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Decay of sperm obtained from epididymes of wild ruminants depending on postmortem time

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Abstract

We have carried out a study on the effect of postmortem time (PT) in some characteristics of epididymal sperm salvaged from hunted Iberian red deer and roe deer. Testis were collected, identified, refrigerated down to 5 °C, and sent to our laboratory by the wardens of the hunting reserves. This way, samples were delivered at different times postmortem. Sperm were extracted from the cauda epididymis by means of cuts. Analyzed parameters were: osmolality, pH, motility—both subjectively and with CASA, HOS test reactivity, acrosomal status and viability (assessed with propidium iodide). Osmolality and pH rose with prolonged postmortem time, possibly due to tissue decomposition. Most sperm quality parameters negatively correlated with PT. Besides, when comparing PT classes (groups of 24 h for red deer and 30 h for roe deer), we could appreciate that motility was more affected by PT than other quality variables. Progressive motility was especially impaired. We also classified the samples in high, medium and low quality for each PT group (considering progressive motility, intact acrosomes and reactivity to the HOS test), and it was clear that after 2 days the number of high quality samples was testimonial, and after several days, we almost found only low quality samples. In conclusion, epididymal sperm from Iberian red deer and roe deer undergo a decrease of quality with PT, but it could stay acceptable within many hours postmortem. There are implications for wildlife conservation programs, as epididymal sperm is a good source of germplasm. If valuable animals die and it is not possible to process their sperm immediately, it may still be possible to obtain viable spermatozoa many hours later.
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Keywords: Red deer; Roe deer; Postmortem recovery; Epididymal sperm; Refrigeration; Epididymal storage

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1. Introduction

The cervid species Iberian red deer (*Cervus elaphus hispanicus*) and roe deer (*Capreolus capreolus*) are appreciated trophies in Spain and are subjected to controlled hunting, both in state hunting reserves and in private properties. Populations are frequently constricted to small areas and separated by fences and other barriers, thus inbreeding and loss of genetic variability are a recurrent hazard [1]. There is also a great interest in keeping good trophies and autochthonous subspecies. Consequently, the interest in developing artificial reproduction techniques and genetic resource banks for these species has been increasing, considering the possibilities that this kind of approach offers [2–4]. Since males are hunted in numbers around the rut season, there is an available source of epididymal sperm.

Postmortem sperm recovery is an useful strategy for germplasm banking [5]. This technique allows to use the epididymal sperm reserves of deceased or hunted males, especially when semen collection by other ways would be difficult or impossible. Sperm stored in the cauda epididymis have usually good quality and a high level of maturation, being able to fertilize oocytes. To date, many studies have demonstrated that it is possible to obtain viable gametes postmortem. Furthermore, successful pregnancies have been achieved in many species using epididymal sperm for artificial insemination [6–10].

However, in order to get good quality samples, sperm collection and processing should be carried out immediately after the death of the animal. This is not always possible, especially regarding wild species. In these circumstances, animal death is generally unpredictable or it happens far away from laboratories and technicians. In the case of Iberian red deer and roe deer in Spain, samples sometimes cannot be delivered immediately to the laboratory, since hunting often takes place in the wild. Even though sperm cells can survive for some time in the epididymes of dead animals, their quality deteriorates with time, because of the changes related to body death and decomposition [8,11].

Therefore, to determine the quality and decay of sperm stored in epididymes post-mortem, some studies have been carried out in a few species, such as mice [8,12–15], boar [16], dog [17], some African wild species [18–20], mouflon [6] and Iberian red deer [21–23]. In general, these works agree that there is a general deterioration in sperm quality depending on time postmortem, specially marked in the first hours, and that refrigeration of the epididymes down to around 5 °C is the best strategy to lower this damage. However, there are many dissimilarities between species, possibly due to differences on cold shock endurance of epididymal sperm.

In this work we have evaluated the quality of sperm samples obtained from Iberian red deer and roe deer epididymes, which were delivered to our laboratory at different times postmortem. Since refrigeration devices are widely available, testicles of hunted animals could be kept at 5 °C during its storage. Our objective was to determine the effect of postmortem time on sperm quality and the characteristics of its decline, in the same conditions that often occur when samples are collected in the wild and immediate transport to the laboratory is not always possible. This work is included in a long-term plan dedicated to setting up a germplasm bank for wild ruminant species in the North of Spain, obtaining sperm samples from hunted animals.

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2. Material and methods

2.1. Sample collection

Samples were collected from recently shot Iberian red deer (*C. elaphus hispanicus*) and roe deer (*C. capreolus*) in the hunting reserves of Ancares, Mampodre and Picos de Europa (León, North of Spain). Collection was carried out during the breeding season (rut) of this species (autumn for Iberian red deer and beginning of summer for roe deer), by the official gamekeepers of the hunting reserves. These hunting reserves are directed by the regional government (Junta de Castilla y León), and hunting is very selective and regulated following population control criteria. Harvest plans followed Spanish Harvest Regulation, Law 4/96 of Castilla y León, which conforms of European Union Regulation, and the Annual Hunting Regulation, which establish species and number of individuals that can be hunted. Furthermore, species and number of individuals that can be hunted, and the exact periods of the year in which hunting can take place, are reviewed each year by the Annual Hunting Regulation. Animal manipulations were 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, genitalia collection and refrigeration were noted and attached to the corresponding sample. Refrigerated samples were sent to our laboratory in the Veterinary Clinic Hospital of the University of León (Spain). Postmortem time (PT) was defined as the interval between the death of the animal and the arrival of the sample to our laboratory. A total of 199 Iberian red deer samples and 72 roe deer samples were delivered.

2.2. Sample processing

Sample processing was performed in a walk-in fridge (5 °C) immediately after its arrival. Epididymes were dissected and cleaned from connective tissue. Sperm were collected performing several incisions in the cauda epididymis with a surgical blade, and taking the white fluid emerging from the cut tubules. To diminish blood contamination, superficial blood vessels were cut previously, wiping its content and drying cauda surface thoroughly. Sperm from both epididymes of the same animal were put together in a plastic cone, conveniently labeled, and used in the subsequent analysis.

2.3. Sperm assessment

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

Osmolality and pH of each sample were measured using a cryoscopic osmometer (Osmomat-030, Gonotec^{TM}; Berlin) and an electronic pH-meter (CG 837, Schott^{TM}; Mainz), respectively.

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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 during a minimum of 20 min, and read within 20 min. We checked that there were not motility variations within this time period. A Makler counting chamber (10 μm depth; Haifa Instruments, Israel), warmed up to 37 °C, was filled with 5 μL of sample and examined with a phase contrast microscope (Nikon Labophot-2; negative contrast optics), 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 subjectively estimated. Besides, a CASA system coupled to the same microscope was used to objectively assess motility (Motility Analyzer v. 7.4G, Hamilton-Thorne Research™), collecting the following parameters: total motility (%), progressive motility (%), average path velocity (μm/s; VAP), straightness (%; STR), and amplitude of the lateral movement of the head (μm; ALH). At least five fields and 200 cells were recorded at 200×, using an image acquisition rate of 25 frames/s and an acquisition time of 0.8 s.

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×) [24]. Acrosomal status (percentage of cells with an intact acrosome) was evaluated counting at least 100 cells.

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. Responsiveness to the test was determined counting a minimum of 100 cells with a phase-contrast microscope (400×). HOS test reactivity was defined as percentage of positive cells (those with a swollen tail) [25].

Sperm viability was assessed using the fluorescent dye propidium iodide (PI). Five microliters of sample were diluted in 500 μL of PI solution (5 μg/mL PI in the same Hepes solution described in motility assessment). Samples were kept 10 min in the dark before being analyzed with a epifluorescence microscope (Nikon Optiphot; 400×, 450–490 nm excitation filter, 510 nm dichroic beam splitter, 520 nm barrier filter). At least 100 cells were counted, and the percentage of non-stained cells (viable spermatozoa) was noted [26].

2.4. Data processing and statistical analysis

Statistical analysis were carried out using the SAS™ package (SAS Institute, Cary, NC). Since collected data was not normally distributed and heavily tailed, non-parametric test were preferred. First, we obtained the Spearman correlation coefficients between PT and sperm parameters, in order to detect possible relationships between them. To study the variation of sperm parameters along PT, we divided it in many intervals, and carried out a comparison. We used 24-h intervals for Iberian red deer (from 0 to 168 h, plus an extra class for PT > 168 h). For roe deer, due to lower number of samples and their unbalanced

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161 distribution among 24-h groups, we used 30-h intervals instead (from 0 to 150, plus and
162 extra class for PT > 150 h). A multiple group comparison was carried out using the
163 Kruskal–Wallis test, followed by a group pairwise comparison (Wilcoxon rank-sum test)
164 when differences were significant ($P < 0.05$). The mean and standard deviation of the PT
165 >168 and >150 h classes were, respectively, 208 ± 57 h and 226 ± 74 h.
166 Besides, in order to describe the evolution of the general quality of the samples and
167 compare both species, the samples were classified accordingly to their quality in 24 h PT
168 groups. Quality was defined by the progressive motility, HOS test reactivity, and acrosomal
169 status of each sample. A sample was included in the high quality group if all the three
170 parameters were equal or higher than 60%. If any of them was lower than 60%, but all of
171 them were at least 30%, the sample was included in the medium quality group. Whenever
172 any parameter was below 30%, the sample was included in the low quality group. The
173 distribution of quality groups between PT classes was compared using the χ^2 test. When the
174 conditions made unsuitable the application of this test, the Fisher's exact test was used
175 instead. $P < 0.05$ was used for statistical significance.

176 ## 3. Results

177 ### 3.1. Data collected

178 Almost all the samples could be analyzed for pH, osmolality, subjective motility
179 assessment, HOS test, acrosome integrity, and viability; however, some samples were
180 rejected because of bad aspect or improper refrigeration (Table 1). CASA was available
181 only for 55% (92) and 39% (26) of red deer and roe deer analyzed samples, respectively,
182 thus there was a lower sample number available in these cases. We have observed a high
183 variation within PT groups, which is evident considering the wide interquartil ranges
184 (Figs. 1–4). It was not clear if this variation corresponded to individual differences between
185 males, to collection and refrigeration conditions, or to other factors.

186 ### 3.2. Correlation analysis

187 Analysis of the correlations between PT and the studied parameters (Table 2) rendered
188 similar results for both species. Motility (except for STR in roe deer), HOS test reactivity,
189 intact acrosomes and viability presented negative correlations with PT. On the other hand,
190 osmolality and pH showed positive correlations. In general, correlations were highly or

Table 1
Number of samples for each species and PT classes

Red deer	[0–24]	]24–48]	]48–72]	]72–96]	]96–120]	]120–144]	]144–168]	>168	Total
n	16	28	39	20	21	12	17	15	168
Roe deer	[0–30]	]30–60]	]60–90]	]90–120]	]120–150]	>150	Total		
n	6	23	8	6	6	18	67		

Note that not all the samples delivered (199 for red deer and 72 for roe deer) were analyzed.

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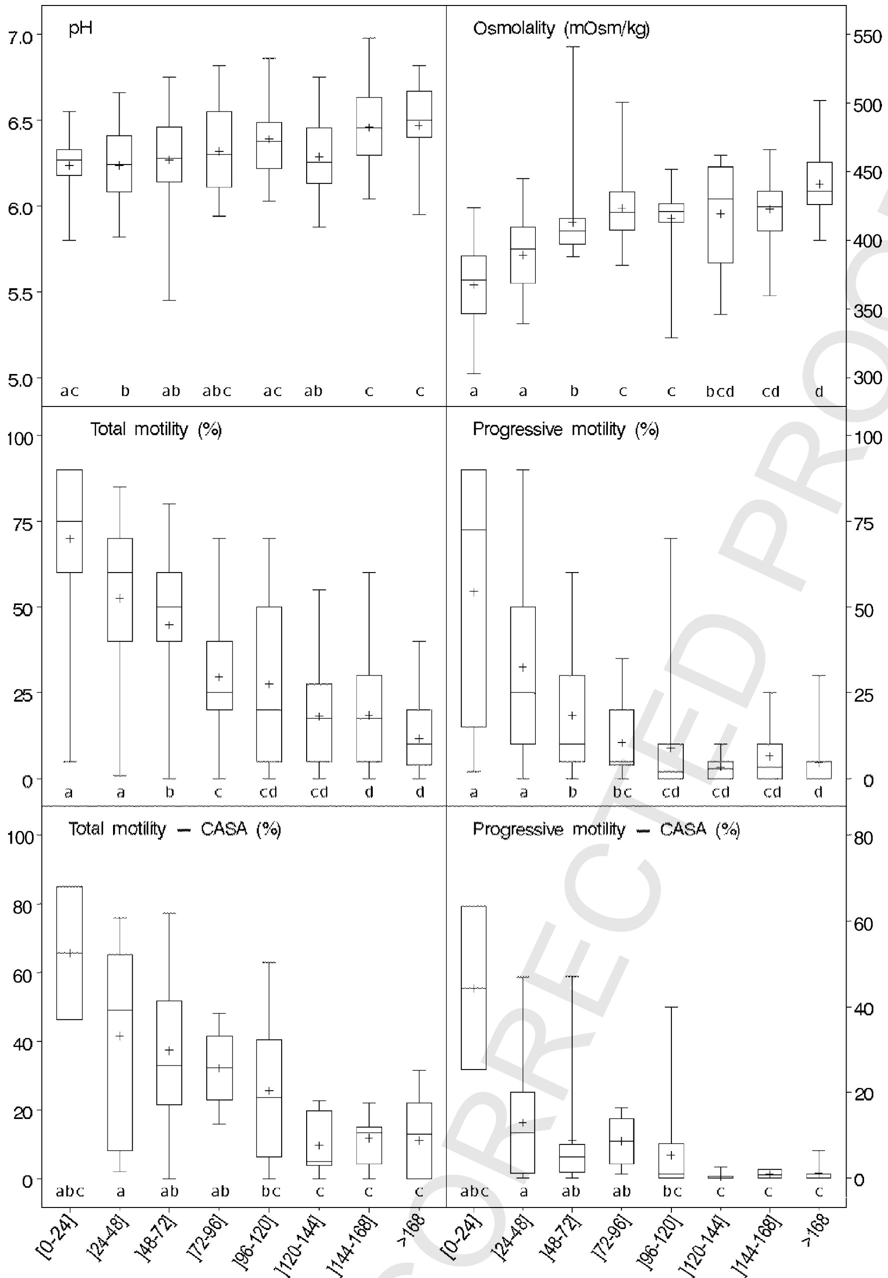

Fig. 1. Red deer: evolution of sperm pH, osmolality, total motility and progressive motility (subjective and CASA) depending on postmortem time (time intervals in hours). Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside the boxes 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 bottom of a plot indicate significant differences between PT classes ($P < 0.05$).

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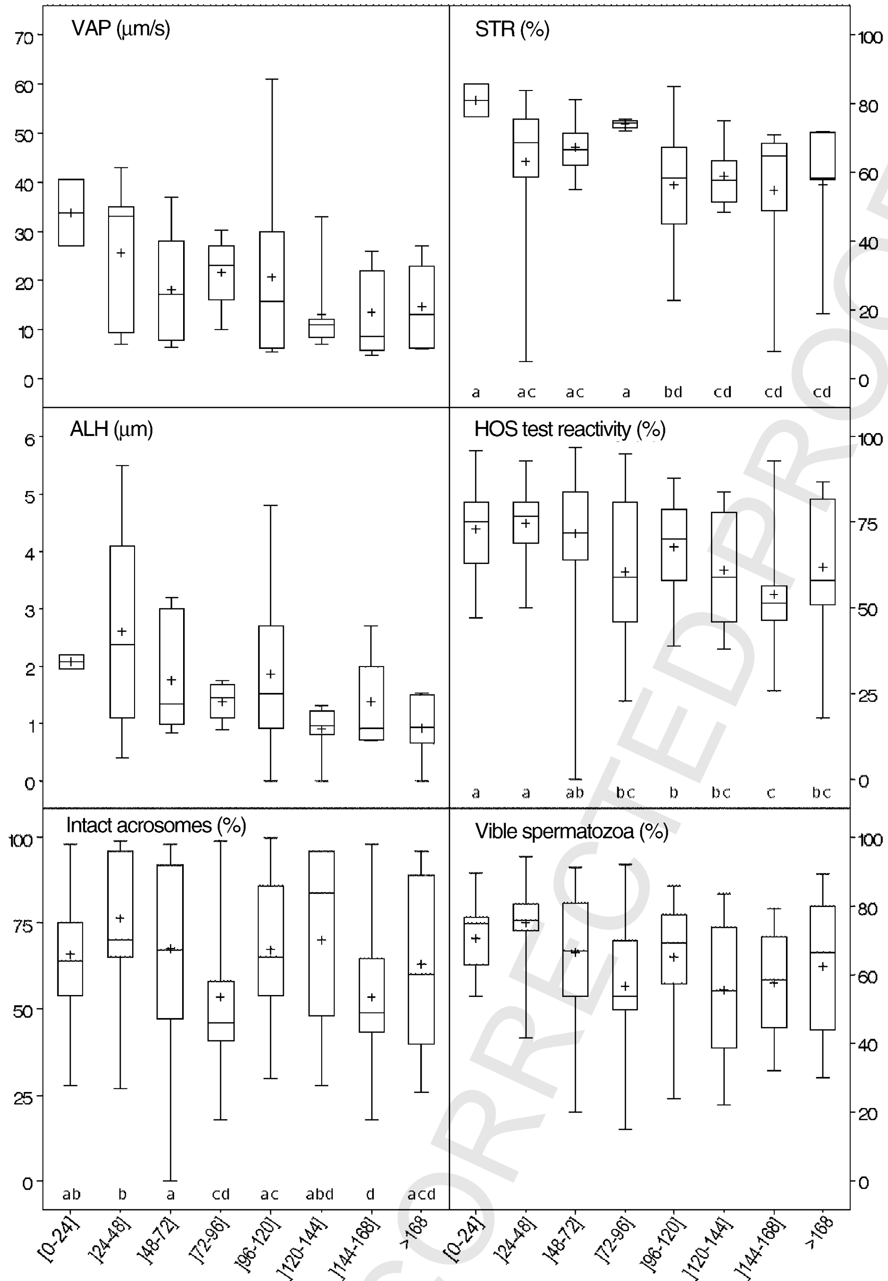

Fig. 2. Red deer: evolution of sperm VAP, STR, ALH, HOS test reactivity, acrosomal integrity and viability depending on postmortem time (time intervals in hours). Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside the boxes indicates 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 bottom of a plot indicate significant differences between PT classes ($P < 0.05$).

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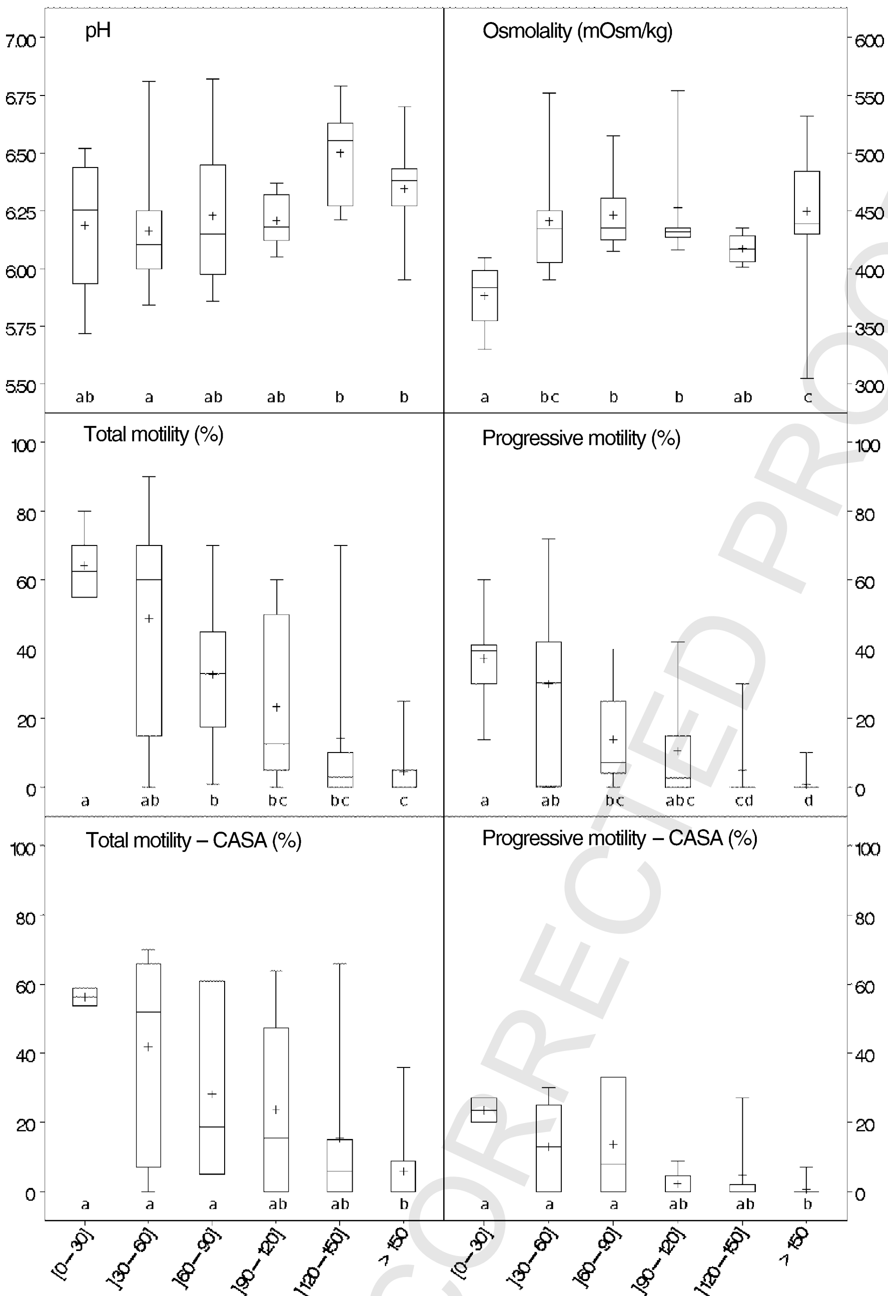

Fig. 3. Roe deer: evolution of sperm pH, osmolality, total motility and progressive motility (subjective and CASA) depending on postmortem time (time intervals in hours). Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside the boxes 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 bottom of a plot indicate significant differences between PT classes ($P < 0.05$).

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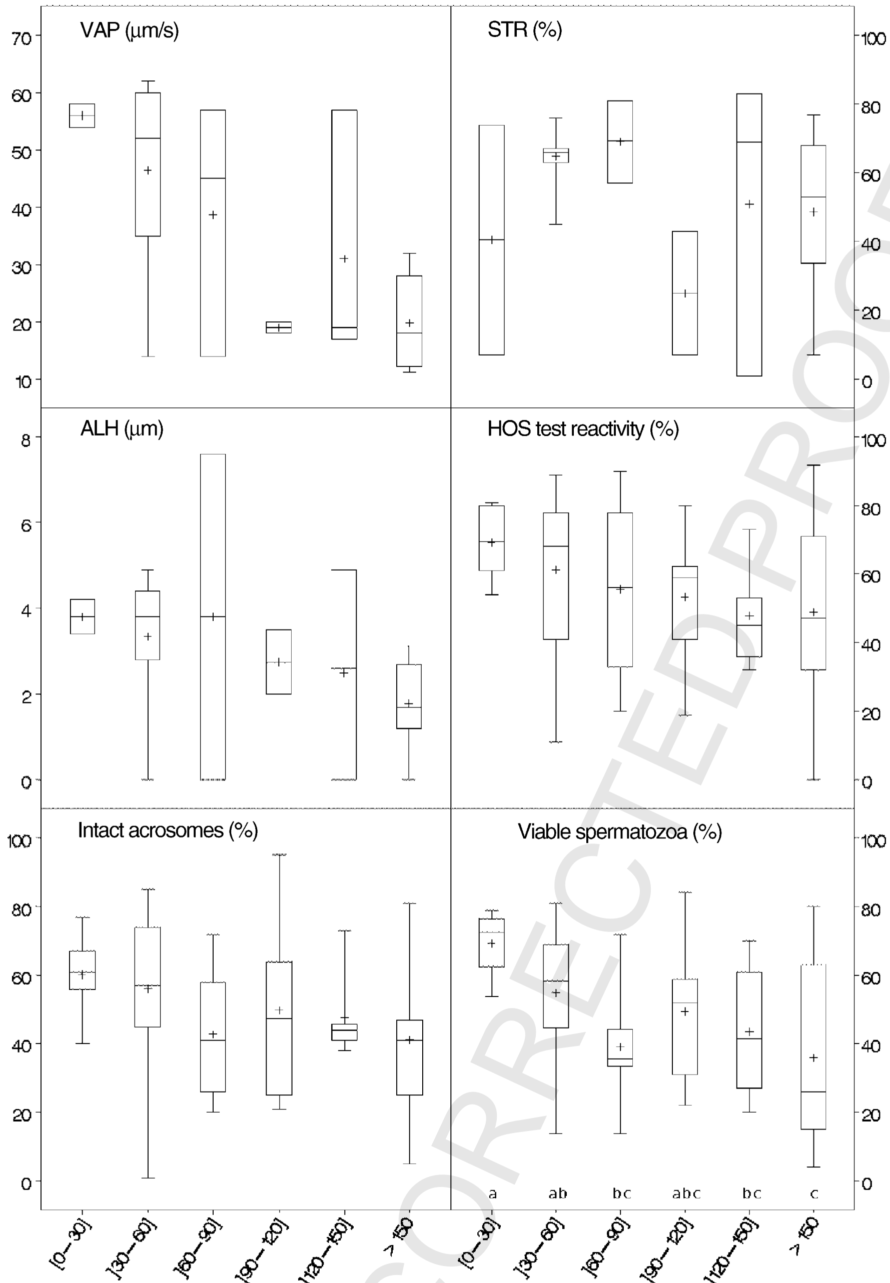

Fig. 4. Roe deer: evolution of sperm VAP, STR, ALH, HOS test reactivity, acrosomal integrity and viability depending on postmortem time (time intervals in hours). Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside the boxes indicates 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 bottom of a plot indicate significant differences between PT classes ($P < 0.05$).

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Table 2
Correlations between PT and sperm parameters (Spearman coefficients and significance)

	pH	Osmolality	TM[a]	PM[b]	TM[a] (CASA)	PM[b] (CASA)
Red deer	0.30***	0.57***	−0.62***	−0.56***	−0.54***	−0.52***
Roe deer	0.39**	0.35**	−0.64***	−0.68***	−0.51***	−0.55***
	VAP	STR	ALH	HOS test reactivity	Intact acrosomes	Viability
Red deer	−0.36**	−0.35***	−0.41**	−0.32***	−0.21***	−0.26***
Roe deer	−0.54**	−0.12	−0.40*	−0.37**	−0.36**	−0.49***

[a] Total motility.
[b] Progressive motility.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

191 very highly significant. Correlation coefficients were low in most cases, though. Remark-
192 ably, PT and total and progressive motility, both by subjective and CASA assessments,
193 rendered correlation coefficients above 0.5.

194 *3.3. Comparison between PT classes*

195 A summary of the data grouped by PT classes is shown in Figs. 1–4. In general, data
196 followed the corresponding trend indicated by the correlation coefficients. However, we
197 did not find significant variations in many cases, especially for roe deer, possibly due to the
198 high variability of the samples. In fact, for roe deer, only the classes]30–60] h and >150 h
199 included more than 10 cases (Table 1), and, as said before, only a fraction of these samples
200 was analyzed by CASA, therefore this could be another reason for the lack of significance.
201 In both species, pH and osmolality varied differently. Whereas pH seemed to rise only
202 slightly, and this rising was only evident in the last PT classes, osmolality increased early
203 (after 48 h for red deer and 30 h for roe deer). Beyond this point, osmolality continued
204 rising clearly in the case of Iberian red deer. However, for roe deer, it stalled and no further
205 increase was noted.
206 Total motility decreased steadily along the studied time periods, but progressive motility
207 dropped around the third day. By the fourth day, progressive motility was practically 0.
208 This was observed both in subjective and CASA parameters. Other motility parameters,
209 yielded by the CASA system, showed a steady decrease, only significant for STR in red
210 deer, though. For roe deer, STR was still high in the last classes.
211 HOS test reactivity and acrosomal status showed significant differences between groups
212 only for Iberian red deer. In this species, it could be appreciated that the proportion of
213 spermatozoa positive to the HOS test decreased with PT (but only in a low percentage).
214 However, in the case of acrosomal status, the results were less evident, since there were
215 many samples with high values in the last PT classes.
216 The case of the viability was completely opposite, because, although both species
217 showed a diminution of viability values through PT, only roe deer data rendered

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218 statistical differences between PT groups. In this case, we found one important difference
219 between the two species, since both of them had similar viability values in the early PT
220 groups (median value above 70%), but it diminished to 66.5% in the >168 h group for red
221 deer, whereas it dropped to only 26% in the >150 h group for roe deer.

222 *3.4. Distribution of quality groups within PT classes*

223 Distribution of the samples in high, medium, and low quality for each PT class,
224 and results of χ^2 test are shown in Fig. 5. For Iberian red deer, nearly 40% of the
225 samples belonged to the high quality group when processed before the first 24 h
226 postmortem, summing more than 60% together with medium quality samples, although
227 differences in the distributions were not still significant. However, in the next 24 h, the
228 proportion of high quality samples was halved, and low quality samples rose up to 50%.
229 After 120 h postmortem, none of the samples could be considered even as of medium
230 quality.
231 Roe deer samples had a similar distribution than those of red deer. However, the
232 distribution is less clear due to the lower n in each PT class (only the]24–48],]48–72], and
233 >168 h classes had more than 10 samples each). Apparently, the increase of the proportion
234 of bad quality samples was slower than in the case of red deer (comparing]48–72] h
235 classes), but from this point forward the trend was almost identical. Before using χ^2 test,
236 those groups that seemed to be homogeneous were joined in order to increase the number of
237 samples in each one and get reliable results. Thus, data from]24–48] and]48–72] h, and
238]72–96],]96–120],]120–144] and]144–168] h were put together. Lack of significance
239 between [0–24] and]24– 72] h groups is logical, as [0–24] h group included only four
240 samples.

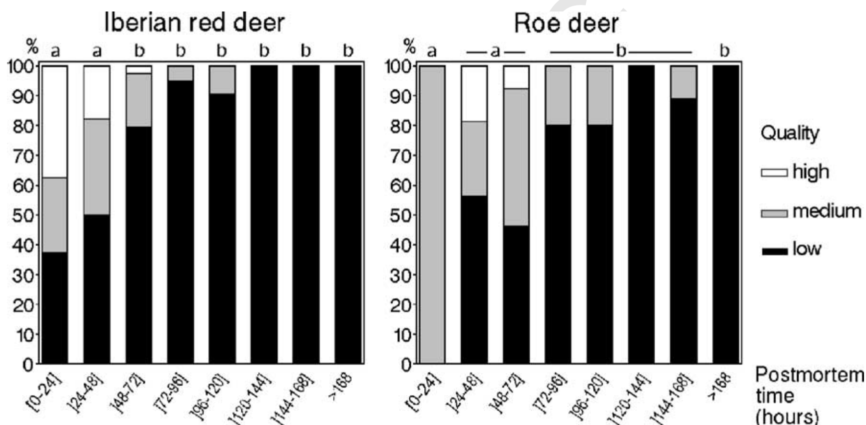

Fig. 5. Distribution of samples in high, medium, and low quality groups for each species and PT classes. High quality: progressive motility, HOS test reactivity and acrosomal status >60%; medium quality: any of these parameters =60% but >30%; low quality: any of these parameters <30%. Different letters indicate statistical differences (χ^2; $P < 0.05$). In the case of roe deer,]24–48] and]48–72], and]72–96],]96–120],]120–144] and]144–168] were considered as single classes for the χ^2 test.

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4. Discussion

In this study we have shown that the quality of the sperm stored in the cauda epididymis of Iberian red deer and roe deer decays with PT. However, for some time, the quality could be high enough to consider the salvage of samples. We have to consider that this work is a preliminary step to establish a germplasm bank for wild ruminants in the North of Spain, and that most of the samples would be obtained from hunted animals. Hunting in that area is very selective, and there is a tendency to collect animals of good aspect, considering trophy and body shape, which are highly heritable traits. Thus, the interest of studying the characteristics of these samples and the impact of postmortem time on their quality.

There are many studies on the deleterious effect of PT in epididymal sperm. Nevertheless, these works also show that it is possible to obtain good quality and even fertile sperm from refrigerated epididymes many hours or days postmortem Garde et al. [21], Sankai et al. [15], Yu and Leibo [17]. Furthermore, some works describe that viable spermatozoa could be retrieved even if the epididymes were stored at ambient temperature for some hours. Christian et al. [27] reported motile spermatozoa in mice 24 h postmortem at room temperature. Other authors confirmed that sperm in these conditions were able to penetrate mice or ewe oocytes [14,28], or produce live mice offspring [8].

Several works on this subject have been carried out in cervids [11,21,23,29], but there are important methodological differences between these other studies and ours. The usual protocol consists in the transport of the genitalia to the laboratory just after the death of the animal, where they are refrigerated in controlled conditions. On the other hand, we planned to carry out this study in the same conditions that usually occur when samples are collected in the wild, and there is no possibility to immediately transport them to the laboratory. Briefly, the testicles are removed from the animal, refrigerated, and delivered to our laboratory as soon as possible, in an insulated container. All this process is carried out by the gamekeepers of the hunting reserves, and the refrigeration and storage are performed in home refrigerators. In this manner, samples arrived to our laboratory one or two times per week during the hunting season, and we processed samples of a wide range of postmortem times, from few hours to a week or more. It was important for us to follow these same collecting and delivery protocol, since in the event of establishing a germplasm bank in this location, the most important source of samples would be this one. This methodology is currently difficult to standardize, thus the interest in knowing what the impact on the samples could be.

As a consequence, we had many sources of variation that could influence the quality of the samples. This can be appreciated in the high variability between different samples. Apart form the individual differences between males, the most important of these sources of variation was the temperature the samples were exposed to during the postmortem time interval. Since hunting takes place in mountainous spots, a wounded animal is often tough to track. Thus, some samples were exposed to ambient temperature for several hours before they could be refrigerated. This should not be worrying in the case of red deer, as it is hunted by autumn, when temperature is usually low in the mountains of the North of Spain, but roe deer is hunted in the summer, when temperatures generally vary from warm to hot.

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285 The refrigeration of the samples is another factor of variation, because we were unaware of
286 the conditions of the gamekeepers' refrigerators and the possible incidents in their
287 operation. Furthermore, there might be a interaction between individuals and the impact
288 of temperature changes on the spermatozoa, so the sperm of one male could endure these
289 temperature variations whereas the sperm of another one could not [30].

290 Nevertheless, we considered that these sources of variation were acceptable, since we
291 wanted to study the quality of the samples that had actually endured the conditions
292 described above. Nevertheless, we removed from our data those samples that were kept at
293 ambient temperature more than 6 h after the death of the animal, or those we considered
294 that had been kept in very bad conditions (frozen or rotten). Anyway, some authors have
295 indicated that there is little decrease in the quality of the samples kept at room temperature
296 for a few hours (6–15 h) [8,15,31]. Recently, Kaabi et al. [32] reported similar cleavage
297 rates for oocytes inseminated with ram sperm from epididymes stored at room temperature
298 or refrigerated, both at 24 and 48 h postmortem. All these studies indicated us that,
299 although we could not control the time between the death of the animal and the
300 refrigeration of the samples, this factor possibly did not affect seriously our samples.

301 As expected, we have detected a decrease of sperm quality along PT. The decrease of
302 sperm quality with PT is due not only to sperm aging, but also to the processes inherent to
303 tissue decomposition after death. Songsasen et al. [8], working with mice, described the
304 degeneration of the epididymal tubules, showing that histological changes start as soon as
305 18 h postmortem. These changes were described as picnosis of the content of epithelial
306 cells to the lumen of the tubules, followed apparently by the disruption of the epithelium
307 some hours later. Hishinuma et al. [11] found similar changes in Sika deer epididymes
308 several days postmortem. In our study, the increase of osmolality reflects the histological
309 changes described by those authors. Concretely, Hishinuma et al. [11] indicated that
310 degeneration at days 4 and 7 was more evident that at day 1, which coincides with our
311 findings regarding a significant increase of osmolality after several days postmortem.
312 Moreover, the positive correlation between pH and PT indicates a loss of buffering capacity
313 of the epididymal fluid. The rise of pH is less obvious than the increase of osmolality, as it
314 was significant only after many days postmortem. This could indicate a good resilience of
315 the buffering system inside the tubules. We have to consider that both osmolality and pH of
316 the surrounding media have a great effect on sperm motility and metabolism [33]. Thus, the
317 alterations of the physical environment of the epididymal sperm may have an important
318 role in the loss of quality that we have observed, adding up to the intrinsic process of decay
319 and aging of sperm due to PT.

320 In general, both Iberian red deer and roe deer epididymal sperm samples underwent a
321 loss of quality when the epididymes were stored for more than 2–3 days at 5 °C.
322 Progressive motility was the parameter that dropped more pronouncedly, followed by
323 total motility, which coincides with results in other species [11,16,17,32]. For the other
324 motility parameters, we think that more consistent results could have been achieved using a
325 modern CASA system, which would have allowed to get sperm subpopulation parameters,
326 instead of mean populational parameters, that can be deceiving [34]. We are preparing a
327 complementary study in which sperm motility will be measured with a state-of-the-art
328 CASA at different postmortem times, in order to perform a more complete study of
329 motility.

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330 Considering recent studies in cervids, in a preliminary report on Iberian red deer [22], we
331 obtained a similar trend on motility (24 h intervals over a 4-day period), with progressive
332 motility dropping to almost nil at the end of the period. Hishinuma et al. [11], working with
333 Sika deer, found that total motility significantly decreases as soon as the first day
334 postmortem, and it continues falling when PT increases. Soler et al. [23] also detected
335 a significant decrease of motility with PT in Iberian red deer, reporting that the sperm
336 motility index (SMI) significantly reduces after 2 days, and it stabilized until the end of the
337 experiment (4 days). Although we used different parameters considering motility (SMI
338 combines total and progressive motility in the same parameter), the trend we reported in the
339 case of Iberian red deer seems to be similar to those of these authors. However, mainly due
340 to the abrupt decrease of progressive motility, our data show an early drop of quality, which
341 marks a difference with the work of Soler et al. [23]. This discrepancy could be due to the
342 differences in the experimental design (the epididymes were taken to the laboratory just
343 after the death of the animals and kept in controlled refrigerated conditions), in the
344 recollection time (November and December), and in the origin of the animals (South of
345 Spain). Unfortunately, none of these studies reported pH or osmolality of the samples
346 (sperm was immediately mixed with media after extraction). Considering our observations,
347 it would be of great interest to extend this study, in order to clarify if the loss of quality of
348 sperm motility is due not only to the normal process of aging, but also to the variations of
349 the physical parameters of the epididymal fluid, and to what extent.
350 On the other hand, neither Hishinuma et al. [11] nor Soler et al. [23], found a reduction in
351 sperm viability during the studied period (7 and 4 days, respectively). We have reported a
352 highly significant negative correlation between viability and PT, but with low correlation
353 coefficients. Comparing PT groups, we did not found significant differences for red deer,
354 but for roe deer, which might indicate a difference between these species. Iberian red deer
355 sperm possibly underwent a decrease in viability, as indicated by the correlation analysis,
356 but it might be much more subtle. This can explain the lack of significant differences in the
357 other studies, which also used a different method for viability assessment (eosin staining).
358 Other quality parameters that endured well the postmortem conditions were the function-
359 ality of the plasma membrane (HOS test reactivity) and the acrosomal status. Both of them
360 had highly significant negative correlations with PT for both species, but box plot
361 distributions showed that differences between PT classes were small. In our previous
362 study on red deer [22], we obtained similar results for HOS test reactivity, and, for
363 acrosomal status and sperm viability, values were lower the fourth day postmortem. This
364 would indicate that in fact these parameters are affected by postmortem time, but not as
365 dramatically as motility.
366 Other authors have reported differences comparing related species. For instance, Sankai
367 et al. [15], working with mouse, and Soler et al. [23], working with red deer, reported
368 increasing numbers of sperm with abnormalities after 4 days postmortem. However, the
369 former indicated a very high increase (more that 60%) of bent tails, whereas the later found
370 a much lower increase (around 20%) of bent midpieces. Furthermore, Lubbe et al. [19], and
371 Killian et al. [20] working with African wild Perissodactyls and African wild ruminants,
372 respectively, reported some differences between similar species, regarding motility
373 diminution along PT. In fact, cold-shock resistance varies pronouncedly between species,
374 even between breeds or individuals [30]. Sankai et al. [15] interpreted the increasing

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375 proportion of sperm with bent tails as a consequence of the hardening of the plasma
376 membrane due to low temperatures. This effect could contribute, at least in some extent, to
377 the differences that we have find between red deer and roe deer, regarding plasma
378 membrane integrity (sperm viability).

379 Finally, the classification of samples according to their quality showed that the
380 proportion of samples classified as of low quality quickly grew with PT for the two
381 species. Using a similar classification, Garde et al. [31], working with Iberian red deer,
382 found that sperm quality seemed to reduce after 12 h postmortem, and the proportion of
383 samples classified as excellent (progressive motility >60%, normal morphology >40%,
384 intact acrosomes >40%, and HOS test reactivity >40%) dropped after that moment,
385 reaching 0% in the 24–36 h period. In our work, our data followed a similar behavior, for
386 both species. The chances of finding samples of acceptable quality before 2 days
387 postmortem (under refrigeration) seemed to be good, but they quickly got much worse
388 with increasing PT.

389 In conclusion, the quality of both Iberian red deer and roe deer epididymal sperm
390 decreased with postmortem time, but this decrease was different for different parameters.
391 Motility was the most affected one, and membrane and acrosomal integrity seemed to
392 endure better the postmortem conditions. Besides, in both species we could still find
393 acceptable samples after several days of refrigeration. Other authors [14,17,23] recom-
394 mended that if samples cannot be processed just after the death of the animal, they should
395 be refrigerated, in order to process them when facilities were available. Considering our
396 results, this advise seems reasonable, as the deleterious effect of storage at 5 °C seems to be
397 acceptable, but only for few days (in our study this could be 2 days). Moreover, Sankai et al.
398 [15] found similar results refrigerating mice epididymes protected either by mineral oil,
399 body fat or inside the whole body. In our case, keeping testis and epididymes inside the
400 scrotum should be enough to protect the cauda epididymis from desiccation, consequently
401 avoiding complication in the collection of the sample in the field.

402 On the other hand, even if the samples have lost its motility because of a long storage, we
403 have found that other characteristics might be still acceptable, so IVF or ICSI could be used
404 to achieve fecundation and pregnancy in the case of valuable individuals or endangered
405 species [13,30]. Nevertheless, further studies on the fertilizing capacity and the effect of
406 frozen–thawing on Iberian red deer and roe deer epididymal spermatozoa, after being
407 refrigerated for many days postmortem, must be carried out in order to confirm this
408 possibility. In this sense, our group is carrying out further experiments in order to assess the
409 suitability of these samples for cryopreservation.

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