAP and ALH were higher in the transition period, whereas LIN and BCF were higher in the rut.

3.3.2. Subpopulation 2 (CL2s)

CL2s was characterized by medium velocity and high linearity, accompanied by low ALH and high BCF. Its presence was important in all the three seasons, increasing from more than one-third of the motile sperm in the rut, to more than two-thirds in the post-rut. Its parameters were also similar in the three seasons, with only a significant decrease in linearity with regard to rut.

3.3.3. Subpopulation 3 (CL3s)

CL3s was similar to CL2s, that is, fast and linear, with lower ALH and higher BCF. Thus, it contained mainly spermatozoa with very linear trajectories, and was found in the three seasons in similar proportions. Its parameters were similar in the transition and post-rut seasons, when they were higher than in the rut.

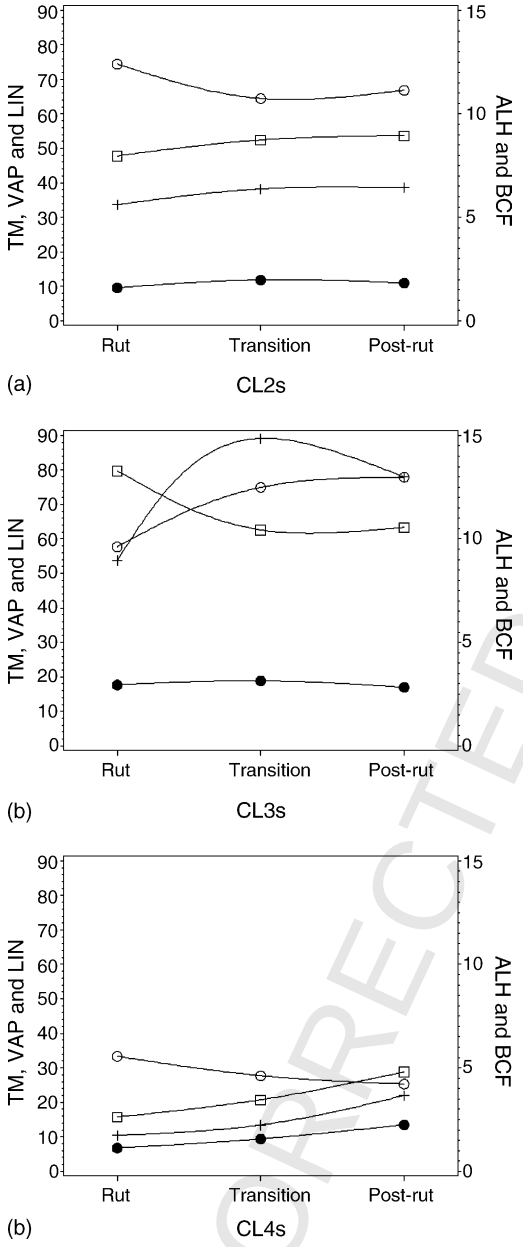

Fig. 5. Motility parameters of CL2s, CL3s and CL4s, depending on season. Note that the scales for VAP (μm/s) and LIN (%), and ALH (μm) and BCF (Hz) are different. VAP: (+); LIN: (○); ALH: (●); BCF: (□).

254

254 *3.3.4. Subpopulation 4 (CL4s)*
255 CL4s included slow and non-linear sperm, with low ALH and BCF. However, its VAP,
256 ALH and BCF improved greatly in the post-rut. This subpopulation included over 15% of
257 spermatozoa in all the three seasons.

258 *3.3.5. Other subpopulations*
259 We found also two extra subpopulations in the rut and other two in the post-rut. In one
260 case, it seemed to be the same subpopulation (medium velocity and non-linear, both in the
261 rut and post-rut), but then other two subpopulations were very different (slow and non-
262 linear in the rut and fast and linear in the post-rut). They also appeared in very low
263 proportions (below 3%). This suggested that these subpopulations were in fact "outlier"
264 clusters, due to the grouping of spermatozoa with extreme values, which segregated in the
265 clustering process as independent clusters and were not included in further analysis.

266 **4. Discussion**

267 It is well known that epididymal sperm motility is deeply affected by storage time.
268 Numerous authors [3,5,6,32], working on different species, have reported that several
269 motility parameters are altered as soon as few hours post mortem, and that these motility
270 changes occurred before other characteristics, such as morphology or viability, were
271 affected. In a prior study on the effect of post mortem time on epididymal sperm from
272 Iberian red deer and roe deer [14], we found that the percentages of motile and progressive
273 spermatozoa dropped just after the first 24 h post mortem (although we did not find
274 significant differences before 48 h). In this work, we have been able to confirm a decrease
275 in motility with post mortem time, not only in the percentage of motile sperm, but also in its
276 quality (subpopulation pattern). We have to point out that our earliest sample was 19 h post
277 mortem, and, ideally, we should recover and process the sperm as soon as possible, since
278 some authors reported loss of quality and fertilizing ability within few hours post mortem
279 [33,34]. However, Kaabi et al. [6] did not find significant differences between the cleavage
280 rates of oocytes fertilized with ram sperm, which had been salvaged from epididymis either
281 <2 or 24 h post mortem, although it was clearly lower when sperm was recovered at 48 h
282 post mortem.
283 The subpopulation pattern that we have found in the first interval of the post mortem
284 time study could be explained considering sperm maturation. Many authors have focused
285 on this subject [35–39], reporting that motility increases during the transit of spermatozoa
286 through the different parts of the epididymes, gaining velocity and linearity. Furthermore,
287 between the proximal and distal cauda there is an increase in linearity, but not of other
288 parameters, due to biochemical changes related to epididymal maturation [37,40].
289 Regarding the first interval, CL2p (medium velocity and linear) would represent a
290 subpopulation containing mature spermatozoa, whereas CL1p (slow and non-linear)
291 would be formed mainly by spermatozoa which had not complete their epididymal
292 maturation. Moreover, CL3p (rather rapid, but little linear) would contain mature
293 spermatozoa undergoing some kind of activation, considering its high VAP, BCF and
294 ALH, and its lower LIN. In fact, Yeung et al. [40] found that an increase in cAMP brought

about the maturation of epididymal sperm, but it also induced hyperactivation in already matured spermatozoa.

The increase in CL1p with post mortem time, and its decreasing quality, reflects the effect of post mortem time on spermatozoa. Not only did the general proportion of motile spermatozoa and its quality decrease, but the effect of time reflected on the subpopulation pattern too. Considering the evolution of CL2p and CL3p, the trend indicated a steady decrease. In fact, the sudden drop in CL2p and increase in CL3p in the 24–48-h interval seemed to be a consequence of individual variation, rather than an actual consequence of post mortem time. This may also be the cause of the alteration of VAP and LIN in CL2p and CL3p in 48–72 h. On the other hand, the drop in CL2p during the last interval would indicate a definitive loss of this subpopulation. Other studies [41] have found motility patterns similar to CL2p to be compatible with the achievement of fecundation, in the case of epididymal sperm. Thus, CL2p could be a marker of good condition for a sample, and the previously-mentioned drop possibly suggests that such a long post mortem time may impair the samples so notably that salvaging would not be worthwhile. However, it would depend on the sample (non-endangered versus endangered species or rare individuals) and the techniques we used (such as ICSI, which does not depend on sperm motility).

The lost of CL2p is related to the appearance of CL4p, in the third interval. In fact, the evolution of the four subpopulations indicated that members of CL2p were mostly shifting to CL4p (very slow but linear), which is similar to CL2p on LIN values. This suggests that CL4p spermatozoa may be exhausted spermatozoa, formerly belonging to CL2p. The noticeable presence of this subpopulation indicates bad quality in a sample, in the same way that CL2p might indicate good quality.

Abaigar et al. [23] carried out an experiment on Mohor gazelle (*Gazella dama mhorr*), also based on the analysis of sperm subpopulations. Part of it consisted of the study of the variation in subpopulations patterns, depending on storage time (from 0 to 96 h). It is difficult to compare our results with those of these authors, since they worked on different species, and used ejaculated semen (with very high velocities and low linearity, in comparison with our samples). However, they also found that the proportions of some populations increased and decreased throughout the storage period. These authors related such variations to functional changes associated with prolonged storage, coinciding with our data. Unfortunately, they only showed the proportions of the subpopulations, but not the values of the motility descriptors for each subpopulation and storage time; thus, we cannot evaluate the implications for our work.

The other part of our work dealt with the effect of seasonality on motility. Considering the general data, it is evident that motility was better in the transition and post-rut periods. In another study [15], we studied some parameters of epididymal sperm from red deer (including visual assessment of motility, viability and acrosomal status) and also obtained better results from the period between mid-October to mid-December than during the rut. There are other references on the increase of the percentage of motile sperm after the rut has finished [42]. A possible explanation is based in the idea of sperm hypermaturation in the epididymis because of prolonged storage. Rodriguez and Bustos Obregon [43] carried out a study on stallion semen, obtained during both the non-breeding and breeding seasons, observing that sperm chromatin packing was more elevated during the non-breeding season. They concluded that this was a consequence of prolonged epididymal storage,

resulting in hypermaturation, which possibly caused faulty male pronuclear formation. In our case, hypermaturation would result in better motility.

Cervids are strongly seasonal. During the rut, spermatogenesis and sexual activity are very elevated; therefore, epididymal reserves are continuously depleted and restored. The hinds can enter several times in estras during the rat season, but this event is less frequent thereafter because most of the females are already pregnant. Therefore, a male has fewer opportunities to mate after the rut. Rapid renewal of epididymal sperm during the rat also occurs in non-dominant males, not only because they might mate but also because deers ejaculate during the rat as part of the usual behavior, not being necessarily associated with mating [44,45]. As a consequence, during the rut, the sperm in the cauda epididymis would consist mostly of ''fresh'' spermatozoa, which would complete their maturation at the moment of contacting seminal plasma rather than in the cauda epididymis. On the other hand, in the transition period and post-rut, sperm remains longer in the cauda epididymis (due to reduced input and output), and would therefore be mostly composed of spermatozoa stored over a long period of time, which would have been overexposed to the epididymal environment. Hypermaturation may become apparent in the form of more motile sperm, with higher motility descriptors than during the rut, but this increase in motility quality may not translate in better fertility.

We have to consider this when analyzing the results of the clustering. The better motile condition is also reflected in the motility descriptors of each subpopulation, which showed more active spermatozoa when the season advanced. CL2s (medium velocity and linear) may be considered a subpopulation of spermatozoa with mature motility, since its proportion increased with the season, but its motility descriptors remained little changed. In addition, CL1s (slow and non-linear) may comprise spermatozoa that have still not achieved mature motility. In fact, not only did CL2s increase after the rut, but it did so at almost at the same pace as CL1s decreased. Therefore, it seems likely that when CL1s spermatozoa mature, they achieve CL2s characteristics, and that this process affects more spermatozoa after the rut. In the post-rut, we did not find CL1s anymore, which is in agreement with very low sexual activity in this period, resulting in long storage periods, thus allowing the maturation of all CL1s spermatozoa in the cauda epididymis. On the other hand, CL3s (fast and linear) and CL4s (very slow and non-linear) may represent subpopulations of very mature spermatozoa and maturation-resilient spermatozoa, respectively. The stability of their proportions throughout the three periods possibly indicates that spermatozoa with these qualities either have highly matured biochemical and morphological characteristics (CL3s), or lacks some of them (CL4s, therefore being unable to accomplish motility maturation). It is possible that CL2s spermatozoa need seminal plasma in order to achieve the maturation degree of CL3s [46].

In conclusion, our study shows that the subpopulation study based on motility data greatly improved the interpretation of motility changes between post mortem intervals and seasons. Our results showed that the subpopulation pattern was deeply altered with post mortem time, and the quality of the samples was severely compromised after several days post mortem (more than 72 h). Furthermore, we found an increase in the proportion of mature sperm in the cauda epididymis after the rut, which may not be necessarily connected to an increase in sample fertility, and could even indicate worse sperm quality. However, these findings must be completed with the study of subpopulation patterns in

ejaculated sperm and recently harvested (less than 19 h) epididymal spermatozoa. The results of this work are based on relatively novel techniques, and our results are still open to a wide range of interpretations. Further research, combined with subpopulation purification techniques and fertility studies, is necessary in order to complete this report.

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