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## Sperm parameters on Iberian red deer: Electroejaculation and post-mortem collection

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### Abstract

Artificial reproductive technologies (ART) for cervids have improved, but a need remains for the collection of basic data. We studied two models of sperm collection in Iberian red deer, post-mortem (PM) in a wild population (179 samples) and by electroejaculation (EE) in a farmed population (37 samples), recording: testicular and epididymal weight, testicular diameter, sperm quantity, pH and osmolality and spermatozoa quality (motility by CASA, abnormal forms, cytoplasmic droplets, viability and acrosomal status). We tested the relationship of these parameters with stag age and compared the two models (PM and EE; medians showed). Genitalia parameters were linearly related to stag age (testicular diameter: 31.5–50.5 mm for 2–9 years). Total number of spermatozoa collected were PM:  $2.5 \times 10^9$  and EE:  $3.6 \times 10^9$  ( $P > 0.05$ ), increasing with age only for PM. We found a positive relationship between testicular size and spermatozoa collected for PM. Osmolality and pH were PM: 6.28 and 378 mOsm/kg; EE: 7.63 and 309 mOsm/kg ( $P < 0.05$ ). The pH increased with age only for EE. Percentage of motile spermatozoa was similar for PM and EE, but motility quality was lower for PM. Abnormal forms, proximal and distal droplets were lower for EE (22%, 1.3%, 1.5% vs. PM: 23%, 4.3%, 83%). Viability was similar (74%) and intact acrosomes were higher for EE (97% vs. 89%). Both PM and EE samples could be used for germplasm banking. This study contributes data for a better understanding of red deer spermatology and for the development of ART in cervids.

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**Keywords:** Red deer; Epididymal spermatozoa; Electroejaculation; Sperm evaluation; Testicular growth

### 1. Introduction

The use of artificial reproductive techniques (ART) on wild ungulates has increased considerably during the last years, not only with conservationist purposes [1–4],

but also because several of them have become the focus of a quickly developing farming business for producing venison, velvet and trophies. Being an important business in several countries [5–7], electroejaculation (EE), estrous synchronization, artificial insemination and embryo transfer have been investigated in several cervid species [5,7–9], and the red deer has received most of the attention of farmers and researchers [10]. Moreover, due to the increased need of management programs for hunting reserves and taking advantage of the availability of samples during the hunting season,

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the post-mortem (PM) collection of spermatozoa has arisen as an alternative to other methods. Indeed, using semen from naturally dead or poached males is considered as a very good option for conservation programs [2,11].

However, there is still a lack of data regarding several basic aspects of semen collection in cervids. In this article, we present data from Iberian red deer (*Cervus elaphus hispanicus*) collected from 1998 to 2003. During this period, we studied two models of sperm collection in this species: post-mortem harvesting from hunted free living stags and electroejaculation of farmed deer. This study deals with semen quality, genitalia morphometry and the effect of stag age on these parameters. To our knowledge, there are no previous studies on red deer presenting this kind of reproductive data and using this approach.

Electroejaculation is the preferred method for semen collection in this species, having been in use for more than 35 years [12,13]. This method allows the collection of high quality semen without noticeable differences from semen collected by natural service [6], but it requires restraint and sedation or anesthesia [7]. In fact, electroejaculated spermatozoa has produced offspring both after either laparoscopic-intrauterine or cervical insemination [1,5,6,10]. The artificial vagina has also been successfully used for obtaining ejaculates of good quality [14,15], but complications (stag training and personnel risks) have limited its use.

Recovery of epididymal spermatozoa has received much less attention. Nevertheless, the number of studies on this topic have recently increased, due to an interest in obtaining doses from dead valuable individuals in farms, and because of its potential use in wildlife conservation programs and for the hunting business [16–18]. Electroejaculation is generally a less convenient option for wild animals, because of the troubles associated with its use. Although epididymal spermatozoa differ from ejaculated ones, this kind of samples have been successfully cryopreserved and used for artificial insemination or *in vitro* fertilization [19–26].

With this study we aimed at contributing to the current knowledge on the reproductive biology of red deer with a triple approach. First, presenting anatomical data we have obtained from wild (hunted) and farmed animals, and studying the predictive value of anatomical data (testicular size) on the sperm quantitative parameters; second, comparing quantitative and qualitative data from spermatozoa collected from the cauda epididymis (wild, post-mortem) and by electroejaculation (farmed); and third, testing the effect of stag age on the aforementioned data. We

present the results of the two models (post-mortem and electroejaculated) together, to highlight the peculiarities of each kind of sample with respect to the other, which was not evident in previous studies. It is important to keep in mind that the two collection methods are closely bound with the rearing conditions of the animals. Therefore, rather than considering the pair post-mortem/electroejaculation, it is more adequate to define both groups as wild/post-mortem and farmed/electroejaculation. We have worked with the Iberian subspecies of red deer (*C. elaphus hispanicus*) which has not been extensively studied despite its importance in the economy and culture of many regions in the Iberian Peninsula [10].

## 2. Materials and methods

### 2.1. Animals

Samples were obtained during the beginning of the breeding season (rut), which in the Iberian Peninsula spans from early September to mid October [18,27]. Ejaculates were obtained from 18 Iberian red deer stags (*C. elaphus hispanicus*, Helzheimer 1909) during five consecutive years (1999–2003). Animals ranged 1.5–7.5 years of age and weighed 147.5 [142.2–178.8] kg. They were housed in a half-freedom system at facilities of the University of Castilla-La Mancha (Albacete, Spain), and they were maintained under natural day length conditions (38°57'N). During the experiment they lived in a meadow of 6500 m<sup>2</sup> composed of *Festuca arundinacea* (52.4%), *Dactylis glomerata* (28.6%), *Medicago sativa* (14.3%) and *Trifolium repens* (4.8%). Fodder was based on barley, alfalfa and oats, and animals had ad libitum access to cereal straw and water. Animal handling was performed in accordance with the Spanish Animal Protection Regulation, RD223/1998, which conforms to European Union Regulation 86/609. Epididymal samples were collected from 185 Iberian red deer stags harvested in the game reserves of Ancares, Mampodre and Picos de Europa (León, Spain) and in several private hunting reserves of the province of Cáceres (Spain). All the animals were adult and lived in a free-ranging regime, and weighed 75.0 [67.5–108.5] kg. Harvest plans followed Spanish Harvest Regulation, Law 4/96 of Castilla y León and Law 19/01 of Extremadura, which conforms 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, were reviewed each year by the Annual Hunting Regulation of the respective regions.

## 2.2. Electroejaculation procedure

Stags were restrained and anesthetized with an intravenous injection of 0.75 mg/kg xilacine (Rompun<sup>®</sup> 2%; Bayer AG, Leverkusen, Germany) and 1.5 mg/kg ketamine (Imalgene 1000<sup>®</sup>; Rhone-Mérieux, Lyon, France). Before performing the electroejaculation, we recorded several morphological parameters: body weight using an electronic scale, and width of each testicle (axis parallel to the cephalocaudal axis) and thickness of the scrotal wall using a caliper. We estimated the testicular width by subtracting the thickness of the scrotal wall. Rectum was cleaned from faeces and the prepuce area was shaved and washed with physiological saline. Electroejaculation was carried out using a rectal probe measuring 375 mm length and 40 mm width; the length of the electrodes was 80 mm. The probe was connected to a power source that allowed to controlling voltage and amperage. Ejaculation occurred at average values of 4.5 V and 90 mA (average time to ejaculation: 3 min 44 s). Anesthesia was reverted with 0.3 mg/kg yohimbine hydrochloride (prepared at 0.9%; Sigma, Madrid, Spain). Samples suspected of urine contamination (abnormal color and observations during the electroejaculation procedure) were discarded. We conducted 37 electroejaculations in the 18 farmed stags.

## 2.3. Post-mortem sperm recovery

The scrotum, including testicles and epididymes, was removed from the carcass and refrigerated at 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). 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. We recorded testicular weight and cauda epididymal weight by using an electronic scale, and the testicular width (axis parallel to the cephalocaudal axis) by using a caliper. Before starting sperm collection from the cauda epididymis, we pierced the superficial blood vessels, wiping out the blood. Spermatozoa were collected making several incisions on the cauda with a surgical blade, and taking the sperm mass emerging from the cut tubules with the aid of the blade. Sample from both epididymes of the same animal was mixed and weighed before further

manipulation. We collected and processed the testicles of 179 stags.

Only 30 post-mortem samples could be processed between 18 and 26 h post-mortem, and these were used for sperm quality analyses (pH, osmolality, motility, abnormal forms, viability). We considered this time as acceptable for maintaining sperm quality, based in previous studies in red deer [17,28].

## 2.4. Spermatozoa quality

All chemicals were of at least Reagent grade, and were acquired from Sigma (Madrid, Spain). For fixing sperm samples, we used a 2% glutaraldehyde solution (146 mM glucose, 34 mM sodium citrate tribasic and 24 mM NaHCO<sub>3</sub>).

Each sample was measured for osmolality and pH, using a cryoscopic osmometer (Osmomat-030, Gonotec<sup>TM</sup>; Berlin), and an electronic pH-meter (CG 837, Schott<sup>TM</sup>; Mainz), respectively. The sperm concentration (spermatozoa/mL) was calculated using a Bürker hemocytometer, after diluting 5 µL of sample in 500 µL of fixing solution. The total number of collected spermatozoa was calculated by multiplying its concentration by the sample volume. For epididymal samples, the volume was estimated from sample weight (density previously determined as 0.94 g/mL).

Aliquots were fixed (5 µL in 500 µL of fixing solution) for studying abnormal forms and cytoplasmic droplets using phase contrast microscopy (×400) and at least 100 cells per sample. Cytoplasmic droplets were classified as proximal or distal. Proximal droplets were considered abnormal in all cases. Distal droplet were considered abnormalities only in the case of ejaculated samples, since the distal droplet is a normal occurrence in the epididymal spermatozoon of ruminants [29]. Other abnormal forms observed were macro and microcephalic spermatozoa, pyriform heads, loose heads, midpiece reflex, segmental aplasia, broken tail or coiled tail. Since we did not observe any special association of the diverse abnormalities with each kind of sample, for simplicity we show only the total percentage.

## 2.5. CASA and fluorescence-based analysis

CASA (Computer Assisted Sperm Analysis) motility assessment was carried out as follow. The sample was diluted down to 20 × 10<sup>6</sup> spermatozoa/mL in PBS (pH 7.5, 320 mOsm/kg; for epididymal sperm we increased osmolality to 380 mOsm/kg with NaCl, isoosmotic for this kind of samples). A 5 µL drop was put on a slide at

37 °C, covered with a 22 × 22 coverslip and examined (5 fields at least) with a phase contrast microscope (Nikon Labophot-2; negative contrast optics) with a warming stage at 37 °C. Analysis was carried out using the Motility Analyzer v. 7.4G (Hamilton-Thorne Research) and the following parameters were used for the study: total motility (%; TM), progressive motility (%; PM), curvilinear path velocity (μm/s; VCL), linearity (%; LIN), amplitude of the lateral movement of the head (μ; ALH) and beat cross frequency (Hz; BCF). A spermatozoa was considered motile when VCL > 10 μm/s, and progressive if VCL > 25 μm/s and STR > 80% (STR, Straightness, was also provided by the CASA). Explanation of the descriptors of sperm movement has been thoroughly reported [30]. We used the following configuration as displayed in the software setup: frames acquired: 20; frame rate: 25/s; minimum contrast: 10; minimum size: 9; Lo/Hi size gates: 0.9/2.1; Lo/Hi intensity gates: 0.4/1.6; non-motile head size: 9; non-motile brightness: 10; medium VAP value: 25; low VAP value: 10; slow cells motile: NO; threshold STR: 80. Thus, the image acquisition rate was 25 frames/s and the acquisition time was 0.8 s.

Sperm viability and acrosomal status were assessed using fluorescent probes (modified from Cheng et al. [31]). Samples were diluted 1:100 in PBS (pH 7.5, 320 mOsm/kg; 380 mOsm/kg for epididymal samples), and stained with prodidium ioide (PI; 25 μg/L) and PNA lectin conjugated with FITC (1 μg/mL). After 10 min, the samples were analyzed with an epifluorescence microscope (Nikon Optiphot; ×400, 450–490 nm excitation filter, 510 nm dichroic-beam splitter, 520 nm barrier filter). At least 100 cells were evaluated, discriminating between red (non-viable, acrosome intact), red-green (non-viable, acrosome damaged), green (viable, acrosome damaged) and non-stained (viable; acrosome intact) spermatozoa. For data analysis, we used the percentage of viable spermatozoa (PI-cells) and the percentage of spermatozoa with intact acrosome (PNA-cells).

## 2.6. Statistical analysis

Results are shown as median and interquartile range [25% quartile–75% quartile]. For the statistical analysis, we used the R statistical environment [32]. We used linear models and regression analysis (Spearman's rank correlation) to study the effect of age and sample source (post-mortem or ejaculated) on the parameters analyzed. Due to the skew of the data, it was subjected to appropriate transformations (logarithmic or arc sine) in

order to normalize it. Whenever the age was considered an unimportant factor, we used the asymptotic Wilcoxon Mann–Whitney rank-sum test for comparing electroejaculation and post-mortem samples. The data collected was very heterogeneous; thus, despite transforming it, we preferred using non-parametric tests in these comparisons.

A testicular production ratio was produced by dividing the number of spermatozoa obtained (from the epididymis or by electroejaculation) by the corresponding testicular diameter (sum of right and left sides), and this parameter was compared between post-mortem (wild) and electroejaculation (farm) groups by using linear models. Moreover, following Malo et al. [33], we calculated a relative testicular size by summing the diameters of both testicles and dividing by the weight of the animal. We used linear models to study the association between testicular size (weight and diameter), epididymal weight and the relative testicular size with the concentration and total number of spermatozoa collected.

## 3. Results

### 3.1. Testicular measurements

In post-mortem samples, stag age was linearly related to testicular weight ( $r^2 = 0.60$ ) and epididymal weight ( $r^2 = 0.53$ ) (Fig. 1;  $P < 0.001$ ). We did not find significant differences between testicular diameter obtained from the electroejaculation experiment and from the post-mortem experiment (Fig. 2), and it was also linearly related to stag age with  $r^2 = 0.57$  ( $P < 0.001$ ) The relative testicular size (ratio testicular diameter:body weight) was not constant throughout age, but adjusted to a negative exponential model in both groups of animals ( $P < 0.05$ ), indicating that body weight and testicular growth followed different trends, with younger animals having faster body growth than testicular growth. The variation of the relative testicular size was similar in the farmed and wild groups ( $P > 0.05$ ).

### 3.2. Sperm data

Sperm concentration (Fig. 3a) was significantly higher for epididymal samples ( $P < 0.001$ ), with a pooled median value of  $4.10 [3.04–4.86] \times 10^9/\text{mL}$ , whereas for electroejaculation samples we obtained  $0.44 [0.29–0.80] \times 10^9/\text{mL}$ . However, the total number of spermatozoa per sample (Fig. 3b) did not differ statistically between epididymal ( $2.50 [1.68–5.03] \times 10^9$ ) and



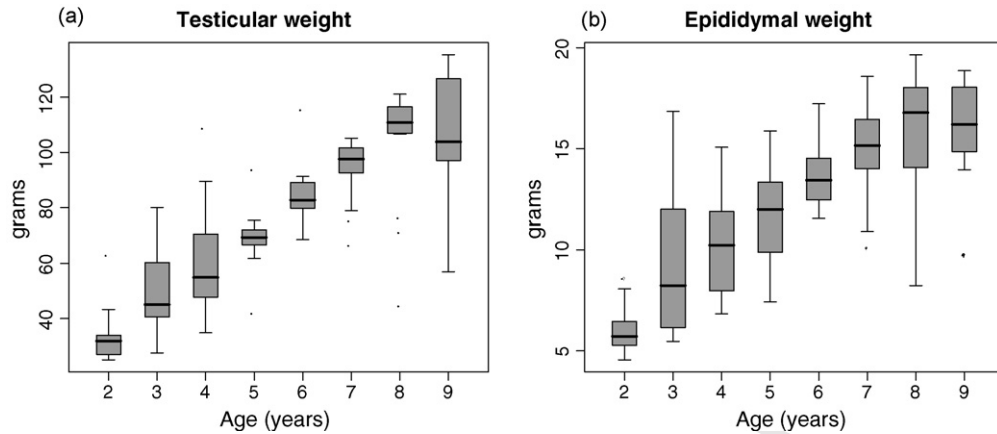


Fig. 1. Testicular weight (a) and epididymal weight (b) from the samples collected post-mortem, showing its variation with the estimated deer age. Testicular and epididymal data are averages of right and left sides. There was a linear relationship of both weights with age ( $r^2 = 0.60$  for testicular weight and  $r^2 = 0.53$  for epididymal weight;  $P < 0.001$ ). The boxes spread from the 25% to the 75% quartiles, and the whiskers extend to 1.5× interquartile range. Observations beyond whisker limits are showed as dots. The median is indicated by a horizontal line.

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343 electroejaculated samples ( $3.60 [1.74–5.55] \times 10^9$ ). The  
344 testicular production ratio (spermatozoa collected/sum of  
345 testicular diameters) did not differed significantly  
346 between post-mortem (40.5 [23.9–55.7]) and ejaculation  
347 groups (34.5 [20.0–65.4]). In post-mortem samples, age  
348 was weakly related with sperm concentration ( $r^2 = 0.07$ ),  
349 total spermatozoa ( $r^2 = 0.11$ ) and testicular production  
350 ratio ( $r^2 = 0.20$ ) ( $P < 0.001$ ). In this group, testicular  
351 diameter was also weakly related with sperm concentra-  
352 tion ( $r^2 = 0.12$ ) and total spermatozoa ( $r^2 = 0.36$ )  
353 ( $P < 0.001$ ), whereas testicular weight was related with  
354 total spermatozoa ( $r^2 = 0.17$ ;  $P < 0.001$ ), but not with

sperm concentration. Such relationships were not found  
in the electroejaculation group.

The pH and osmolality varied greatly between  
epididymal and electroejaculated samples (Fig. 3c and  
d). Ejaculated semen was more alkaline and had a lower  
osmolality than the samples collected from the cauda  
epididymis ( $P < 0.001$ ). Pooled pH was 6.28 [6.19–6.52]  
for epididymal samples and 7.63 [7.16–8.10] for  
ejaculates. The pH of ejaculates also increased with  
age ( $r^2 = 0.12$ ,  $P = 0.028$ ), from 6.71 at 2 years of age to  
8.19 at 7 years of age. We detected a sudden fall in pH  
measurements for 6-year-old stags; however, an analysis  
using linear mixed models (using deer and collection year  
as random effects) showed that this was rather an effect of  
collection year. Osmolality measurements yielded 378  
[356–395] mOsm/kg for epididymal samples and 309  
[321–332] mOsm/kg for ejaculated semen.

Spermatozoa obtained by electroejaculation differed  
from those obtained from the cauda epididymis (Fig. 4).  
Although the percentage of motile spermatozoa was  
high and roughly similar for both kind of samples  
( $P = 0.051$ ), the quality of movement was much better  
for ejaculated samples. For instance, the median value  
of sperm velocity (VCL) and linearity (LIN) for  
ejaculated semen approximately doubled those of the  
epididymal samples (112  $\mu\text{m/s}$  and 81% vs. 53  $\mu\text{m/s}$   
and 44%). The percentage of abnormal forms and  
proximal droplets was slightly larger for epididymal  
spermatozoa, although significant (abnormal forms:  
29% vs. 18.8%; proximal droplets: 3% vs. 0.6%;  
 $P < 0.01$ ), but the difference in the percentage of  
spermatozoa bearing a distal cytoplasmic droplet was  
considerable (83% vs. 1.5%;  $P < 0.001$ ). This is an

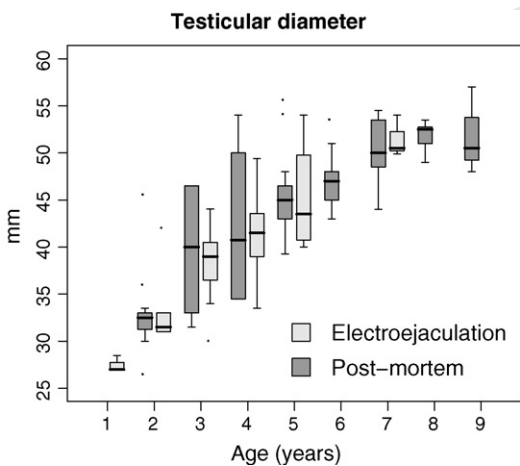


Fig. 2. Increase of testicular diameter (average of right and left sides) with increasing male age, for post-mortem samples and electroejaculated animals. There was a very significant linear relationship between both parameters ( $r^2 = 0.69$ ;  $P < 0.001$ ). The data from electroejaculated and post-mortem samples did not differ significantly. See Fig. 1 for a description of box plot elements.

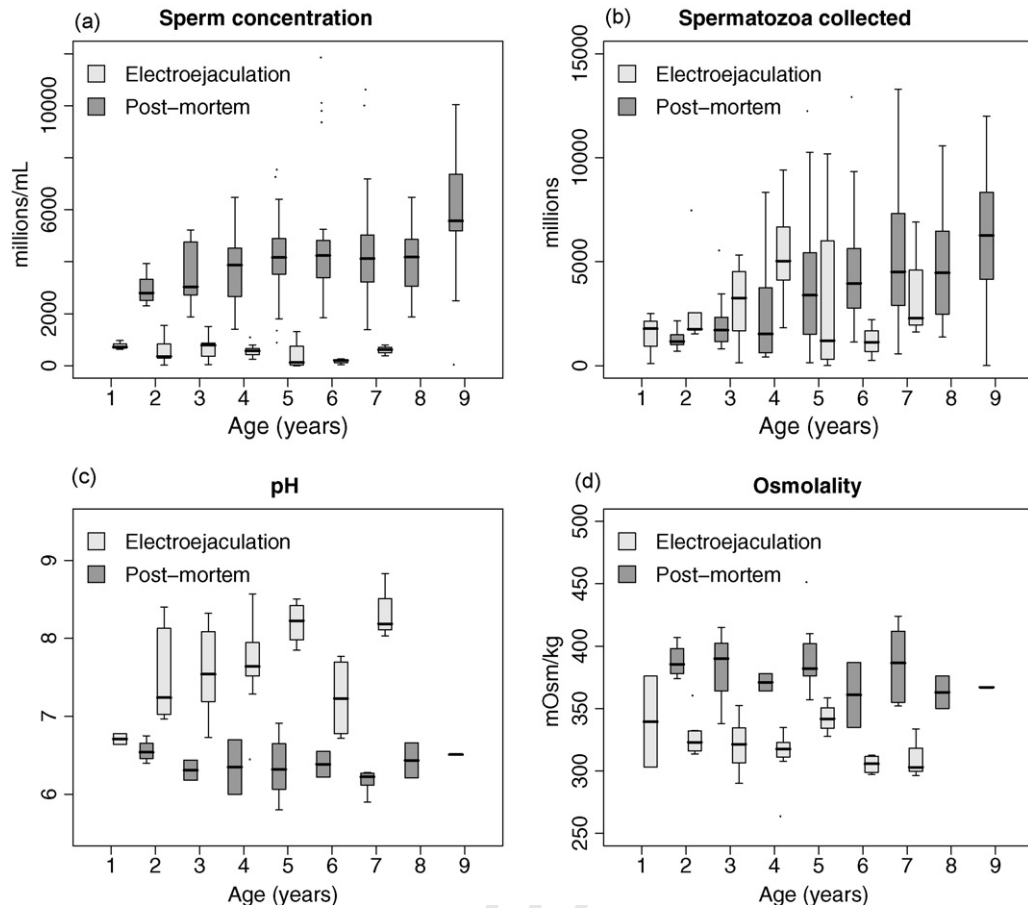


Fig. 3. Sperm concentration, total spermatozoa collected, pH and osmolality of electroejaculated and post-mortem samples, classified by age. Concentration, pH and osmolality were significantly different between post-mortem and ejaculated samples ( $P < 0.001$ ). Age was significantly related to concentration and number of spermatozoa from post-mortem samples ( $P < 0.001$ ), and to the pH of ejaculated semen ( $P = 0.028$ ). See Fig. 1 for a description of box plot elements.

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388 expected result when dealing with epididymal samples,  
389 since in ruminants the cytoplasmic droplet remains  
390 attached to the midpiece of the spermatozoa while in the  
391 epididymis, shedding when diluting in seminal plasma  
392 during the ejaculation [29]. The fluorescence probes PI  
393 and PNA-FITC indicated that the samples from both  
394 sources had a high percentage of membrane-intact  
395 (viable) and acrosome-intact spermatozoa. There were  
396 no significant differences between sources regarding  
397 viable spermatozoa (overall median: 74%). Considering  
398 viable spermatozoa, ejaculated samples showed a  
399 higher proportion of intact acrosomes (97% for  
400 ejaculate vs. 90% for epididymal sperm;  $P = 0.002$ ).

#### 4. Discussion

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402 In this study, we show novel data on Iberian red deer  
403 spermatology regarding two practical situations for

403 sperm collection: post-mortem recovery from wild deer  
404 and electroejaculation of farmed stags. It could be  
405 argued that the environmental and rearing differences  
406 would affect the sperm characteristics in each group.  
407 Nevertheless, we have considered that in the practice  
408 each collection method is inseparable from the  
409 situation of the animals: post-mortem collection is  
410 the most convenient method for wild animals,  
411 especially when subjected to hunting programs,  
412 whereas electroejaculation is routinely used for farmed  
413 animals. Binding each collection method to the wild  
414 and farmed populations is not confounding, but indeed  
415 much more realistic that if both methods had been  
416 tested in the same population. It would be possible to  
417 perform a direct comparison of the post-mortem and  
418 electroejaculation methods, conducting electroejacu-  
419 lation and castration in the same animals, but this  
420 implies many difficulties.  
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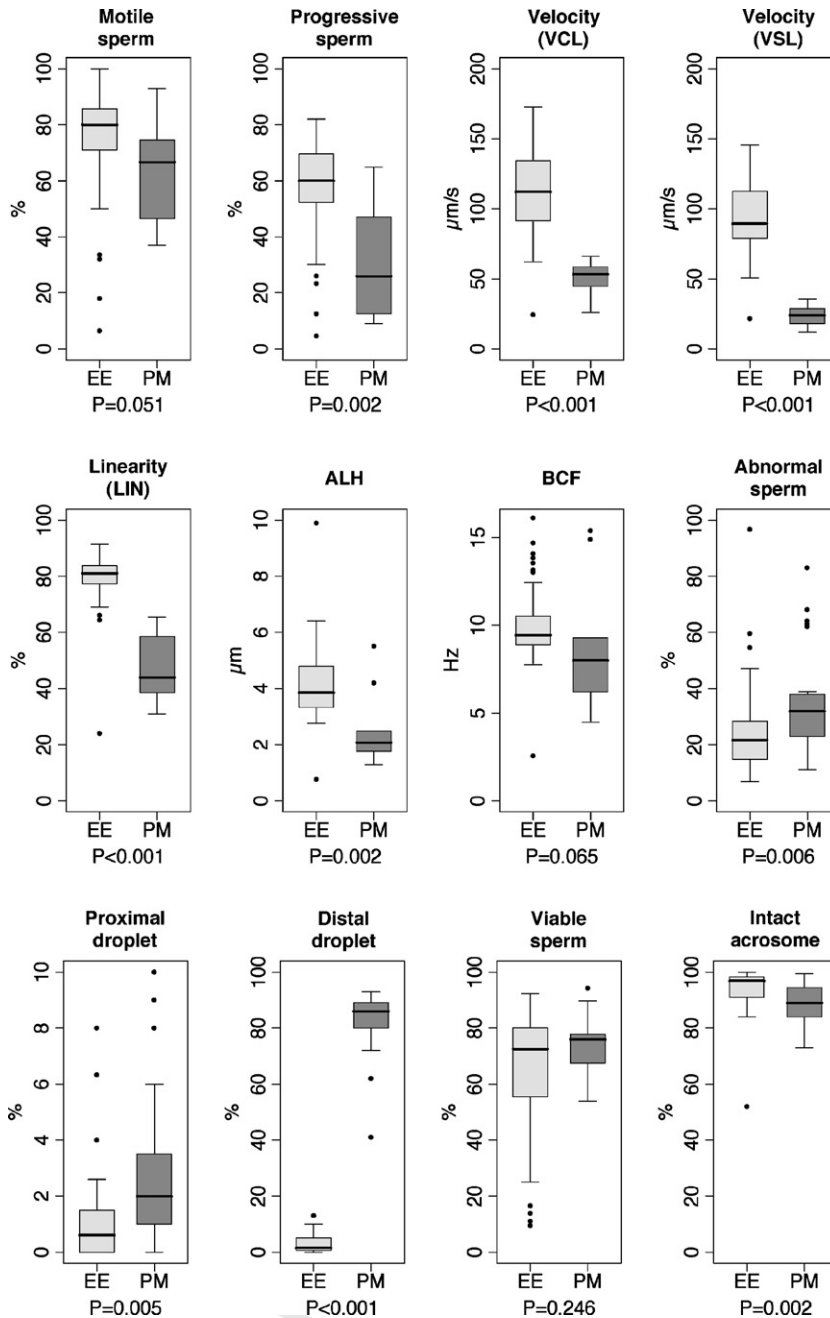


Fig. 4. Comparison of spermatozoa characteristics between electroejaculated samples (EE) and epididymal samples (PM). The P values from the between-groups comparison are shown under each plot (asymptotic Wilcoxon Mann–Whitney rank-sum test). See Fig. 1 for a description of box plot elements. In the proximal droplets plot, a data point with value 54 (PM) was intentionally left out of the graph.

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We have reported morphometric data regarding the testicular size of Iberian red deer. Although there are many differences with other subspecies (smaller size, adaptation to a dry environment, etc.) [10], our results might be generalizable. Few studies have considered the effect of age in testicular size of red deer. Lincoln [34] and Gomez et al. [35] worked only with young stags,

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aiming to study changes during and just after puberty. Lincoln [34] reported a testicular weight of ~32 and ~40 g at 2 and 3 years of age, respectively. These weights are within the interquartile range reported in this study for these ages, even though Lincoln [34] worked with a different subspecies (possibly *C. elaphus scoticus*). Gomez et al. [35] used Iberian red deer, and

435 reported a mean testicular diameter of 3 cm in yearlings  
436 and of 3.75 cm in 2-year old stags, which approximate  
437 our mean results for these ages (3.1 and 3.5 cm,  
438 respectively). In our study, we have demonstrated for  
439 the age range 1–9 years a linear relationship between  
440 age and both testicular size and epididymal weight. The  
441 data obtained from 9-year-old deer (Figs. 1 and 2)  
442 suggest that testicular growth may slow down after 8  
443 years of age, as showed in studies in bull [36] and  
444 buffalo [37,38]. This hypothesis should be tested in  
445 future studies including older animals. Moreover, the  
446 testicular and body growth rates were different. Body  
447 weight increased faster than testicular size in young  
448 males (1–3 year old), and then at a similar rate at older  
449 ages. This result differs from Malo et al. [33], who did  
450 not found any effect of age upon relative testicular size,  
451 although it is possible that most animals in that study  
452 were adult, thus presenting a constant relationship  
453 between both growth rates.  
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455 The increasing number of spermatozoa collected  
456 post-mortem in older animals could not be due only to  
457 the increase of testicular size, as showed by the low  
458 determination coefficients, but maybe by an improve-  
459 ment of spermatogenetic efficiency with age. Interest-  
460 ingly, a relationship between spermatozoa number and  
461 age was not found in electroejaculated samples. The  
462 reason might be that, whereas the collection technique  
463 allowed to recover most of the sperm reservoir in the  
464 cauda epididymis [39], the electroejaculation technique  
465 is bound to high variability in their results because of  
466 many factors (mainly due to the animal, such as stress,  
467 response to anesthesia, response to the stimulation,  
468 etc.). Although the yields of both techniques were not  
469 statistically different, we found a very high variability  
470 on the number of spermatozoa collected by electro-  
471 ejaculation. Unfortunately, we could not control for  
472 ancillary variables, because of restrictions imposed by  
473 animal rearing. From a practical point of view,  
474 spermatozoa yield was high enough to prepare a  
475 considerable number of insemination doses per sample.  
476 From the interquartile values and using  $100 \times 10^6$   
477 spermatozoa per dose [24], we could prepare 16–50  
478 doses per epididymal sample or 17–55 doses per  
479 ejaculate. We obtained similar results in a previous  
480 study on epididymal spermatozoa [18], confirming  
481 post-mortem recovery as an adequate source of sperm  
482 doses in hunting areas. Gizejewski et al. [16] could  
483 collect  $8.8 \pm 3.9 \times 10^9$  spermatozoa (mean  $\pm$  S.D.)  
484 from wild European red deers (*C. elaphus carpathicus*  
485 *var. montanus*) in the rut. The difference may rest in the  
486 larger size of these stags compared with the Iberian  
487 subspecies. In fact, the testicular weight reported by

488 these authors was  $155.9 \pm 26.8$  g (mean  $\pm$  S.D.) for a  
489 median age of 9 years, much larger than the weights  
490 reported by us (mean weight at 9 years: 106.1 g).  
491 However, in general, our results seem to be higher than  
492 those obtained by other authors. Thus, Malo et al. [40]  
493 (*C. elaphus hispanicus*) and Hishinuma et al. [41] (*C.*  
494 *nippon*) using a similar method for recovering  
495 epididymal spermatozoa, accounted for  $1.9 \pm 0.1$   
496  $\times 10^9$  and  $1.3 \pm 0.5 \times 10^9$  (mean  $\pm$  S.E.M.) sperma-  
497 tozoa, respectively, whereas we were able to collect  
498  $3.9 \pm 0.2 \times 10^9$  spermatozoa per sample. The differ-  
499 ence with the study of Malo et al. [40] can be explained  
500 by the fact that it was carried out after the rut (October–  
501 December), when epididymal sperm concentration is  
502 lower (previously showed in [18]). Regarding electro-  
503 ejaculation, Umaphy et al. [42] (*Axis axis*) and  
504 Monfort et al. [43] (*C. eldi thamin*) obtained in average  
505 less than  $2.0 \times 10^9$  spermatozoa per ejaculate, whereas  
506 we obtained a mean value of  $3.4 \times 10^9$  spermatozoa. In  
507 contrast, Mulley et al. [44] and Asher et al. [45] reported  
508 collecting more than  $4.0 \times 10^9$  spermatozoa from  
509 *Dama dama*. A possible cause for these differences  
510 might be the lack of standardized protocols among  
511 studies.

512 In this study, we have showed that epididymal  
513 present higher concentration, have a lower pH and  
514 higher osmolality than ejaculated semen. Fernandez-  
515 Santos et al. [46] and Martinez-Pastor et al. [23] found  
516 that epididymal spermatozoa from Iberian red deer  
517 should be frozen in media of higher osmolality, more  
518 similar to epididymal fluid, rather than in the  
519 conventional one, isoosmotic to ejaculated spermat-  
520 ozoa. Moreover, we have found that electroejaculates  
521 increased their pH values with age. The explanation of  
522 this fact is beyond the scope of the present study,  
523 although we hypothesize that it might be due to changes  
524 in the secretions contributing to the seminal plasma.  
525 The ramifications of such a study would be many, since  
526 it could be aimed not only to identify the nature and the  
527 cause of this change of pH, but also the physiological  
528 source and why it was related to age.

529 We did not find any significant effect of age in the  
530 sperm quality parameters. However, Garde et al. [11]  
531 reported that epididymal samples from young stags (2  
532 and 3 years of age) had lower motility, more abnormal  
533 spermatozoa and less reactivity to the HOS test than  
534 older males. These authors proposed that, since the  
535 samples had been collected at the very beginning of the  
536 breeding season, these young animals had not reached  
537 the testosterone peak yet [34,47], thus the worse results  
538 regarding sperm quality. It is possible that, in our study,  
539 most of the young stags were in the same conditions that



539 the older males, not presenting prominent differences in  
540 their semen quality. Considering motility, although  
541 epididymal and ejaculated spermatozoa were similar  
542 regarding the percentage of motile and viable sperma-  
543 tozoa, CASA parameters (velocity, linearity, etc.) were  
544 lower for epididymal samples. We have to point out that  
545 these differences were not due to the different ability of  
546 the CASA system to deal with different kind of samples  
547 (analyses were confirmed by using the “replay” option  
548 of the software and by visual observation). Studies  
549 using CASA to analyze red deer motility are scarce, and  
550 only Malo et al. [40] showed CASA parameters in their  
551 study, which were higher than the ones showed here.  
552 There are two possible reasons for this difference: that  
553 study was carried out after the rut, when motility  
554 parameters are higher [18,27]; and the samples could be  
555 analyzed shortly after the death of the males. In our  
556 case, samples could not be processed immediately, and  
557 were transported refrigerated to lengthen their viability,  
558 which might have depressed their motility. We cannot  
559 forget that refrigerated storage of the testicles is very  
560 common when collecting samples from wild animals  
561 and the processing facilities are beyond several hours  
562 from the collection site, and therefore our results should  
563 rather be used as a reference for these situations.  
564 Nevertheless, it has observed previously that deer  
565 epididymal spermatozoa seemed to improve their  
566 motility once extended, suggesting that motility  
567 depression observed in freshly collected samples might  
568 often be temporal [39,48,49]. Moreover, Martínez-  
569 Pastor et al. [23], comparing many freezing protocols,  
570 found similar motility for both electroejaculated and  
571 epididymal samples after thawing, even though the  
572 epididymal spermatozoa had undergo refrigerated  
573 storage for about 20 h. Furthermore, Soler and Garde  
574 [50] found a lack of correlation between classical  
575 parameters and penetration scores, motility among  
576 them, for red deer epididymal spermatozoa. Thus, it is  
577 possible that sperm motility of epididymal spermatozoa  
578 do not reflect its real ability on fertilization or  
579 cryopreservation when measured just after collection.

581 In conclusion, in this study we have demonstrated  
582 that stag age affects testicular and epididymal size and  
583 several sperm parameters, both in electroejaculated and  
584 post-mortem samples, but it does not seem to influence  
585 sperm quality. We also found that the growth rates of  
586 body weight and testicular size differed. Furthermore,  
587 we have presented data regarding many reproductive  
588 parameters of Iberian red deer, which may be of  
589 application on other subspecies and even in other cervid  
590 species, and have showed that it is possible to obtain a  
591 large number of spermatozoa by both electroejaculation

591 or post-mortem collection. Moreover, we want to  
592 highlight that, despite the differences reported here and  
593 in previous studies, both electroejaculated and epidid-  
594 ymal spermatozoa (considering quantity and quality)  
595 should be equally valid for establishing germplasm  
596 banks, either for conservation or selection. We expect  
597 that this study would help to improve the development  
598 of assisted reproductive techniques on this species,  
599 mainly regarding sperm collection, its manipulation and  
600 its cryopreservation. In this regard, the development of  
601 new cryopreservation protocols should take into  
602 account the differences between epididymal and  
603 electroejaculated spermatozoa, since they may behave  
604 differently depending on extender composition, freez-  
605 ing/thawing rates and other variables.  
606

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