UNIVERSIDAD DE LEÓN

FACULTAD DE CIENCIAS BIOLÓGICAS Y AMBIENTALES

Departamento de Biología Celular y Anatomía

Obtención postmortem y calidad de los espermatozoides de la cola del epidídimo del ciervo rojo ibérico (*Cervus elaphus hispanicus*)

Post-mortem recovery and quality of the spermatozoa obtained from the cauda epididymis of Iberian red deer (*Cervus elaphus hispanicus*)

por

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Memoria presentada como parte de los requerimientos para optar al título de Doctor.

León, 7 de octubre de 2004

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A mi familia y amigos

A todos aquellos que luchan
para que la investigación tenga
un mejor fundamento
que becas
sin derechos laborales

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Lista de acrónimos

A continuación se muestran las definiciones de una serie de acrónimos comunes a varios de los trabajos compilados en este documento.

VCL *Velocity-CurviLinear (track velocity)*, velocidad respecto a la trayectoria real. Es la relación entre la longitud de la trayectoria real (curvilínea) del espermatozoide y el tiempo invertido en el recorrido.

$$VCL = \frac{\sum_{i=1}^{n} e_i}{\Delta t} \quad e_i = \sqrt{(x_i - x_{i+1})^2 + (y_i - y_{i+1})^2}$$

Donde n es el número de imágenes de la secuencia a analizar, Δt es el tiempo total, y x_i e y_i son las coordenadas del espermatozoide en cada imagen.

VSL *Velocity-Straight Line (straight line velocity)*, velocidad respecto a la trayectoria recta o lineal. Es la relación entre la longitud del segmento que une el punto inicial y el punto final de la trayectoria del espermatozoide y el tiempo invertido en el recorrido.

$$VSL = \frac{d}{\Delta t} \quad d = \sqrt{(x_n - x_1)^2 + (y_n - y_1)^2}$$

Donde d es la distancia entre el punto inicial y final de la trayectoria, n es el número de imágenes de la secuencia a analizar, Δt es el tiempo total, y (x_1, y_1) y (x_n, y_n) son las coordenadas del espermatozoide en la primera y última imagen, respectivamente.

VAP *Velocity-Average Path (smoothed path velocity)*, velocidad respecto a la trayectoria «media» o suavizada. Es la relación entre la longitud de la trayectoria real del espermatozoide, tras ser suavizada por un algoritmo matemático, y el tiempo invertido en el recorrido.

$$VAP = \frac{\sum_{i=1}^{n} e_{m_i}}{\Delta t}$$

Donde n es el número de imágenes de la secuencia a analizar, Δt es el tiempo total, y e_{m-i} es la distancia en cada imagen de cada uno de los segmentos que componen la trayectoria suavizada.

LIN *LINearity*, índice de linealidad. Es la relación entre VSL y VCL, expresada como porcentaje, e indica la similitud de la trayectoria real con una línea recta.

$$LIN = \frac{VSL}{VCL} \times 100$$

STR *STRaightness*, índice de rectitud. Es la relación entre VSL y VAP, expresada como porcentaje, e indica la similitud de la trayectoria suavizada con una línea recta.

$$LIN = \frac{VSL}{VAP} \times 100$$

WOB *WOBble*, índice de oscilación. Es la relación entre VSL y VCL, expresada como porcentaje, e indica la similitud de la trayectoria suavizada con la trayectoria real, lo cual ofrece una estimación de la sinuosidad de la trayectoria real.

$$LIN = \frac{VAP}{VCL} \times 100$$

ALH *Amplitude of Lateral Head displacement*, amplitud media del desplazamiento lateral. Es el valor medio del desplazamiento que realiza la cabeza del espermatozoide en su trayectoria curvilínea de un lado a otro de la trayectoria lineal.

$$ALH = \frac{\sum_{i=1}^{n-1} alh_i \times 2}{n-2}$$

Donde n es el número de imágenes de la secuencia a analizar, y alh_i es la distancia desde el centroide de la cabeza del espermatozoide hasta la trayectoria suavizada, en cada imagen.

BCF *Beat Cross Frecuency*, frecuencia de batido. Es la relación entre el número de veces que la trayectoria real cruza la trayectoria suavizada y el tiempo total. Indica la frecuencia con la que la cabeza cruza la trayectoria suavizada, y es una estimación de la intensidad del batido flagelar.

CCC *Cubic Clustering Criterion*. Uno de los estadísticos devueltos por los procedimientos FASTCLUS y CLUSTER (SAS/STAT^{TM}), que puede ser utilizado para determinar el número final de clústers.

Capítulo 1

Sumario

La creación de bancos de germoplasma para especies silvestres es un proceso complejo y que requiere una buena planificación, por lo cual es necesario contar con conocimientos suficientes sobre la especie en cuestión y sobre el material a preservar. Sólo de esta manera es posible diseñar una estrategia efectiva. En este trabajo se presentan los resultados de varios estudios realizados con muestras espermáticas extraídas post-mortem de la cola del epidídimo del ciervo rojo ibérico. Estos estudios han sido realizados dentro del contexto de la creación y mantenimiento de bancos de germoplasma (incluidos dentro de los bancos de recursos genéticos), por lo que contemplan aspectos interesantes para su funcionamiento, sobre todo en relación con la preservación de muestras de rumiantes silvestres. Dado que en nuestra región las muestras proceden de actividades cinegéticas, un primer enfoque fue el estudio del efecto del tiempo post-mortem (1–7 días) en la calidad seminal de muestras refrigeradas. Tal como era de suponer, esta calidad decreció apreciablemente, especialmente la movilidad espermática, existiendo correlaciones negativas entre casi todas las variables cualitativas y el tiempo post-mortem. Parámetros físicos como la osmolaridad y el pH también sufrieron variaciones (menos evidentes para el segundo). Tras 3 días post-mortem, apenas hubo muestras de calidad aceptable. La estacionalidad fue otro aspecto importante objeto de estudio, dividiendo para ello el período de recogida de muestras en varios períodos: berrea (mediados de septiembre a mediados de octubre), resto de la estación reproductiva (mediados de octubre a mediados de diciembre) y fuera de la estación reproductiva (febrero). Las dimensiones y peso de testículos y epidídimo fueron mayores durante la berrea, así como la cantidad de muestra seminal recuperada. La calidad espermática fue mejor en el período de la estación reproductora posterior a la berrea (aunque esta conclusión fue condicionada por los resultados obtenidos en otro experimento, como se explica más abajo), y mucho peor fuera de la estación.

Por otra parte, para determinar subpoblaciones espermáticas a partir de datos de movilidad proporcionados por CASA, se propuso un modelo compuesto por una serie de procedimientos estadísticos multivariantes. Este método fue utilizado para analizar los ocho descriptores de movilidad por espermatozoide en muestras epididimarias, durante un protocolo estándar de criopreservación. El análisis indicó la presencia de tres subpoblaciones diferentes, cuyas proporciones y características

variaron dependiendo del momento en que se analizó la muestra (tras la extracción, antes de congelar y después de descongelar). La movilidad espermática se incrementó en la pre-congelación respecto al análisis inicial, a costa de una caída de la progresividad. Mediante un análisis discriminante, el número de descriptores fue reducido a tres, y, tras repetir el análisis de subpoblaciones, apareció un patrón muy similar, donde la subpoblación de mayor calidad detectada en el primer análisis (rápida y linear) se dividió en dos (de velocidades media y rápida, respectivamente). Por otra parte, otro análisis de clústers permitió clasificar a los machos utilizados en el estudio según la congelabilidad de sus espermatozoides. Además, hubo una correlación significativa entre la proporción de la subpoblación linear antes de la congelación y la recuperación de viabilidad tras la descongelación.

El método estadístico para determinar subpoblaciones se utilizó también para analizar un conjunto de muestras procesadas a distintos tiempos post-mortem (1–4 días) y en distintas épocas de la estación reproductora (mediados de septiembre-mediados de octubre; mediados de octubre-noviembre; primera quincena de diciembre). En el análisis de intervalos post-mortem, la variación del patrón de subpoblaciones (que indicaba un predominio de subpoblaciones de mala calidad según aumentaba el tiempo post-mortem) sugirió cambios críticos después de 72 h post-mortem. En cuanto a la estacionalidad, aparecieron cuatro subpoblaciones, cuya evolución aparentemente indicaba mejor calidad cuanto más avanzada estaba la estación. Sin embargo, la subpoblación que incrementó su presencia posiblemente agrupaba espermatozoides con una maduración epididimaria excesiva. Esto podría indicar que, después de la berrea, debido a la menor utilización de las reservas epididimarias, los espermatozoides podrían sufrir fenómenos de hipermaduración en la cola del epidídimo, lo cual sería negativo.

Finalmente, se realizó una comparación entre dos métodos habituales de obtención de las reservas espermáticas de la cola del epidídimo, con el fin de determinar el efecto del método en los espermatozoides recuperados. Las muestras utilizadas fueron procesadas de manera que, de cada par de testículos correspondientes a un mismo animal, una de las colas fuese procesada por medio de cortes (diluyendo inmediatamente la masa seminal en diluyente de congelación), mientras que la otra fue separada del resto del epidídimo y perfundida con diluyente de congelación desde el conducto deferente. Las muestras obtenida con cada método fueron analizadas para determinar: rendimiento de la extracción, calidad espermática (incluyendo análisis de subpoblaciones) y nivel de contaminación celular (sangre y tejido epididimario). Las muestras fueron además criopreservadas para estudiar el efecto del método en la congelabilidad. El rendimiento fue similar para ambas técnicas, pero la calidad fue mejor en las muestras obtenidas por perfusión (sobre todo teniendo en cuenta el análisis de subpoblaciones espermáticas). La contaminación celular fue más importante en las muestras obtenidas por cortes, especialmente al considerar el recuento de eritrocitos.

Estos experimentos nos dan información muy interesante para la conservación de especies silvestres mediante el uso de bancos de germoplasma. Así, al menos para el caso del ciervo rojo ibérico y especies similares, sería aconsejable procesar y congelar las muestras cuanto antes, en todo caso antes de 48–72 h, para prevenir alteraciones posiblemente críticas en la calidad espermática. En

cuanto a la estacionalidad, la mejor calidad encontrada después de la berrea sugiere que esas muestras son potencialmente utilizables. Sin embargo, tras el análisis de subpoblaciones y la sospecha de que puedan sufrir hipermaduración, está claro que tales muestras deben ser analizadas más en profundidad, de manera que se confirme si serían aptas para su uso en técnicas de reproducción asistida. En todo caso, por rendimiento y calidad, las muestras recogidas fuera de estación (febrero) no parecen apropiadas para ser incluidas por rutina en un banco de recursos genéticos. Por otra parte, la comparación de las dos técnicas de extracción indicó que la perfusión del epidídimo debería ser utilizada preferentemente para la obtención de muestras, aunque no se descartaría la técnica de cortes en determinadas ocasiones, por su sencillez. Finalmente, la aplicación del método estadístico propuesto para el análisis de subpoblaciones, tanto para complementar los anteriores estudios como en el análisis del proceso de criopreservación, indica que esta metodología podría utilizarse rutinariamente y aportar información valiosa, no disponible con otros métodos de análisis espermático.

Capítulo 2

Summary

Setting up germplasm banks for wildlife is a complex process, requiring a good planning, thus we need enough knowledge about the species and the material we want to preserve, in order to design an effective strategy. In this Thesis, I present the results of several experiments carried out on sperm samples recovered post-mortem from the cauda epididymis of Iberian red deers. These studies have been performed taking into account the founding and management of germplasm banks (within the genetic resource banks), therefore considering some interesting aspects related to them, especially concerning samples from wild ruminants. Since the samples collected in our region come from hunting activities, a first study dealt with the effect of post-mortem time (1-7 days) on the quality of the sperm from refrigerated samples. As expected, this quality decreased sensibly, especially sperm motility, finding negative correlations between almost every qualitative parameters and post-mortem time. Physical parameters, osmolality and pH underwent variations too (being less evident for the latter). After 3 days post-mortem, there was hardly any sample with acceptable quality. Seasonality is another important factor, which was also studied, splitting the period when sample recovery took place in: rut (mid-September to mid-October), post-rut (mid-October to mid-December) and non-breeding season (February). The size and weight of testicles and epididymis were higher in the rut, such as the quantity of recovered sperm sample. Sperm quality was higher in the post-rut (however this conclusion was conditioned by the results obtained in other experiment, as explained below), and much worse in the non-breeding season.

Moreover, a method including several statistical multivariate procedures was proposed to identify sperm subpopulations from CASA motility data. This method was then used to analyze eight descriptors per spermatozoa from epididymal samples, through a typical cryopreservation protocol. This analysis identified 3 subpopulations, whose proportions and characteristics varied depending of the moment the sample was analyzed (just after recovery, pre-freezing or post-thawed). Sperm motility increased pre-freezing, but progressivity dropped. A discriminant analysis reduced the number of descriptors to 3, which were used to repeat the subpopulation analysis, which rendered a similar pattern, where the subpopulation with higher quality (rapid and linear) had split in two (medium velocity and rapid). On the other hand, another cluster analysis allowed me to classify

the males according to the freezability of their sperm. Moreover, there was a significant correlation between the proportion of the linear subpopulation and the viability recovery after freezing-thawing.

This statistical method was also used to analyze samples processed at different post-mortem times (1–4 days) and in different moments of the mating season (mid-September to mid-October; mid-October-November; first fortnight of December). The post-mortem time analysis showed a variation of the subpopulation pattern, displaying an increase of the proportion of bad-quality subpopulations, which suggested some critical changes after 72 h post-mortem. Regarding seasonality, the statistics rendered four subpopulations, whose evolution indicated apparently that quality increased after the rut. However, the subpopulation which increasing proportion was composed probably by spermatozoa excessively mature, and this may indicate that, after the rut (with a decreasing utilization of epididymal reserves), spermatozoa might undergo hypermaturation in the cauda epididymis, being this a negative fact.

Finally, two routine methods for recovering spermatozoa from the cauda epididymis were compared, in order to determine the effect on sperm quality. Of each pair of testicles, one cauda epididymis was processed by means of cuts (diluting the sample immediately in extender), and the other was flushed from the deferent duct with extender. Both samples were analyzed for: yield of the method, sperm quality (including subpopulation analysis) and contamination extent (blood and epididymal tissue). The samples were cryopreserved too, in order to determine the impact of the extraction technique in their freezability. Yield was similar for both techniques, but quality was better for flushing. Samples obtained by cuts were more contaminated, especially taking into account erythrocyte counts.

These experiments have rendered very interesting information concerning wildlife conservation using germplasm banks. Thus, at least in the case of the Iberian red deer and similar species, it would be advisable to process and cryopreserve sperm samples before 48–72 h, preventing changes of sperm quality that might be critical. Regarding season, samples collected post-rut are possibly usable because of their better quality. However, the subpopulation analysis showed that these samples might have underwent hypermaturation, thus further analyses are necessary to clarify if they are adequate for use in assisted reproduction protocols. Nevertheless, due of its poor yield and quality, samples collected in the non-breeding season might not be routinely included in germplasm banks. On the other hand, the comparison between the two techniques for recovering epididymal sperm showed that flushing must be used preferably for this task, although cuts may not be discarded for some situations, due of its simplicity. Finally, the application of the proposed statistical method for subpopulation analysis, not only for complementing these experiments, but also for studying the cryopreservation process, indicate that this kind of analysis might be used on a routine basis, contributing with worthy information which would not be available using other analytical methods.

Capítulo 3

Introducción general

A partir de la Revolución Industrial, el ser humano ha adquirido una capacidad sin precedentes para alterar el medio natural. De hecho, el desarrollo disfrutado por los países más avanzados económicamente ha sido posible a costa de la modificación de toda la biosfera. Esto ha producido una acelerada degradación de la biosfera, lo cual ha sido motivo de preocupación según se han ido vislumbrando sus posibles consecuencias.

Uno de los efectos más evidentes de este comportamiento agresivo ha sido el aumento en la tasa de extinción de las especies silvestres. En este momento, la lista roja de la IUCN (International Union for Conservation of Nature and Natural Resources) [1] recoge un total de 22.424 especies (animales y vegetales). Es significativo que más del 80 % estén catalogadas bajo algún tipo de amenaza. De ellas, 58 especies están ≪extinguidas en estado silvestre≫, y 2.249 se encuentran ≪en estado crítico≫. Además, 762 se consideran definitivamente extinguidas. En este panorama habría que incluir aquellas especies calificadas como de ≪preocupación mínima≫ globalmente, pero que se encuentran localmente amenazadas, contando con subespecies y poblaciones con distintos grados de vulnerabilidad.

La constatación de este problema, y de que el medio natural es fuente de riqueza no sólo por las materias primas que proporciona, sino *per se*, ha planteado la necesidad de proteger los distintos hábitats y la biodiversidad que albergan [2–5]. Como consecuencia, han surgido diversas iniciativas conservacionistas, incrementándose los esfuerzos para gestionar adecuadamente las áreas naturales, se han potenciado los parques zoológicos y reservas naturales, y se han propuesto nuevas estrategias para evitar el declive de las poblaciones silvestres. Efectivamente, uno de los problemas más acuciantes es, aparte de las amenazas directas, la fragmentación de poblaciones, que suele acarrear fenómenos de consanguinidad y deriva genética, con consecuencias frecuentemente desastrosas [6]. La solución a este problema no es sencilla, y los sistemas de corredores o la traslocación entre parques zoológicos son, con frecuencia, complicados, onerosos o inviables [7].

En este contexto surgió el concepto de banco de recursos genéticos, que posteriormente se ha ampliado con la acuñación del término ≪banco de recursos biológicos≫ (BRB). En estos centros se almacenan a medio o largo plazo tejidos, gametos o embriones de las especies de interés, los

cuales pueden ser utilizados para diversos usos, desde estudios epidemiológicos a experiencias de clonación. Dentro de estos centros tienen especial importancia los bancos de germoplasma, que almacenan a muy baja temperatura espermatozoides, gametos y embriones, a la espera de su utilización en programas de reproducción asistida [8–11]. Las ventajas de este tipo de bancos son evidentes, especialmente en lo que concierne a la enorme economía de espacio y recursos (un sólo tanque de nitrógeno líquido de tamaño medio puede almacenar miles de dosis seminales). El tamaño efectivo de las poblaciones silvestres puede ser incrementado sin dificultad, y es posible disponer simultáneamente de germoplasma procedente de varias generaciones diferentes [6]. Por otra parte, el transporte de estas dosis es relativamente sencillo, salvando las correspondientes regulaciones nacionales e internacionales [12]. Esto permite que los programas de recuperación puedan ser diseñados con gran flexibilidad [13, 14]. Por otra parte, estos bancos son útiles no sólo para la conservación de las especies en peligro, sino también para aquellas que cuentan con censos suficientes, ya que permite realizar estudios sobre la especie y responder ante riesgos futuros.

Sin embargo, antes de poder iniciar un proyecto de este tipo, es necesario contar con unos protocolos eficientes para evaluar y criopreservar el material a salvaguardar, sean espermatozoides, ovocitos o embriones [6, 15]. También se debe tener en cuenta cuál será la utilización final de este material, inseminación artificial, FIV, ICSI, transferencia embrionaria, clonación, etc. [16, 17]. No obstante, no es posible la aplicación directa de las técnicas de reproducción asistida que se han utilizado exitosamente en especies domésticas, y su adaptación, o la creación de otras nuevas, específicas, requiere un conocimiento exhaustivo de la biología reproductiva de las especies en cuestión y del tipo de muestras con las que se está trabajando [18, 19].

En este trabajo se describen varias experiencias realizadas con espermatozoides de ciervo rojo ibérico (incluyendo en algunas de ellas muestras de rebeco y corzo) recogidos post-mortem. Estas experiencias han sido realizadas teniendo en cuenta su futura aplicación en la creación de bancos de germoplasma para éste y otros rumiantes silvestres. La importancia de estas especies en España, y en nuestra región concretamente, es considerable, no sólo por su valor cinegético y turístico, que se refleja en el desarrollo de las zonas donde estas actividades se realizan, sino también por su valor ecológico [20–22]. Aunque muchas de las técnicas de obtención de gametos utilizadas en ganadería han podido aplicarse a estas especies silvestres [23–27], esto es complicado en la práctica, debido precisamente a su carácter silvestre. En el caso de las especies cinegéticas, tenemos la gran ventaja de contar con una fuente estacional de gametos, procedente de los animales abatidos en actividades de caza controlada.

La mejor fuente de gametos masculinos en muestras post-mortem, en caso de poder optar a ella, es la cola del epidídimo. El epidídimo es un tubo largo y contorneado que conecta los vasos eferentes, a la salida del testículo, con el conducto deferente [28, 29]. Se divide en tres zonas, cabeza a la proximal, cuerpo a la intermedia y cola a la distal. Su función es sumamente importante, ya que los espermatozoides sufren un proceso de maduración durante el trayecto epididimario [30–33]. De esta manera, si bien nos encontramos con una limitada funcionalidad a la salida del testículo, en la

cola del epidídimo los espermatozoides son casi equivalentes a los del eyaculado, aunque no hayan completado su evolución funcional y morfológica [34–36]. Recogiendo los espermatozoides de la cola del epidídimo podemos conseguir dosis seminales de alta calidad, en las que se puede basar, o complementar, un banco de recursos genéticos de la especie en cuestión [37–39].

No obstante, el aprovechamiento de este recurso nos enfrenta a varios problemas. Uno de los principales aspectos que hay que considerar es la pérdida de calidad de las muestras en función del tiempo que transcurre desde la muerte del animal hasta que es procesada [40–42]. Hay que tener en cuenta que las actividades cinegéticas transcurren en entornos alejados de los laboratorios, y frecuentemente agrestes, así que no es extraño que puedan pasar varias horas hasta que la muestra es criopreservada. Aunque la calidad espermática y la fertilidad de la muestra puedan permanecer inalteradas cierto tiempo, la extensión de este período es perjudicial [39, 43–47]. El mayor o menor tiempo que haya transcurrido desde la muerte de un animal debe influir en el criterio para procesar la muestra en cuestión. Por otra parte, un aspecto positivo de los espermatozoides epididimarios es que son más resistentes que los espermatozoides del eyaculado [48].

No menos importante es la época de recogida, ya que la mayoría de las especies silvestres presentan una marcada estacionalidad reproductiva [49–51]. El ciervo rojo es una de estas especies, en la cual el celo aparece concentrado en una época del año (la berrea), con evidentes cambios en la producción y calidad espermática a lo largo del año [27, 52–55]. Es indudable que la estacionalidad debe ser tenida también en cuenta para una correcta administración de un banco de recursos genéticos, definiendo períodos óptimos de recogida de muestras.

El proceso de disección y extracción de la muestra seminal va a suponer en mayor o menor medida su contaminación con sangre y tejido epididimario. Esta contaminación depende del método utilizado para extraer los espermatozoides, y su efecto sobre la muestra (durante la congelación o en su posterior utilización en técnicas de reproducción asistida) es incierto [56, 57]. Sin embargo, este aspecto ha sido objeto de poca atención [58], a pesar de ser potencialmente crítico para el éxito de un banco de germoplasma, ya que la elección de un método u otro puede influir en la cantidad de muestra obtenida, en la viabilidad espermática, en la resistencia a la criopreservación y, finalmente, en el resultado de la aplicación de técnicas de reproducción asistida.

Además, como se señaló anteriormente, es necesario tener un amplio conocimiento de la biología espermática. Para ello, es fundamental disponer de técnicas analíticas que permitan su estudio y evaluación, de manera que se puedan tomar las decisiones adecuadas lo antes posible. Un tipo de análisis relativamente reciente, y que presenta grandes posibilidades, tanto para la evaluación de la calidad como para la investigación básica, es la determinación de las proporciones y características de las subpoblaciones espermáticas presentes en una muestra [59–61]. El interés de este tipo de análisis reside en la posibilidad de que la fertilidad de una muestra espermática pueda depender de su composición poblacional, y más concretamente, de la existencia de un tipo de subpoblación con unas características definidas.

La determinación de estas subpoblaciones es posible gracias a la aparición de técnicas de análisis

morfológico o de movilidad que son capaces de distinguir espermatozoides individuales y asignar a cada uno una serie de descriptores. De esta manera, cada espermatozoide puede ser asociado con otros, formando grupos con una cierta homogeneidad interna. Esta asignación es llevada a cabo por algoritmos especialmente dedicados a tal fin. Sin embargo, no hay todavía un análisis estadístico estándar, y el tipo de algoritmo y su combinación con otros procedimientos estadísticos varían dependiendo de los autores. De todas maneras, en los últimos años se han propuesto diferentes metodos, tanto utilizando datos morfológicos [62, 63] como de movilidad [59, 60, 64–67].

Estos tres factores, efecto del tiempo post-mortem y estacionalidad, el método de obtención de muestra epididimaria y el análisis de subpoblaciones espermáticas, han constituido buena parte de mi trabajo de investigación en los últimos años. En esta memoria se presentan los resultados obtenidos. Debemos considerar que las experiencias descritas han sido realizadas con la intención de que sus conclusiones tengan un significado en el contexto de la creación de bancos de germoplasma, no sólo para el ciervo rojo ibérico, sino también para otras especies cercanas.

Efectivamente, en caso de que se estableciese un banco de germoplasma para rumiantes silvestres, habría que tener en cuenta todos los factores objeto del presente estudio. Así, debemos saber el efecto que el tiempo post-mortem ha producido sobre las muestras que lleguen al laboratorio. Aunque se organizase un sistema eficaz de recogida y transporte, y este tiempo fuese muy reducido (situación idónea), no sería extraño que un buen número de muestras tuviesen que esperar más de lo aconsejable. Dado lo montañoso del territorio, podrían pasar muchas horas desde la recogida de la muestra hasta que ésta es entregada al responsable de transportarla al laboratorio (como suele suceder cuando la pieza se cobra temprano por la mañana pero la partida no regresa hasta la tarde); y hay que contar además con los animales heridos que no son localizados sino hasta el día siguiente. Este aspecto se trata en el capítulo 6.

El estudio sobre el efecto de la estacionalidad se presenta en el capítulo 7. La temporada de caza abarca un período más amplio que el celo de los animales, por lo que un banco de germoplasma recibiría muestras no sólo durante la época de celo, sino durante otras épocas del año. Por lo tanto, debemos saber cuál va a ser el rendimiento de las muestras (cantidad de espermatozoides obtenidos), y su calidad. En principio, asumimos que la época de apareamiento debería ser la idónea. Sin embargo, teniendo posibilidad de añadir muestras en otros momentos del año, tal vez de animales valiosos, debemos estudiar las características de esas muestras, a fin de poder decidir adecuadamente en cuanto a su inclusión en el banco.

El análisis de subpoblaciones espermáticas es un método novedoso que permite obtener una gran cantidad de información de una muestra de semen, haciendo muy atractiva la aplicación de esta técnica en el análisis de las muestras de ciervo. Sin embargo, en la bibliografía, los métodos estadísticos utilizados no están descritos por lo general con el suficiente detalle como para poder ser replicados con absoluta fiabilidad. Otros autores utilizan paquetes de software especializados en el análisis multivariante, como PATN (http://www.patn.com.au/) [64, 65], cuyos procedimientos estadísticos son descritos vagamente y no tienen un equivalente exacto en el software estadístico mul-

tipropósito que suele estar disponible en cualquier centro de investigación. El capítulo 8, aborda el primer paso de la investigación sobre el análisis de subpoblaciones espermáticas. Éste fue orientado fundamentalmente a la aplicación de una serie de procedimientos estadísticos, cuya entrada fuesen los datos de movilidad proporcionados por CASA, y en su salida asignase cada espermatozoide a una subpoblación.

El programa propuesto fue utilizado además para realizar un estudio complementario sobre el efecto de la estacionalidad y el tiempo post-mortem. El primer estudio (capítulos 6 y 7) se realizó con muestras cuya movilidad había sido estimada bien subjetivamente o bien con un sistema CASA que proporcionaba únicamente datos medios de la muestra. Disponiendo de un sistema CASA cuya salida identificaba a cada espermatozoide individualmente, fue posible analizar estos dos factores desde una nueva perspectiva, identificando el patrón de subpoblaciones de las muestras y observando cómo ese patrón se modificaba según el tiempo post-mortem o el período del año que se tratase. En el capítulo 9 se aborda este nuevo enfoque.

Finalmente, la falta de trabajos comparativos entre métodos de extracción, y su importancia, decidió el tema de la última parte del trabajo expuesto aquí. Si pretendemos dotar a los bancos de germoplasma de unos protocolos estandarizados, debemos establecer qué métodos de extracción se deben utilizar en cada circunstancia concreta. En el capítulo 10, se describe la comparación de dos métodos frecuentemente utilizados, extracción mediante cortes y extracción mediante perfusión de la cola del epidídimo. Otros métodos, como la disección de la cola en el seno de medio tamponado o la extracción mediante aplastamiento de la cola, no merecieron mayor atención, dado que son más apropiados para especies de tamaño mucho menor, y resultarían ineficientes en ciervo.

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Capítulo 4

Objetivos

El sujeto de estudio en los siguientes objetivos consistió en muestras de espermatozoides de la cola del epidídimo, obtenidas post-mortem de machos adultos de ciervo rojo ibérico. Los objetivos fundamentales definidos en el plan de trabajo de esta Tesis fueron:

1. Determinar el impacto del tiempo post-mortem sobre muestras obtenidas de genital, analizando la disminución de la calidad seminal en distintos intervalos post-mortem y las características de esta disminución.

2. Comparar varias características testiculares, epididimarias y seminales en distintos períodos de la temporada de caza, especialmente la producción y la calidad espermática en cada uno de esos períodos.

3. Proponer una metodología de análisis de subpoblaciones espermáticas a partir de datos de movilidad proporcionados por CASA, y determinar su utilidad analizando muestras durante un protocolo estándar de criopreservación, evaluando su capacidad para detectar cambios en el patrón de subpoblaciones y su posible relación con la calidad tras la descongelación.

4. Estudiar la variación del patrón de subpoblaciones espermáticas, utilizando la metodología propuesta en el punto anterior, en muestras recogidas en distintos intervalos post-mortem y en distintas épocas del año.

5. Comparar dos métodos de recuperación de la muestra espermática a partir de la cola del epidídimo del ciervo rojo ibérico, mediante cortes o mediante perfusión, estudiando las diferencias en producción, calidad espermática (incluyendo el análisis de subpoblaciones espermáticas) y el grado de contaminación de la muestra obtenida.

Capítulo 5

Objectives

The subject of the study in the following objectives consisted in sperm samples from the cauda epididymis, obtained post-mortem from adult male Iberian red deer. The fundamental objectives defined in this Thesis were:

1. To assess the effect of post-mortem time on samples obtained from genitalia, analyzing the decrease of sperm quality in different intervals post-mortem and the characteristics of this decrease.

2. To compare many testicular, epididymal and seminal characteristics in different periods of the hunting season, especially sperm the sperm yield and quality in each of these periods.

3. To propose a method for the analysis of sperm subpopulations using motility data from CASA; additionally, to use this method to analize samples during a cryopreservaton protocol, in order to evaluate its efficiency detecting changes in the subpopulation pattern and its relationship with post-thawning quality.

4. To analyze the variation of the sperm subpopulation pattern, using the method proposed above, between different post-mortem periods and different periods of the year.

5. To compare two protocols for the recovery of sperm samples from the cauda epididymis of the Iberian red deer, by means of cuts or by flushing the cauda, studying differences of yield, sperm quality (including subpopulation analysis), and contamination level.

Capítulo 6

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

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Theriogenology

Aceptado el 20 de marzo de 2004
En prensa desde el 9 de junio de 2004

6.1 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 ioide). 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 two 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.

Keywords: red deer, roe deer, postmortem recovery, epididymal sperm, refrigeration, epididymal storage

6.2 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 postmortem, 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.

6.3 Material and Methods

6.3.1 Sample collection

Samples were collected from recently shot Iberian red deer (*Cervus elaphus hispanicus*) and roe deer (*Capreolus 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. 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.

6.3.2 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.

6.3.3 Sperm assessment

All chemicals were adquired 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™; Berlin) and an electronic pH-meter (CG 837, Schott™; Mainz), respectively.

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 5 fields were observed at $\times$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 5 fields and 200 cells were recorded at $\times$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 μL were put on a microscope slide, covered with a coverslip and observed with a phase contrast microscope ($\times$400) [24]. Acrosomal status (% 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 μL of sample were diluted in 500 μL of a hypoosmotic sodium citrate solution (100 mOsm/kg). After 18 minutes 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 ($\times$400). HOS test reactivity was defined as % of positive cells (those with a swollen tail) [25].

Sperm viability was assessed using the fluorescent dye propidium iodide (PI). Five μL 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 minutes in the dark before being analyzed with a epifluorescence microscope (Nikon Optiphot; $\times$400, 450–490 nm excitation filter, 510ñm dichroic-beam splitter, 520ñm barrier filter). At least 100 cells were counted, and the percentage of non-stained cells (viable spermatozoa) was noted [26].

6.3.4 Data processing and statistical analysis

Statistical analysis were carried out using the SAS^{TM} 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-hour intervals for Iberian red deer (from 0 to 168 h, plus an extra class for PT$>$168 h). For roe deer, due to lower number of samples and their unbalanced distribution among 24-hour groups, we used 30-hour intervals instead (from 0 to 150, plus and extra class for PT$>$150 h). A multiple group comparison was carried out using the Kruskal-Wallis test, followed by a group pairwise comparison (Wilcoxon rank-sum test) when differences were significant ($P<0.05$). The mean and standard deviation of the PT$>$168 h and PT$>$150 h classes were, respectively, 208$\pm$57 h and 226$\pm$74 h.

Besides, in order to describe the evolution of the general quality of the samples and compare both species, the samples were classified accordingly to their quality in 24 h PT groups. Quality was defined by the progressive motility, HOS test reactivity, and acrosomal status of each sample. A sample was included in the high quality group if all the three parameters were equal or higher than 60 %. If any of them was lower than 60 %, but all of them were at least 30 %, the sample was included in the medium quality group. Whenever any parameter was below 30 %, the sample was included in

Table 6.1 Number of samples for each species and PT classes. Note that not all the samples delivered (199 for red deer and 72 for roe deer) were analized.

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		

the low quality group. The distribution of quality groups between PT classes was compared using the χ^2 test. When the conditions made unsuitable the application of this test, the Fisher's Exact Test was used instead. P<0.05 was used for statistical significance.

6.4 Results

6.4.1 Data collected

Almost all the samples could be analyzed for pH, osmolality, subjective motility assessment, HOS test, acrosome integrity, and viability; however, some samples were rejected because of bad aspect or improper refrigeration (Table 6.1). CASA was available only for 55 % (92) and 39 % (26) of red deer and roe deer analyzed samples, respectively, thus there was a lower sample number available in these cases. We have observed a high variation within PT groups, which is evident considering the wide interquartil ranges (figures 6.1, 6.2, 6.3, and 6.4). It was not clear if this variation corresponded to individual differences between males, to collection and refrigeration conditions, or to other factors.

6.4.2 Correlation analysis

Analysis of the correlations between PT and the studied parameters (Table 6.2) rendered similar results for both species. Motility (except for STR in roe deer), HOS test reactivity, intact acrosomes and viability presented negative correlations with PT. On the other hand, osmolality and pH showed positive correlations. In general, correlations were highly or very highly significant. Correlation coefficients were low in most cases, though. Remarkably, PT and total and progressive motility, both by subjective and CASA assessments, rendered correlation coefficients above 0.5.

6.4.3 Comparison between PT classes

A summary of the data grouped by PT classes is shown in figures 6.1, 6.2, 6.3, and 6.4. In general, data followed the corresponding trend indicated by the correlation coefficients. However, we did not find significant variations in many cases, especially for roe deer, possibly due to the high variability of the samples. In fact, for roe deer, only the classes]30–60] h and >150 h included more than

Table 6.2 Correlations between PT and sperm parameters (Spearman coefficients and significance).

	pH	Osmolality	TM[1]	PM[2]	TM[1] (CASA)	PM[2] (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***

[1] Total motility; [2] Progressive motility

* P< 0,05; ** P< 0,01; *** P< 0,001

10 cases (Table 6.1), and, as said before, only a fraction of these samples was analyzed by CASA, therefore this could be another reason for the lack of significance.

In both species, pH and osmolality varied differently. Whereas pH seemed to rise only slightly, and this rising was only evident in the last PT classes, osmolality increased early (after 48 h for red deer and 30 h for roe deer). Beyond this point, osmolality continued rising clearly in the case of Iberian red deer. However, for roe deer, it stalled and no further increase was noted.

Total motility decreased steadily along the studied time periods, but progressive motility dropped around the third day. By the fourth day, progressive motility was practically 0. This was observed both in subjective and CASA parameters. Other motility parameters, yielded by the CASA system, showed a steady decrease, only significant for STR in red deer, though. For roe deer, STR was still high in the last classes.

HOS test reactivity and acrosomal status showed significant differences between groups only for Iberian red deer. In this species, it could be appreciated that the proportion of spermatozoa positive to the HOS test decreased with PT (but only in a low percentage). However, in the case of acrosomal status, the results were less evident, since there were many samples with high values in the last PT classes.

The case of the viability was completely opposite, because, although both species showed a diminution of viability values through PT, only roe deer data rendered statistical differences between PT groups. In this case, we found one important difference between the two species, since both of them had similar viability values in the early PT groups (median value above 70 %), but it diminished to 66.5 % in the $>$168 h group for red deer, whereas it dropped to only 26 % in the $>$150 h group for roe deer.

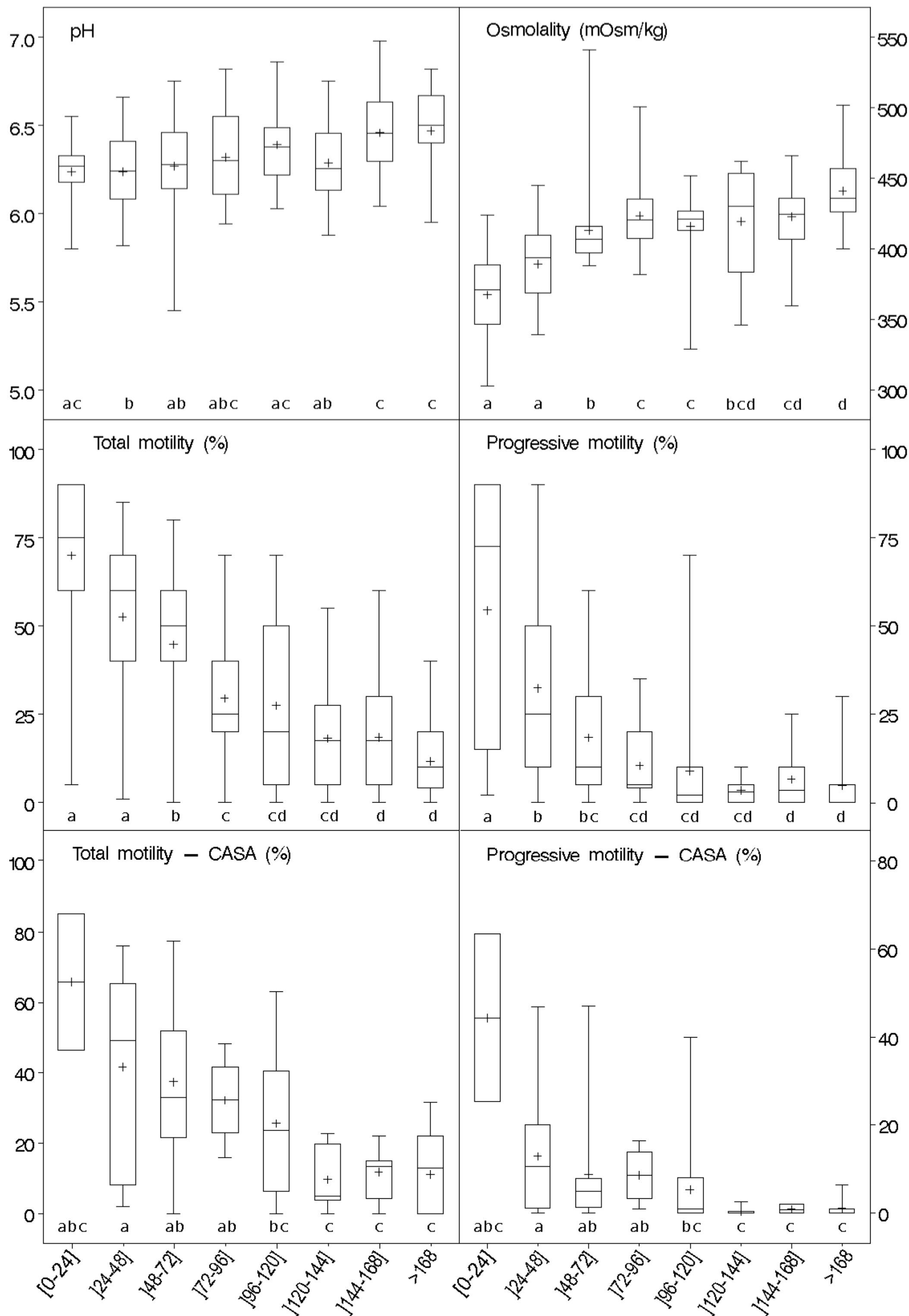

Figure 6.1 Red deer: evolution of sperm pH, osmolality, total motility and progressive motility (subjective and CASA) depending on postmortem time (time intervals in hours). Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside the boxes 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).

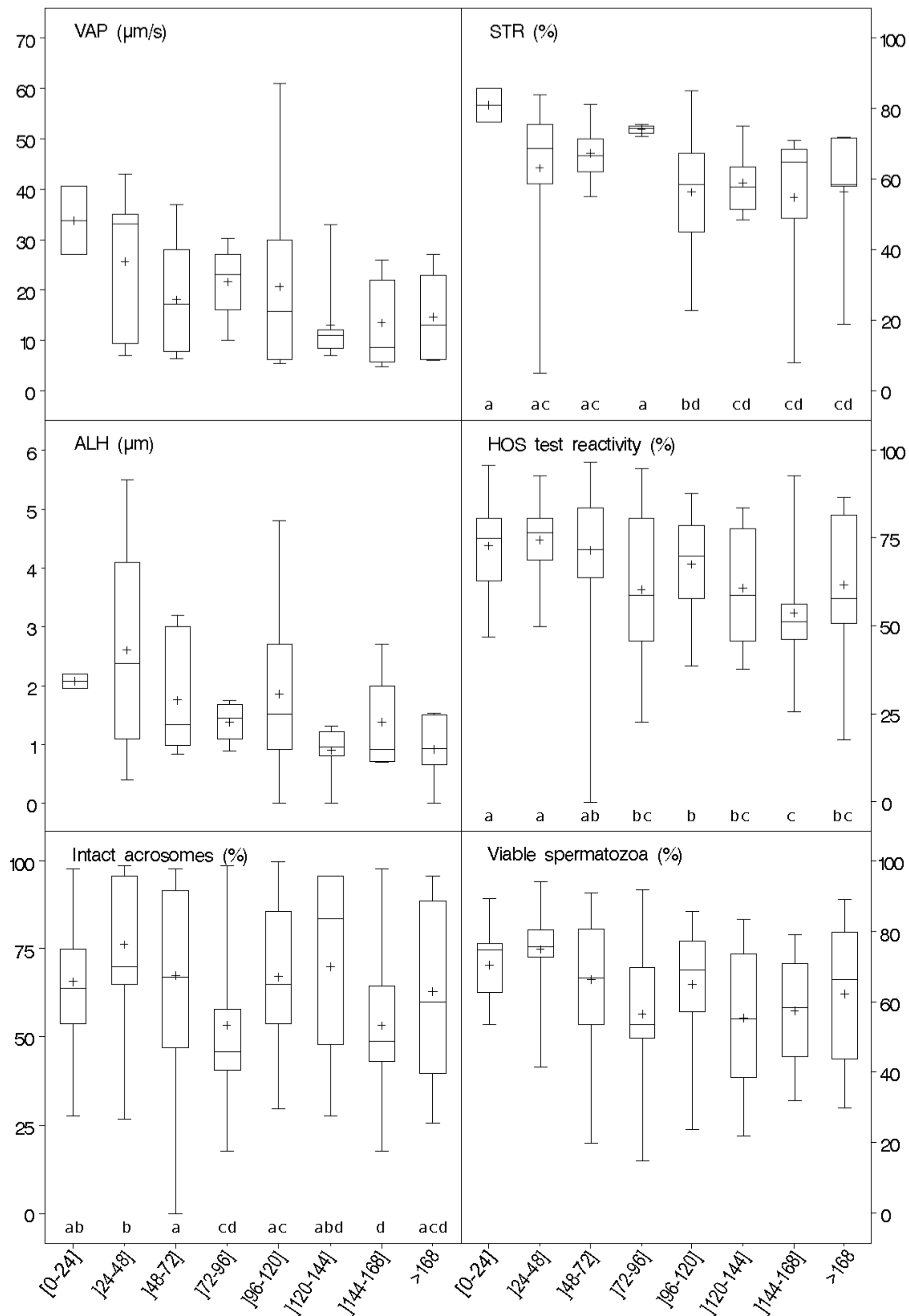

Figure 6.2 Red deer: evolution of sperm VAP, STR, ALH, HOS test reactivity, acrosomal integrity and viability depending on postmortem time (time intervals in hours). Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside the boxes indicates 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 ($P<0.05$).

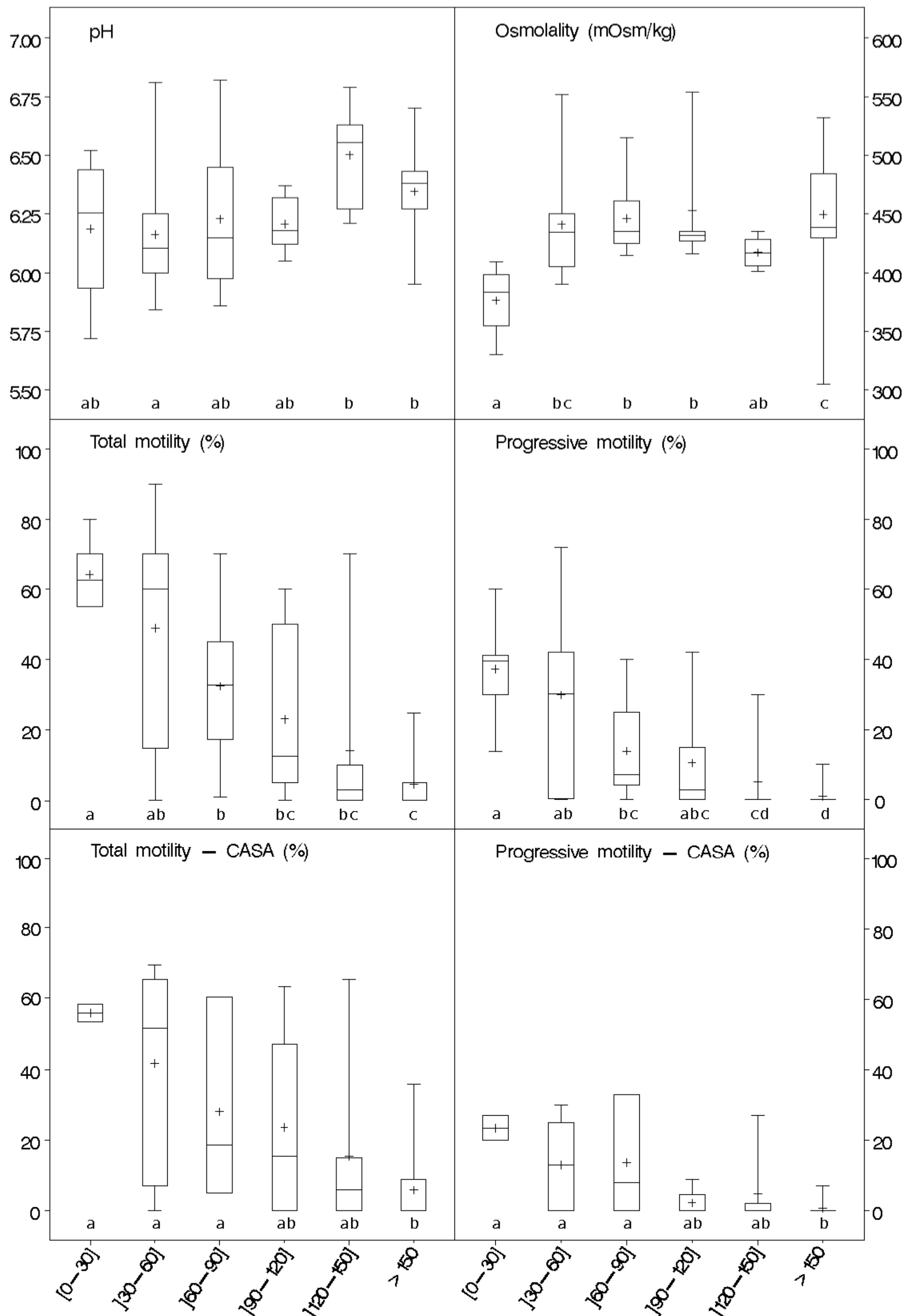

Figure 6.3 Roe deer: evolution of sperm pH, osmolality, total motility and progressive motility (subjective and CASA) depending on postmortem time (time intervals in hours). Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside the boxes 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 ($P<0.05$).

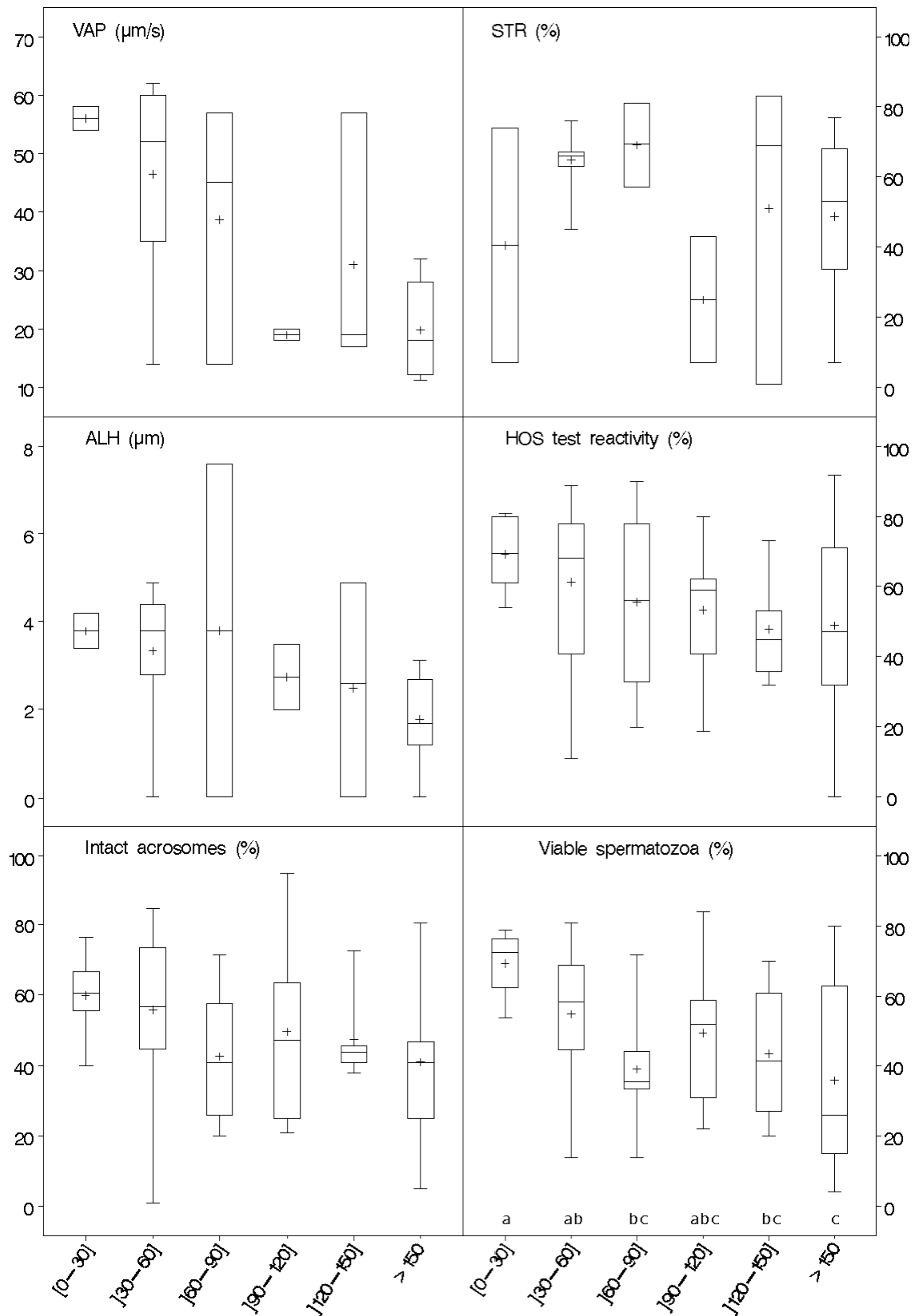

Figure 6.4 Roe deer: evolution of sperm VAP, STR, ALH, HOS test reactivity, acrosomal integrity and viability depending on postmortem time (time intervals in hours). Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside the boxes indicates 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 ($P<0.05$).

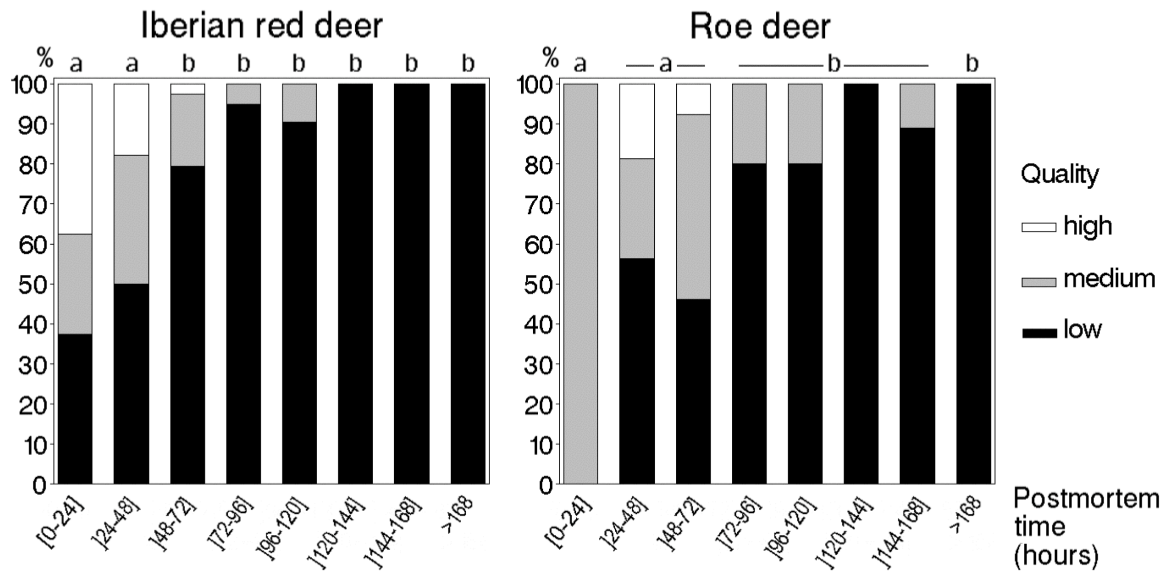

Figure 6.5 Distribution of samples in high, medium, and low quality groups for each species and PT classes. High quality: progressive motility, HOS test reactivity and acrosomal status >60 %; medium quality: any of these parameters ⩽60 % but >30 %; low quality: any of these parameters <30 %. Different 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.

6.4.4 Distribution of quality groups within PT classes

Distribution of the samples in high, medium, and low quality for each PT class, and results of χ^2 test are shown in Figure 6.5. For Iberian red deer, nearly 40 % of the samples belonged to the high quality group when processed before the first 24 h postmortem, summing more than 60 % together with medium quality samples. However, in the next 24 h, the proportion of high quality samples was halved, and low quality samples rose up to 50 %, although differences in the distributions were not still significant. After 120 h postmortem, none of the samples could be considered even as of medium quality.

Roe deer samples had a similar distribution than those of red deer. However, the distribution is less clear due to the lower n in each PT class (only the]24–48],]48–72], and >168 h classes had more than 10 samples each). Apparently, the increase of the proportion of bad quality samples was slower than in the case of red deer (comparing]48–72] h classes), but from this point forward the trend was almost identical. Before using χ^2 test, those groups that seemed to be homogeneous were joined in order to increase the number of samples in each one and get reliable results. Thus, data from]24–48] and]48–72] h, and]72–96],]96–120],]120–144] and]144–168] h were put together. Lack of significance between [0–24] and]24–72] h groups is logical, as [0–24] h group included only 4 samples.

6.5 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 Sankai et al. [15], Yu and Leibo [17], Garde et al. [21]. 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 hours 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. The refrigeration of the samples is another factor of variation, because we were unaware of the conditions of the gamekeepers' refrigerators and the possible incidents in their operation. Furthermore, there might be a interaction between individuals and the impact of temperature changes on the spermatozoa, so the sperm of one male could endure these temperature variations whereas the sperm of another one could not [30].

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

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

In general, both Iberian red deer and roe deer epididymal sperm samples underwent a loss of quality when the epididymes were stored for more than 2–3 days at 5 °C. Progressive motility was the parameter that dropped more pronouncedly, followed by total motility, which coincides with results in other species [11, 16, 17, 32]. For the other motility parameters, we think that more consistent results could have been achieved using a modern CASA system, which would have allowed to get

sperm subpopulation parameters, instead of mean populational parameters, that can be deceiving [34]. We are preparing a complementary study in which sperm motility will be measured with a state-of-the-art CASA at different postmortem times, in order to perform a more complete study of motility.

Considering recent studies in cervids, in a preliminary report on Iberian red deer [22], we obtained a similar trend on motility (24 h intervals over a 4-day period), with progressive motility dropping to almost nil at the end of the period. Hishinuma and Sekine [11], working with Sika deer, found that total motility significantly decreases as soon as the first day postmortem, and it continues falling when PT increases. Soler et al. [23] also detected a significant decrease of motility with PT in Iberian red deer, reporting that the sperm motility index (SMI) significantly reduces after 2 days, and it stabilized until the end of the experiment (4 days). Although we used different parameters considering motility (SMI combines total and progressive motility in the same parameter), the trend we reported in the case of Iberian red deer seems to be similar to those of these authors. However, mainly due to the abrupt decrease of progressive motility, our data show an early drop of quality, which marks a difference with the work of Soler et al. [23]. This discrepancy could be due to the differences in the experimental design (the epididymes were taken to the laboratory just after the death of the animals and kept in controlled refrigerated conditions), in the recollection time (November and December), and in the origin of the animals (South of Spain). Unfortunately, none of these studies reported pH or osmolality of the samples (sperm was immediately mixed with media after extraction). Considering our observations, it would be of great interest to extend this study, in order to clarify if the loss of quality of sperm motility is due not only to the normal process of aging, but also to the variations of the physical parameters of the epididymal fluid, and to what extent.

On the other hand, neither Hishinuma and Sekine [11] nor Soler et al. [23], found a reduction in sperm viability during the studied period (7 and 4 days, respectively). We have reported a highly significant negative correlation between viability and PT, but with low correlation coefficients. Comparing PT groups, we did not found significant differences for red deer, but for roe deer, which might indicate a difference between these species. Iberian red deer sperm possibly underwent a decrease in viability, as indicated by the correlation analysis, but it might be much more subtle. This can explain the lack of significant differences in the other studies, which also used a different method for viability assessment (eosin staining). Other quality parameters that endured well the postmortem conditions were the functionality of the plasma membrane (HOS test reactivity) and the acrosomal status. Both of them had highly significant negative correlations with PT for both species, but box plot distributions showed that differences between PT classes were small. In our previous study on red deer [22], we obtained similar results for HOS test reactivity, and, for acrosomal status and sperm viability, values were lower the fourth day postmortem. This would indicate that in fact these parameters are affected by postmortem time, but not as dramatically as motility.

Other authors have reported differences comparing related species. for instance, Sankai et al. [15], working with mouse, and Soler et al. [23], working with red deer, reported increasing numbers

of sperm with abnormalities after four days postmortem. However, the former indicated a very high increase (more that 60 %) of bent tails, whereas the later found a much lower increase (around 20 %) of bent midpieces. Furthermore, Lubbe et al. [19], and Kilian et al. [20] working with African wild Perissodactyls and African wild ruminants, respectively, reported some differences between similar species, regarding motility diminution along PT. In fact, cold-shock resistance varies pronouncedly between species, even between breeds or individuals [30]. Sankai et al. [15] interpreted the increasing proportion of sperm with bent tails as a consequence of the hardening of the plasma membrane due to low temperatures. This effect could contribute, at least in some extent, to the differences that we have find between red deer and roe deer, regarding plasma membrane integrity (sperm viability).

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

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

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

6.5.1 Acknowledgements

This study has been supported by Junta de Castilla y León (AB29). The authors thank Juan José Martínez, César Gómez, Juan Carlos Peral, the Territorial Service of Environmental Affairs of León, and the gamekeepers of the hunting reserves of Picos de Europa, Mampodre and Ancares (León, Spain) for their collaboration in the collection of the samples used in this work; and Vanesa García, Mercedes Álvarez, Juan Daniel Muro, and Patri Martínez for their help in the processing of the samples.

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Capítulo 7

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

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Aceptado el 21 de agosto de 2004

7.1 Abstract

Seasonality deeply affects the physiology and behavior of many species, and must be taken into account when Biological Resource Banks (BRBs) are established. We have studied the effect of seasonality on many reproductive parameters of free-ranging Iberian red deer, roe deer and Cantabrian chamois, living in Spain. Testicles from hunted animals were collected and sent to our laboratory at different times during the year. We recorded the weight and volume of testis, the weight of the epididymis and its separate parts (caput, corpus, and cauda), the weight of the sperm sample collected from the cauda epididymis, and several sperm parameters (sperm concentration, spermatozoa recovered, motility, HOS test reactivity, acrosomal status, and viability). We studied the data according to several periods, defined accordingly to each species. For red deer, we defined rut (mid-September to mid-October), post-rut (mid-October to mid-December), and non-breeding season (February). For roe deer, they were pre-rut (June), rut (July), post-rut (first fortnight of August), and non-breeding season (September). For chamois: non-breeding season (June to mid-September) and breeding season (October and November). The rut/breeding season yielded significantly higher numbers for almost all parameters. However, in the case of red deer, sperm quality was higher in the post-rut. For roe deer, testicular weight was similar in the pre-rut and in the rut, and sperm quality did not differ significantly between these two periods, although we noticed higher values in the rut. In the case of chamois, sperm quality did not differ significantly from the breeding season, but data distribution suggested that in the non-breeding season there are less males with sperm of good quality. On the whole, we find these results of interest for BRB planning. The best season to collect sperm in this species would be the breeding season. However, post-rut in red deer, pre-rut in roe deer, and non-breeding season in chamois could be used too, because of the acceptable sperm quality, despite the lower quantity salvaged. More in-depth research needs to be carried out on the quality of sperm salvaged at different times of the year in order to confirm these findings.

Keywords: Iberian red deer, roe deer, chamois, post-mortem recovery, epididymal spermatozoa, seasonality

7.2 Introduction

Biological Resource Banks (BRBs) are important tools for the conservation of species and valuable breeds, and have been strongly developed during the last decade. The term BRB comprises many techniques and protocols, the purpose of which is to collect, preserve and utilize tissues and germplasm of selected individuals in order to ensure the continuity and the genetic variability of breeds, populations and species [1–3]. One simple method to achieve the objectives of BRB is the collection of sperm from males, followed by its cryopreservation and its use in AI when needed. This way, the genetic variability of a population can be maintained in an easy and inexpensive manner [4]. However, there are many drawbacks, some of them related to the collection of the sperm

sample, which is a difficult task in wild species. Indeed, one of the most attractive uses of BRBs is the preservation of wild species, since they can be of use in management programs dedicated to protecting not only endangered species but also those that could be at risk in the future [2].

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

The influence of season on sperm production and quality has been largely considered, as a factor of high impact. Most species, at least in non-tropical latitudes, present a circannual cycle, undergoing more or less marked variation in their behavior, body condition and reproductive parameters. There are great differences between species, even between those closely related [10]. In fact, some mammals undergo a complete reproductive arrest in their annual cycle, in which the males present testicular quiescence and lack of sperm, followed by another period of testicular recrudescence and sexual activity (such as the members of the Cervidae) [11, 12]. On the other hand, other species maintain some level of spermatogenic activity throughout the whole year, however it is much more intense during their breeding season [13–15]. Seasonality is less pronounced between males of domestic species, but there are still differences in behavior and sperm characteristics depending on the time of the year [13, 16–20]. Photoperiod, mediated through the hormone melatonin, is the main factor triggering events related with season [21, 22]. There is abundant literature in this respect, and numerous studies have been carried out on circannual variations of many hormones and its importance on body, gonadal and gametogenesis changes [8, 13, 22–24]. In cervids (and in other mammals too), there are also very pronounced changes in body condition and morphology throughout the year, which are strongly related to sexual activity. In fact, the same hormones control both kinds of changes, with testosterone being an important regulator both of antler growth and spermatogenesis [25–27].

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

7.3 Material and Methods

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

7.3.1 Genitalia collection

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

Harvest plans followed Spanish Harvest Regulation, Law 4/96 of Castilla y León and Law 19/01 of Extremadura, which 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, are reviewed each year in the Annual Hunting Regulation of the respective regions. Animal handling was performed in accordance with the Spanish Animal Protection Regulation, RD223/1998, which conforms to European Union Regulation 86/609 and adheres to guidelines established in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the American Society of Andrology.

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

7.3.2 Measurements of testicles, epididymis and sperm sample

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

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

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

7.3.3 Sperm quality assessment

Only those samples with a post-mortem time of 24–48 h were analized for quality. The reason for this limitation is that post-mortem time has a deleterious effect in the quality of the samples, therefore, which we tried to reduce. We chose this period because there were an adequate number of samples in it, for most seasons and species, and the variation of sperm quality during this period seemed to be small and steady, according to the bibliography [28], whereas choosing longer post-mortem intervals would had been an undesirable source of variation in the study.

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

The functional integrity of the sperm plasma membrane was evaluated using the hypoosmotic swelling test (HOS test). Five μL of sample were diluted in 500 μL of a hypoosmotic sodium citrate

solution (100 mOsm/kg). After 18 minutes at room temperature, samples were fixed with a drop of glutaraldehyde solution. Response to the test was determined by counting 100 cells with a phase-contrast microscope (×400). The percentage of positive cells (those with a swollen flagelle) was recorded for each sample [29].

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 μL were put on a microscope slide, covered with a coverslip and observed with a phase contrast microscope (×400) [30]. Acrosomal status (% of cells with an intact acrosome) was evaluated counting at least 100 cells.

The fluorescent dye propidium ioide (PI) was used to assess sample viability. Five μL of sample were diluted in 500 μL of PI solution (25 μg/L PI in Hepes solution, see above). Samples were kept in the dark at room temperature for 10 minutes before being analized with an epifluorescence microscope (Nikon; ×400). At least 100 cells were counted and the percentage of non-stained cells (viable cells) was noted [31].

7.3.4 Statistical analysis

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

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

7.4 Results

7.4.1 Testicular and epididymal characteristics and sperm recovery between seasons

Figure 7.1 displays the distribution of individual testicular weights throughout the year, showing how its distribution varied between the chosen periods. The comparison of different periods of the year showed very clear differences regarding to the quantitative parameters. Figures 7.2–7.5 summarize the measurements of the testis, epididymis and the quantitative data for salvaged sperm (weight of collected sperm, concentration, and spermatozoa$\times$g/mL), for Iberian red deer, roe deer and chamois. Median values are detailed in Table 7.1.

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

7.4.2 Sperm quality between seasons

Figures 7.5 and 7.6 show the variation in sperm quality parameters between the corresponding seasons for each species. Median values are detailed in Table 7.1. Red deer sperm yielded the highest quality values in the post-rut period. Comparing with the rut period, acrosomal integrity and viability where significantly higher. In the non-breeding season, motility parameters dropped, and, in fact, most samples had very poor or no motility at all, but acrosomal integrity and HOS test results were similar to those recorded during the rut.

In the case of Chamois, we found no significant differences, although median values seemed lower in the non-breeding season. In this sense, dispersion of data was higher during the non-breeding season, which suggested the existence of different groups within the class. Indeed, considering individual samples, all those studied during the breeding season rendered good motility results (TM>60 %) together with good acrosomal integrity, HOS test and viability values. On the other hand, almost half of the samples collected during the non-breeding season had almost no motility,

Table 7.1 Results for each species and season (Medians).

	Red deer			Roe deer				Chamois	
Param.[1]	NB	Rut	PostR	PreR	Rut	PostR	NB	NB	BS
TESW	23.9	83.8	39.3	20.6	21.7	14.4	7.6	15.5	26.8
TESV	24	81.5	37.5	15	24.3	15	6.5	17	25
EPIW	5.9	13.7	7.7	1.9	2.4	2.1	1.6	2.4	3.7
CAPW	2.0	5.2	2.8	0.9	1.1	1.0	0.7	1.0	1.6
CORW	0.7	2.1	1.0	0.3	0.3	0.2	0.2	0.4	0.6
CAUW	2.8	6.2	3.8	0.8	0.9	0.9	0.6	1.0	1.6
SSW	0.1	0.9	0.4	0.1	0.3	0.2	0.1	0.2	0.5
SPZC	0.3	2.9	1.9	2.7	3.6	3.4	0.8	2.2	8.2
CONC	17.6	42.2	34.8	22.5	27.0	29.4	11.3	22.0	41.7
SPZ	2.1	35.1	13.2	3.4	7.2	6.4	1.2	3.9	25.9
TM	12.5	60	60	40	55	30[2]	10[2]	37.5	70
PM	2.5	13.2	40	8	34.6	10[2]	0[2]	25	40
HOST	72.5	76	78.5	62	70.5	80[2]	88[2]	55	69
ACR	60.5	67	88.5	49	56.5	64[2]	79[2]	36.5	74
VIAB	—	73.5	82	51	67	4.5[2]	—	46.5	67

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

[2] only one sample.

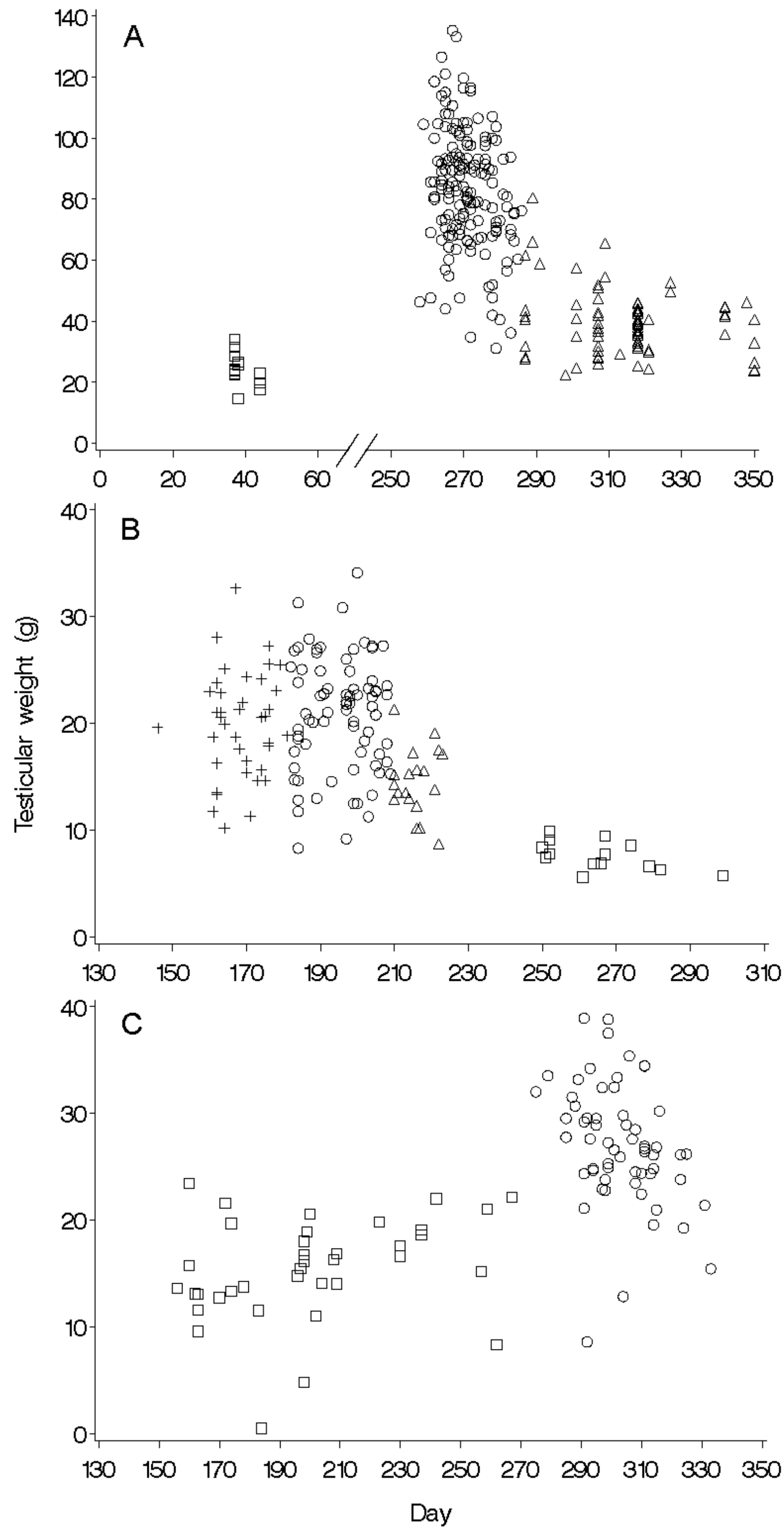

Figure 7.1 Distribution of testicular weights along the year, for Iberian red deer (A), roe deer (B), and chamois (C). Each mark corresponds to one sample (mean value of the two testicles). Figures identify each season (+: pre-rut; ○: rut/breeding season; △: post-rut; □: non-breeding season).

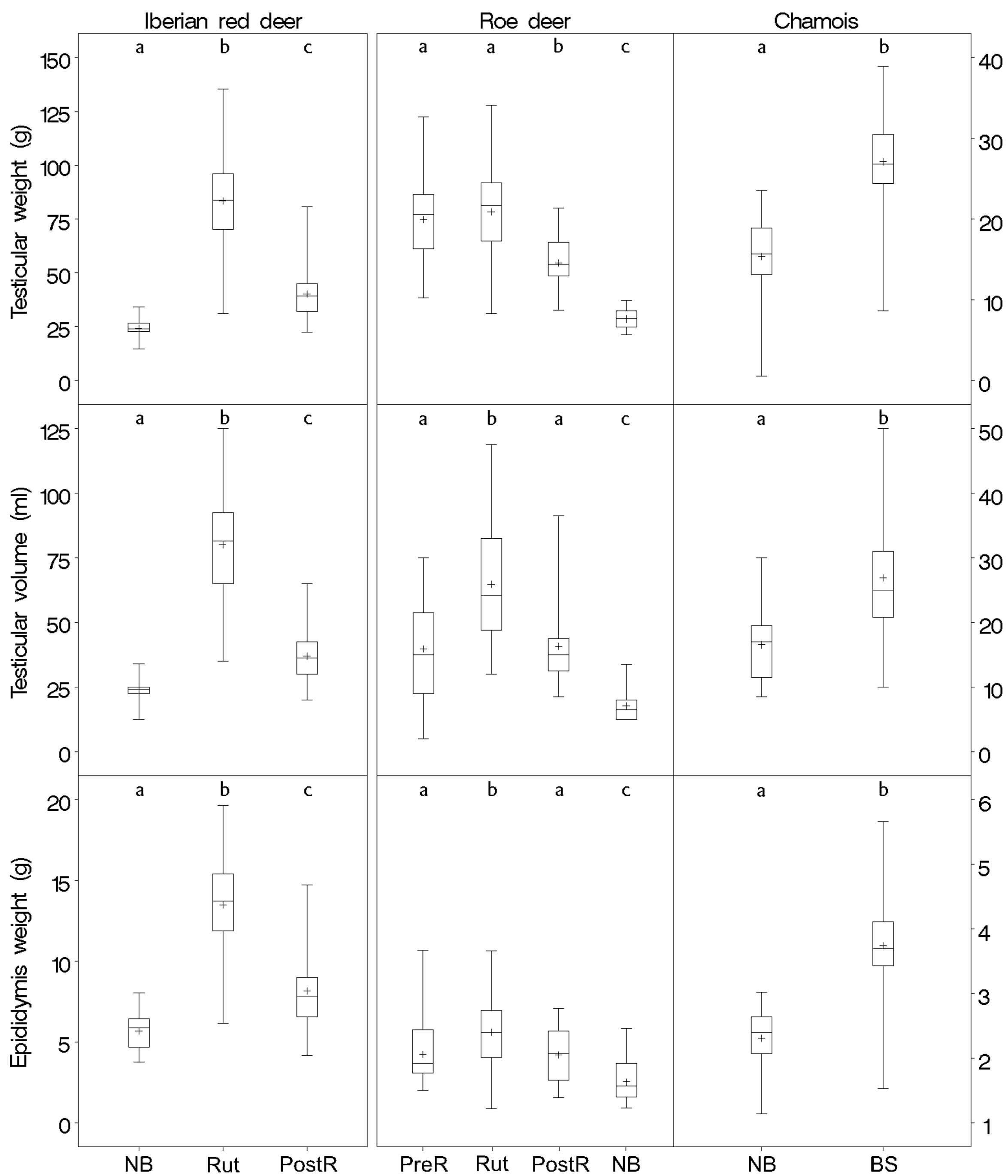

Figure 7.2 Evolution of testicular weight, testicular volume, and epididymal weight depending on season (NB: non-breeding; PreR: pre-rut; PostR: post-rut; BS: breeding season). Left scale refers to red deer, whereas right scale refers to roe deer and chamois. Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line insideindicates the median score. The whiskers reach the maximum and minimum values of the range. The mean is shown with a cross. Different letters on the top of each plot indicate significant differences between seasons ($P<0.05$).

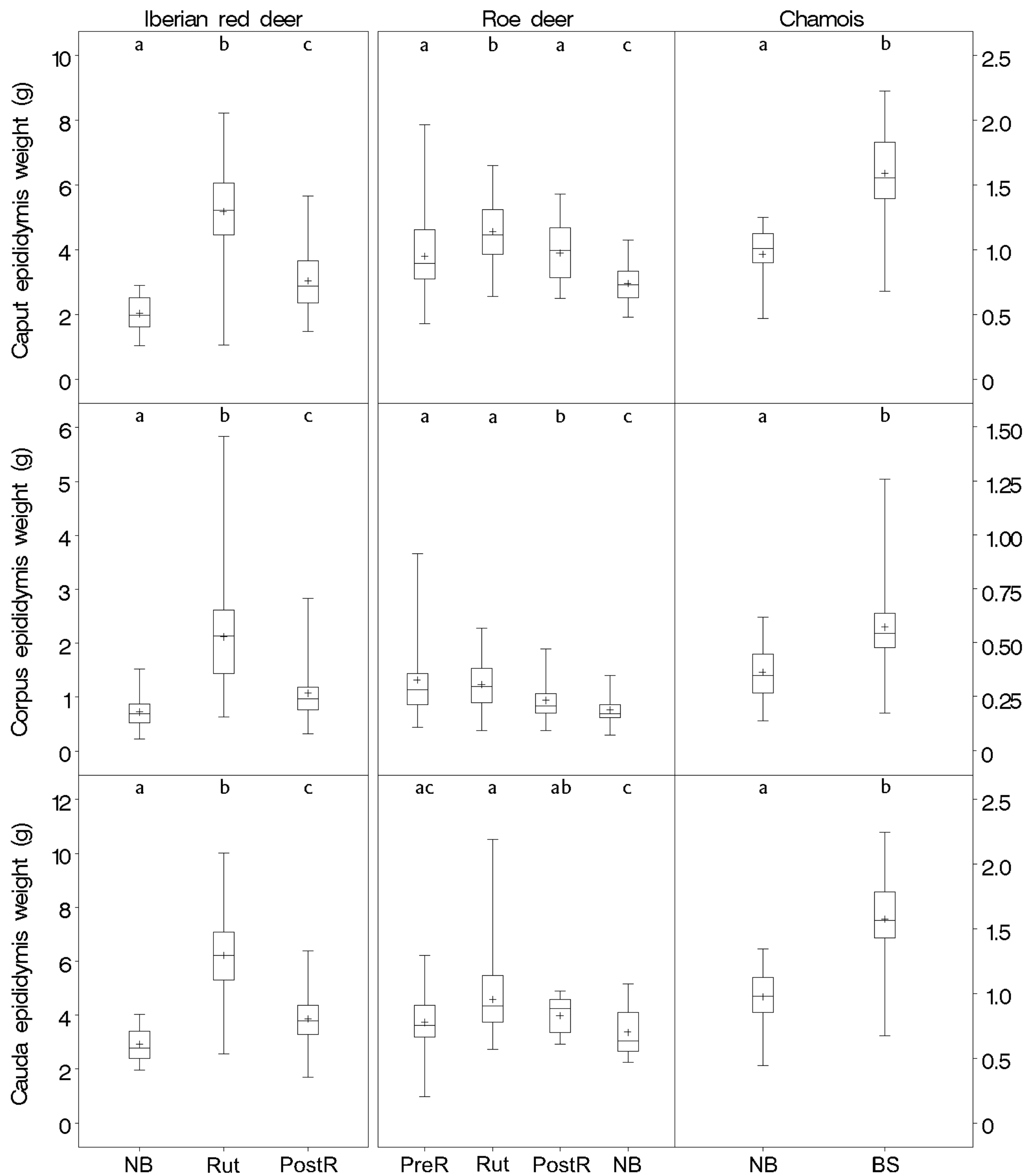

Figure 7.3 Evolution of caput, corpus and cauda epididymis weights depending on season (NB: non-breeding; PreR: pre-rut; PostR: post-rut; BS: breeding season). Left scale refers to red deer, whereas right scale refers to roe deer and chamois. Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside indicates the median score. The whiskers reach the maximum and minimum values of the range. The mean is shown with a cross. Different letters on the top of each plot indicate significant differences between seasons ($P<0.05$).

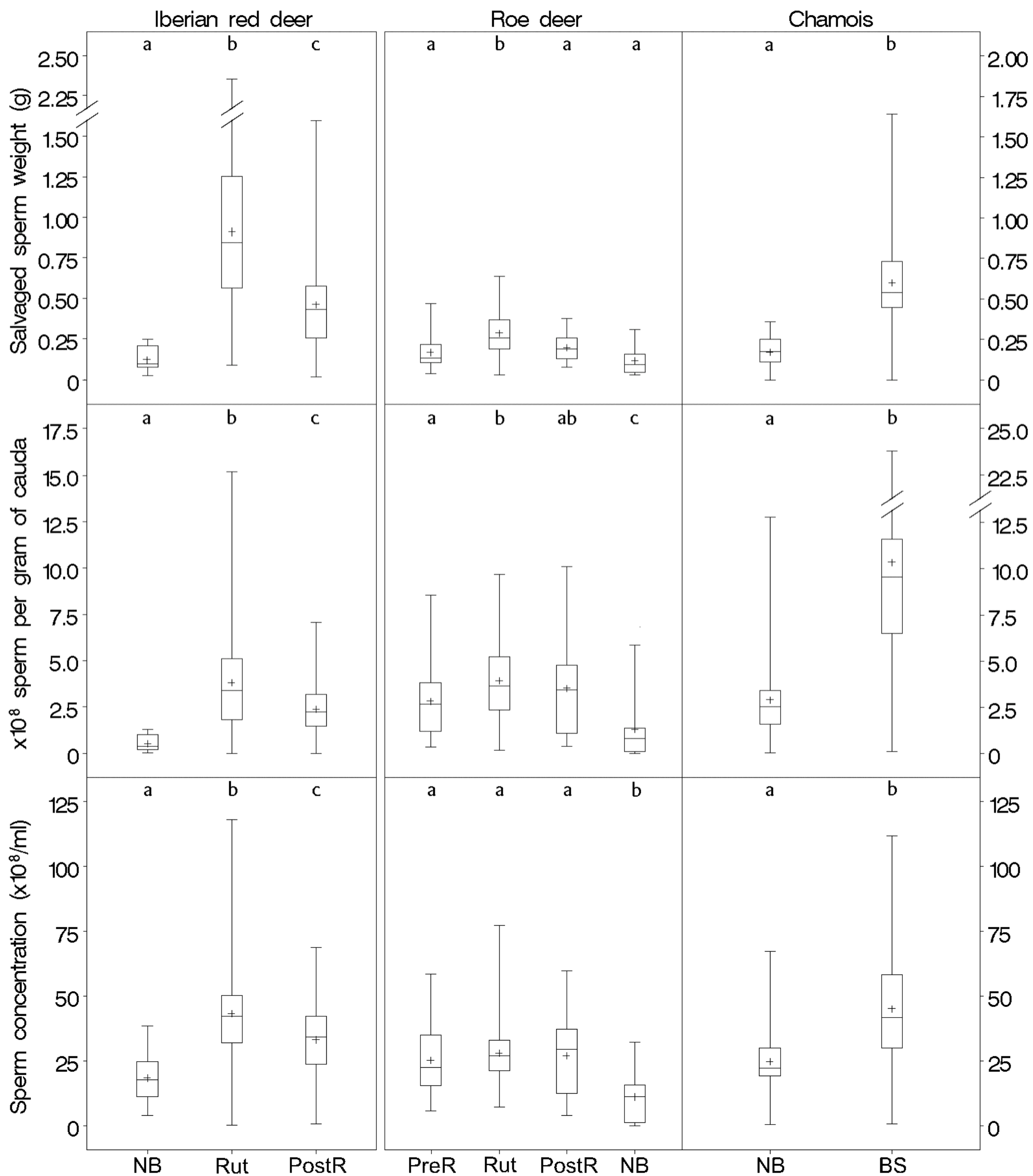

Figure 7.4 Evolution of sperm sample weight, number of sperm per gram of cauda, and sperm concentration depending on season (NB: non-breeding; PreR: pre-rut; PostR: post-rut; BS: breeding season). Left scale refers to red deer, whereas right scale refers to roe deer and chamois. Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside indicates the median score. The whiskers reach the maximum and minimum values of the range. The mean is shown with a cross. Different letters on the top of each plot indicate significant differences between seasons ($P<0.05$).

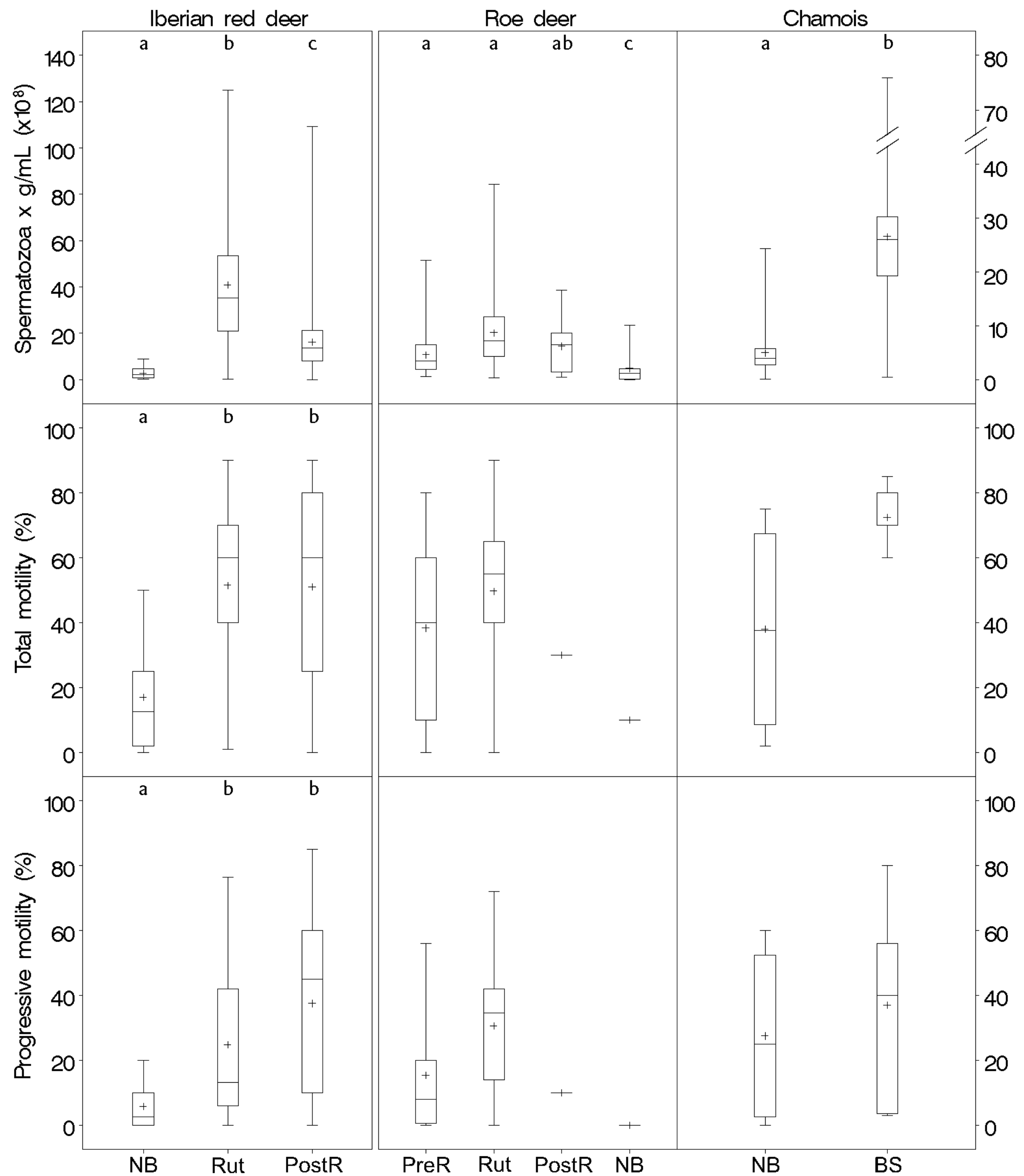

Figure 7.5 Evolution of spermatozoa×g/mL (approximately, total spermatozoa in the sample), total motility, and progressive motility depending on season (NB: non-breeding; PreR: pre-rut; PostR: post-rut; BS: breeding season). Left scale refers to red deer, whereas right scale refers to roe deer and chamois. Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside indicates the median score. The whiskers reach the maximum and minimum values of the range. The mean is shown with a cross. Different letters on the top of each plot indicate significant differences between seasons ($P<0.05$).

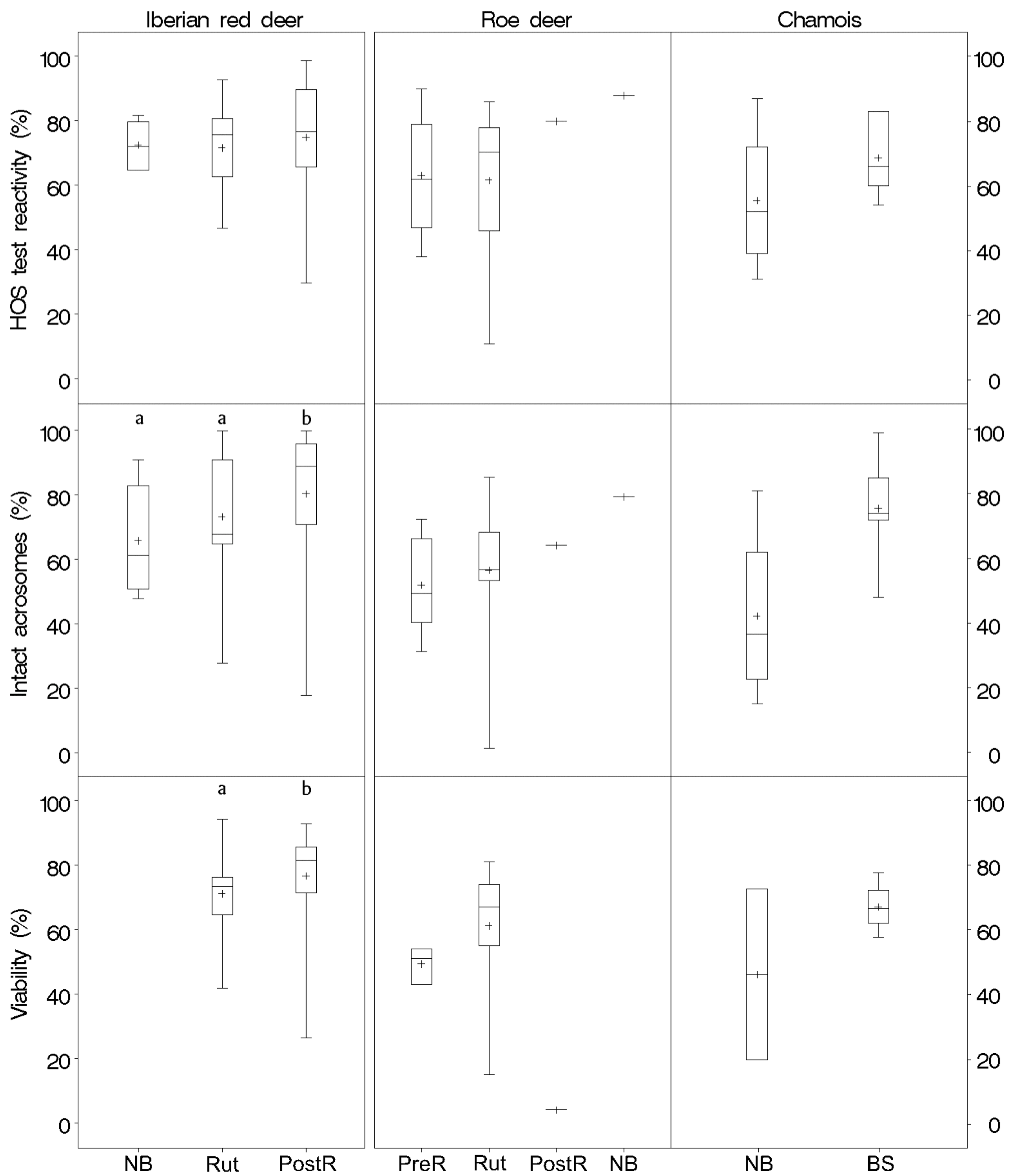

Figure 7.6 Evolution of HOS test reactivity, acrosomal status, and sperm viability depending on season (NB: non-breeding; PreR: pre-rut; PostR: post-rut; BS: breeding season). Left scale refers to red deer, whereas right scale refers to roe deer and chamois. Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside indicates the median score. The whiskers reach the maximum and minimum values of the range. The mean is shown with a cross. Different letters on the top of each plot indicate significant differences between seasons ($P<0.05$).

and also low acrosomal integrity, HOS test and viability results. Interestingly, the rest of the samples in the non-breeding season were comparable to those of the breeding season.

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

7.5 Discussion

In this study we have shown that many reproductive parameters of Iberian red deer, roe deer and chamois vary between different periods of the year. The morphology of testicles and epididymis, and the quantity and quality of the salvaged sperm underwent important variations between these periods, as expected in seasonal species. There is ample bibliography about this subject on the red deer and roe deer, containing much information on behavior, body condition, and hormonal and reproductive status in different periods of the year. Besides, this subject has been studied in other cervids. On the other hand, to our knowledge, there are no studies on the reproductive cycle of male chamois, apart from a recent report by our group [32]. However, this study was limited by the extent of the hunting seasons for each species, which determined the availability of samples.

Seasonality is controlled by fluctuations in the hormone melatonin, which is produced by the pineal gland during the night. Hence, depending on the photoperiod, the concentration of melatonin in the body rises or lowers, and regulates the production of GnRH. One of its final effects is the control of testosterone levels, through LH pulses, which rise before the breeding season and peak during that period, dropping afterwards [13]. Since testosterone is needed for testicular recrudescence and adequate spermatogenesis, this hormonal cycle synchronizes the sexual activity of the males. Consequently, sperm is produced only in the right period of the year.

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

In the case of roe deer, the rut and mating period is much shorter. According to Blottner et al. [12, 23], spermatogenesis and other reproductive parameters peak just before the breeding season of roe deer, and fall quickly afterwards. These authors worked in a higher latitude than us (Germany), and reported that the breeding season of the roe deer comprises from mid-July to mid-August. In the North of Spain, rut occurs in July, but it seems to have a similar length. Thus, we can consider that there is a shift in the sexual cycle of the roe deer because of the different environments (latitude, climate), which could be of use for populational studies. The samples that fell out of the breeding season were included either in a pre-rut or post-rut period, or in a non-breeding season, if they were collected much later (early autumn). The classification was much simpler with the chamois, as we only distinguished two periods, breeding and non-breeding.

The differences we found between seasons, regarding morphological measurements and quantitative sperm parameters, were expected. Many studies have shown that seasonal mammals undergo a cycle of involution and growth that affects not only testicles, but also other components of the genital tract [8], and secondary sexual characteristics, very evident in cervids [33]. In the case of Iberian red deer, our results suggests a very rapid involution of spermatogenic tissues after rut, possibly due to the decrease in the plasma testosterone levels, which has been reported to occur after the rut in similar species. According to these studies [10, 11, 33, 34], plasma testosterone concentration peaks just before the moment of most intense mating activity in the rut, and falls quickly afterwards, so it presents low values only one month after the end of the rut. Seminiferous tubules, which had reached their maximum development, regress, following testosterone drop. Our data collected in February (non-breeding season) showed lower values for all parameters, corresponding with a situation of long-term low testosterone levels. Other authors have obtained similar results studying red deer and other cervid species. For instance, Hochereau-de Reviers and Lincoln [35], working with red deer, found a mean weight of the left testis of 24.4 g in the non-breeding season (February to April), and of 70.7 g in the breeding season (August to October). Comparing with our results, the lower weight obtained by these authors in the breeding season could be due, apart from other factors, to the longer period considered as breeding season, which may include the pre-rut period, when testes have not reached their maximum weight yet. Furthermore, Suzuki et al. [11], studying Sika deer, determined a testicular size coefficient by means of multiplying the three testicular axes and calculating the cubic root of the resulting number. In this way, the relation of the mean values obtained in the rut and in the non-breeding season was 1.46, and between the rut and the post-rut was 1.13. Treating our numbers in the same way, we have 1.5 and 1.3. Other studies in Eld's deer showed similar results [10]. Moreover, our coefficients for roe deer are 1.55 and 1.17. Therefore, different species of cervids possibly follow the same trend.

Roe deer reproductive seasonality has been thoroughly described in many articles. Blottner et al. [23], and Blottner and Roelants [36, 37] indicated that spermatogenesis in roe deer increases considerably immediately prior to the rut, decreasing quickly thereafter. These authors found that sperm production depends not only on the changes in testis mass, but also on mitotic and

meiotic activity. Spermatogenic activity during the non-breeding season is exclusively limited to spermatogonia proliferation, whereas sperm production is activated due to a testosterone increase in the pre-rut period. Testosterone reaches its maximum during the rut and falls abruptly afterwards [38]. In this sense, we had noted a clear differentiation between the rut and the pre- and post-rut periods, considering morphometric parameters. Testicular weight did not changed between the pre-rut and rut, but testicular volume increased. This could indicate that, during the pre-rut, testicular tissues reach their full growth, but other changes, related to their full activation, may occur in these tissues only during the rut (testosterone peak). Blottner et al. [23] described the evolution of testis mass throughout the year, with a trend similar to the one shown here. These authors also studied the number of spermatozoa per gram of testis, which marked a peak in the rut, reaching almost 100×10^6, whereas it was around 50×10^6 sperm/g testis just before or after the rut, and nearly 0 during the winter. These data are in accordance with the number of sperm we salvaged from the cauda epididymis in each season (twice during the rut than during the pre-rut, and six times lower in the non-breeding season). The similarity of our results in the rut and post-rut could be due to a delay between the lowering of testicular spermatozoa and the depletion of sperm reserves in the cauda epididymis. In fact, the stability of the relative sperm content of the cauda (millions of spermatozoa per gram of cauda) between these two seasons does support this idea. We have to keep in mind that all the other studies considered here [23, 36–38] used animals living in a higher latitude than ours (around 52° N vs. 42° N in the North of Spain), where rut takes place between July and August, instead of July, so there is about a 15-day shift we have to take into account when comparing results. Goeritz et al. [8] carried out an ultrasonographic study in roe deer and found that testicular volume and size varied very little from May to September, contrarily to our findings (comparable to a period between April and August, in our work). Another difference is that corpus epididymis reached its maximum diameter between September and October (between the post-rut and non-breeding season), whereas we have found its maximum weight in the pre-rut and the rut, being lower in the post-rut. These differences are possibly due to the different methodology (ultrasounds vs. direct measuring of the testis and epididymis), and the different kind of animals used (captive vs. free-ranging; different subpopulations). Nevertheless, cauda epididymis volume and number of spermatozoa collected —by electroejaculation— agreed with our results.

Chamois reproductive biology has been little studied. In a preliminary study [32], we compared some morphometric and seminal parameters between breeding and non-breeding periods, finding results for testicular and cauda epididymis weights, and salvaged sperm similar to the ones shown here. However, sperm concentration between the two periods was not different, but in this study, using more extensive data, we have found that it was significantly higher in the breeding season than in the non-breeding season. Comparing with red deer and roe deer, it can easily be seen that, even though results for the breeding season were higher, differences between both periods were much smaller than in the case of cervids. In fact, seasonality affects less dramatically to members of the Bovidae family. Nevertheless, its effect on genitalia and sperm production is important, even

in domestic species [13, 20, 39]. According to our results, it is evident that spermatozoa production is clearly depressed during the non-breeding season, although a certain amount of sperm can be collected from the cauda epididymis. Lincoln [22], working with mouflon (*Ovis musimon*), described rapid testicular development preceding the breeding season. This process could be similar in the case of the chamois, with a rapid increase of testicular weight and size prior to the rut. As we did not find differences between the samples collected in different periods within the non-breeding season, we treated this entire interval as homogeneous. However, we could expect some variation in the samples collected just before the breeding season, in September. Unfortunately, we only had four samples collected in September, and during the first fortnight, which did not allow us to carry out such a study. The analysis of samples collected in September would be interesting in order to complement the present study.

These results are of great interest when considering strategies for setting up BRBs of these and similar species. Most reports regarding seasonality and sperm collection in cervids either omit data on the quantity of sperm salvaged from the cauda epididymis, or deal with semen obtained by electroejaculation. In the case of chamois, information is almost nonexistent. Our study shows that, for red deer, samples collected during the post-rut can yield an appreciable amount of sperm, much less than during the rut, though. Indeed, after the rut, spermatogenesis lowers rapidly, but there is still enough activity to maintain spermatogenesis for many weeks [11]. Even though the total number of spermatozoa salvaged in this period was around one third of the value obtained during the rut, these samples could have being processed into many seminal doses for conservation in BRBs. On the other hand, sperm collected in February would have meant that only very few doses could be produced. In the case of roe deer, the most favorable seasons were the rut and the post-rut. There were acceptable numbers, at least in many samples, during the pre-rut, and again very low quantity in the non-breeding season. Similarly, the breeding season of chamois is much more favorable for sperm collection, although an appreciable amount of sperm can be salvaged in the non-breeding season.

Furthermore, the study of sperm quality in each season is compulsory in order to establish a good collection strategy. In the case of red deer, quality is clearly poor in the non-breeding season. Monfort et al. [10], working with Eld's deer, indicated that motility and acrosomal status were better around the rut (winter solstice and spring equinox), and the number of abnormal forms was higher in the non-breeding period (summer solstice and autumn equinox). Interestingly, in our study, progressive motility, HOS test reactivity and acrosomal status yielded better results in the post-rut season than in the rut. In this sense, increased numbers of motile sperm in the post-rut have been noted by other authors [39], and would be a consequence of spermatogenetic changes at the end of the rut, with lower numbers of sperm but of better quality. Recently, Gizejewski [40] studied the characteristics of red deer semen collected with artificial vagina along the year and found some traits that could relate with our results. First, the author studied the different fractions of the ejaculate ("grey", "white" and "yellow") during the pre-mating (August), mating (September to December), transition

(December to February) and post-mating (February to April) periods. He found that, during the mating season (rut and post-rut seasons in our study), the fractions were yellow and white, the latter rich in spermatozoa. However, during the post-mating period (including February) he could obtain only grey fractions (very poor in spermatozoa). Furthermore, he obtained higher sperm concentration in the first part of the mating period, corresponding to the moment of higher libido (corresponding to the rut season in this study). However, sperm motility was better in that period than in the rest of the mating period, contrarily to our findings in epididymal sperm. This issue deserves more attention, and may be related to the interaction of seminal plasma with spermatozoa after leaving the epididymis. In fact, Strzezek et al. [41] reported important changes in seminal plasma composition, even during the mating season.

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

We faced the same problem with chamois, since medians are lower in the non-breeding season, but we did not found significant differences with the breeding season. However, Anel et al. [32] found differences studying motility and acrosomal status. Again, the problem seems to be due to individual differences. Due to the low number of samples analyzed (because of the restriction of 24–48 h post-mortem), we cannot reach definite conclusions, but probably some animals can yield acceptably good sperm during the non-breeding season, whereas other undergo higher variation between both seasons. We have to highlight the presence of samples of good quality in the non-breeding season, contrarily to the case of cervids, where no samples gave acceptable quality results in that period.

In conclusion, we have found important differences between the breeding season and other periods of the year, regarding both testicular and epididymal morphometry and sperm parameters. Our results mostly agree with other studies in cervids, and contribute with novel data for chamois, which has received little attention regarding its reproductive biology. We want to highlight the consequences for BRBs funding based on post-mortem sperm recovery (for these or similar species), since season modified sperm availability and quality. Considering quality and quantity, breeding season was the better period to collect samples. For the other periods, post-rut for Iberian red deer, pre-rut for roe deer, and the non-breeding season for chamois (at least the intervals studied here)

seemed to offer good opportunities to collect samples, although at the cost of less quantity and possibly lower quality (excepting red deer, with very good quality in the post-rut). The post-rut of the roe deer may be discarded because of the low quality of the samples. In the same sense, the non-breeding season is not a good period to collect samples from cervids, considering the very low sperm numbers and quality of the samples. Nevertheless, in the case of endangered species or valuable individuals, it may be worthwhile to collect sperm even in the worst season, since techniques such as sperm selection, IVF or ICSI can overcome the low numbers or the lack of functionality of sperm. However, the quality of the sperm collected at different times during the year should be tested in depth, especially its fertility, in order to confirm our findings.

7.6 Acknowledgements

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

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Capítulo 8

Sperm subpopulations in Iberian red deer epididymal sperm and their changes through the cryopreservation process

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Biology of Reproduction

Aceptado el 14 de septiembre de 2004
Publicado *online el 22 de septiembre de 2004*
(http://www.biolreprod.org/pap.shtml)

8.1 Abstract

We have applied a statistical protocol based on principal component analysis, clustering methods and discriminant analysis for the identification of sperm subpopulations in CASA data. Samples were obtained from the cauda epididymis of 11 Iberian red deers, and cryopreserved following a standard protocol. Motility by CASA was analyzed just after sperm recovery, just before freezing and after thawing, and eight motility descriptors for each individual spermatozoon were recorded. Sperm viability and acrosomal status were also assessed. Subpopulation analysis was performed in four sequential steps: principal component analysis using the 8 motility descriptors; non-hierarchical clustering analysis (k-means) using the first two principal components; hierarchical clustering analysis (UPGMA); and selection of the final number of clusters. Three clusters were obtained for each motility analysis: slow and non-linear; rapid and linear; and rapid, high ALH, non-linear. We detected variations in the clusters between treatments (initial, pre-freezing and post-thawed). Indeed, motility increased and linearity decreased in the pre-freezing analysis. A discriminant analysis isolated three descriptors that were used again in the same statistical analysis, giving four clusters that resembled the pattern found in the first classification. We also performed a clustering analysis of the males according to pre-freezing/post-thawed variation of total motility, viability and acrosomal status. The proportion of the linear subpopulations in the pre-freezing treatment, in both clustering analyses, correlated positively with post-thawed viability recovery. Our results show that clustering analysis of CASA data gives useful and practical information that is not obtained by conventional sperm analysis.

Keywords: Iberian red deer, cryopreservation, epididymal sperm, CASA, sperm subpopulations, cluster analysis

8.2 Introduction

The presence of several distinct subpopulations within sperm samples is nowadays widely accepted by the scientific community. Although the mechanism of formation of these subpopulations and their physiological role are not yet clear [1, 2], many authors have found that susceptibility to capacitation and fertilizing ability varies depending on the subpopulation studied [3–5]. Therefore, the development of experimental techniques and statistical analyses aimed at identifying and isolating subpopulations depending on several characteristics is of great interest, not only because of the study of sperm biology, but also because of practical and economical reasons [6, 7]. In fact, many sperm separation methods, such as density gradient centrifugation or the swim-up are based on the isolation of sperm subpopulations with certain abilities, specially related to fertility [2].

Subpopulation identification has been carried out according to very different sperm characteristics, such as biochemical parameters [8–11], functional tests [12–18] or depending on of sperm morphology [2, 19–21]. These studies have frequently reported a relationship between detected

subpopulations and sperm quality, fertility or its ability to resist cryopreservation.

Improvements in CASA devices have also enabled individual sperm to be distinguished in motility analysis, and subpopulations characterised by motility descriptors to be identified by means of clustering algorithms. However, few studies have considered the existence of sperm subpopulations [22], implying that useful information may have been overlooked. The use of the mean values of motility descriptors oversimplifies the motility analysis, considering the whole sample as being an homogeneous one. Consequently, its internal variability is not taken into account, thus impairing the analysis of the relationship between motility and sperm quality, and, ultimately, fertility [6, 23].

Many researchers have reached interesting conclusions after carrying out subpopulation studies. A large number of these studies were performed using the PATN software, a powerful collection of statistical procedures aimed to the extraction and display of patterns in multivariate data. For instance, Abaigar et al. [22] showed that the addition of caffeine or bicarbonate or cryopreservation with different extenders altered the subpopulation pattern of boar and gazelle semen. Another study on gazelle semen [24] indicated that the subpopulation pattern was altered depending on the voltage used during electroejaculation, seminal fraction, body weight and storage time. Thurston et al. [25] included a multivariate analysis of boar semen, obtaining three subpopulations with different motility qualities. In general, the analysis performed with this software consisted of a so-called non-hierarchical clustering step followed by a hierarchical one, which rendered the final clusters.

Other researches have used other statistical methods based on less specific software, but not less efficient. Most of them performed non-hierarchical clustering on the CASA data, using the k-means model, although others [6] also explored some hierarchical procedures. Amongst the studied species are human [26, 27], common marmoset [6], stallion [28] and boar [6, 7]. These studies reported some interesting data on sperm subpopulation variations with regard to resistance to cryopreservation [27], presence of stimulants [6], storage and fertility [28] and between-boar variability [7].

The aim of the present study was mainly to develop a multi-step statistical protocol that would enable the subpopulation composition of a sperm sample to be determined utilizing the motility data obtained from CASA analysis. We also intended this protocol not to be dependent on one particular statistical package, but to be easily portable to any software capable of performing principal component analysis and clustering procedures. We based the protocol on two successive steps of non-hierarchical and hierarchical clustering procedures, in order to combine the advantages of both techniques. However, as stated by other authors [22, 28], most motility descriptors are highly correlated, and present different scales of measure, which are considerable problems when performing certain statistical clustering methods. Therefore, prior to the clustering steps, we performed a principal component analysis, reducing the number of descriptors to a few, uncorrelated and standardized, variables (principal components). We then used a statistical method based on the clustering history of some statistics to determine the more suitable number of final clusters. Furthermore, a discriminant analysis determined which motility descriptors were the most important discriminating between the found clusters, and another clustering analysis was performed in order to determine if reliable

subpopulations could be obtained by reducing the initial amount of descriptors.

For this study, we used Iberian red deer epididymal sperm, which was analyzed for motility just after being obtained, before freezing and after thawing. Our intention was not only to determine if we could separate sperm subpopulations, but also study the variation of the subpopulation structure amongst the three treatments (initial, pre-freezing and post-thawed), and its relationship with the whole sperm population, the subpopulation of “progressive” spermatozoa, and the subpopulation of “rapid” spermatozoa (obtained apart from the clustering analysis). Finally, we investigated the relationship between sperm “freezability” (taken as the difference between post-thawed and pre-freezing values of some parameters) and the motility subpopulations found in the study.

8.3 Material and methods

All chemicals were obtained from Sigma (Madrid, Spain). Media were not bought as such, but prepared in our laboratory as referred. Table 8.1 shows a list of acronyms frequently used throughout this work.

8.3.1 Genitalia collection and sperm recovery

Genitalia were collected from 11 Iberian red deer (*Cervus elaphus hispanicus*) harvested in several private hunting reserves in the region of Cáceres (Spain). All the animals were adults and lived in a free-ranging regime. Sample collection was carried out during the first fortnight of December.

Harvest plans followed the Spanish Harvest Regulation, 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 times of the year when hunting can take place, are reviewed each year by the Annual Hunting Regulation of the respective regions. 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, collection and refrigeration were recorded 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 about 24 hours postmortem.

Sample manipulation was carried out in a walk-in fridge (5 °C). Testicles with epididymes and vas deferens attached were isolated from scrotum and other tissues. Epididymes were dissected free from the testicles, and cleaned of connective tissue. To avoid blood contamination, superficial blood vessels were previously cut, wiping their contents and drying thoroughly the surface of the cauda. Sperm was 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.

8.3.2 Cryopreservation protocol

Sperm (still at 5 °C) was diluted 1:1 with Tes-Tris-Fructose extender, containing 10 % egg yolk and 4 % glycerol [29]. After resting 2 h at 5 °C, the sample was further diluted with the same extender down to 100×10^6 sperm/mL and packed in 0.25 mL French straws. Freezing was carried out using a a programmable biofreezer (Planner MRII®), at -20 °C/min down to -100 °C, and then transferred to liquid nitrogen containers. Thawing was performed by dropping the straws in water at 65 °C for 6 s.

8.3.3 Computer assisted sperm analysis

Samples were analyzed just after recovery (initial assessment), just before freezing (pre-freezing assessment), and after thawing (post-thawed assessment). We will refer to these three stages as treatments.

Sperm were diluted down to $10–20\times10^6$ 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 minutes. Then, a prewarmed Makler counting chamber (10 μm depth) was loaded with 5 μL of sample. The CASA system consisted of an optical phase contrast microscope (Nikon Labophot-2) (endowed with negative phase contrast objectives and a warming stage at 37 °C), a Sony XC-75CE camera, and a PC with the Sperm Class Analizer software (SCA2002, Microptic, Barcelona, Spain). The magnification was $\times10$. All samples were analyzed at least twice, in order to discard errors due to incorrect sampling. At least 5 fields per sample were acquired, recording at least 100 motile sperm. Image sequences were saved and analyzed afterwards. CASA acquisition parameters were: 25 images acquired, at an acquisition rate of 25 images per second.

Samples were corrected and analyzed using the editing facilities provided by SCA2002. Events other than spermatozoa were removed, and settings were adjusted in each case in order to assure a correct track analysis. Whenever a field was considered to have incorrect analyzed tracks and correction was not suitable, it was removed. After each analysis, data were saved in an Excel file (Microsoft, Redmon, WA). For each sperm analyzed, the SCA2002 rendered the following data: VCL (velocity according to the actual path; μm/s), VSL (velocity according to the straight path; μm/s), VAP (velocity according to the average —smoothed— path; μm/s), LIN (linearity; %), STR (straightness; %), WOB (wobble; %), ALH (amplitude of the lateral displacement of the sperm head; μm), and BCF (frequency of the flagellar beat; Hz). Detailed explanation of these descriptors of sperm movement is provided elsewhere [27, 30–32]. Spermatozoa were considered motile when VCL$>10\mu$m/s.

8.3.4 CASA data preprocessing

Data from SCA were processed with the help of Excel 4 macros programmed ad hoc. Excel files from SCA2002 were modified in order to give each observation (individual spermatozoa) two labels, iden-

tification of the animal and treatment. Then, files were concatenated and the resulting file was used in further analysis. Data produced by statistical procedures were also processed with the aid of macros in order to assign cluster ownership. Also, we defined other two sperm subpopulations, independently of cluster analysis: "rapid" sperm subpopulation (each spermatozoa with VCL>75μm/s) and "progressive" sperm subpopulation (progressive spermatozoa, each spermatozoa with VCL>25μm/s and STR>80 %). These subpopulations will be referred to as PSP and RSP, respectively. We chose these parameters and these values because they were assigned in that way in the configuration of our CASA system for deer. For each subpopulation, we calculated the proportion of spermatozoa it comprised respect to the total number of motile spermatozoa in the sample (SM), or respect to the total number of spermatozoa (either motile or inmotile) in the sample (ST).

8.3.5 Evaluation of sperm viability and acrosomal status

Samples (pre-freezing and post-thawed) were diluted in buffered media (1:100, same composition that the one used for motility analysis), 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 using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). We used a 15 mW argon-laser that provided an excitation wavelength of 488 nm, using the FL3 photodetector channel to read the red emission light of PI (650 long pass filter), and the FL1 photodetector channel to read the green emission light of FITC (530/30 band pass filter). At least 10000 events (spermatozoa detected, after discarding debris) were acquired. Considering that PI stains cells with damaged plasma membrane, and that PNA-FITC stains acrosome-reacted cells, viability and acrosomal status were defined as the percentage of PI-unstained and FITC-unstained cells in the sample, respectively.

8.3.6 Statistical analysis

All statistical analyses were carried out using the SAS/STAT^{TM} package V. 8 (SAS Institute, Cary, NC) [33]. The main objective of the analysis was to extract sperm subpopulations using the motility data obtained from each treatment, by means of a clustering procedure, and then compare subpopulations and treatments using χ^2 and general linear models. P<0.05 was used for significance.

As a first step, we used the PRINCOMP procedure in order to perform a principal component analysis (PCA) of the motility data. The purpose of PCA is to derive a small number of linear combinations (principal components) from a set of variables, that retain as much of the information in the original variables as possible. This allows to summarize many variables in few, jointly uncorrelated, principal components. A good result is when we obtain few principal components accounting for a high proportion of the total variance. In order to select the number of principal components that should be used in the next step of our analysis, we followed the criterion of selecting only those with an eigenvalue (variance extracted for that particular principal component) higher than 1 (Kaiser criterion).

As a second step, we performed a non-hierarchical cluster analysis using the FASTCLUS procedure, and the selected principal components as variables. This procedure performs a disjoint cluster analysis on the basis of distances computed from one or more quantitative variables, using euclidean distances (k-means model) to calculate cluster centers. This clustering method is often used with large data sets before trying a hierachical one, in order to reduce the data to a few initial clusters and then pass them to the hierarchical procedure. After trying many options, we selected a maximum number of clusters of 15, as this number represents a fairly high number of initial clusters for the subsequent hierarchical procedure (too few initial clusters may have impaired the results of the last clustering step), but is not high enough to complicate the follow-up of the hierarchical clustering. We also took advantage of this step to detect outliers, since this clustering procedure is very sensitive to them, which appear as clusters with only one member. Following detection, outliers can be removed and the procedure rerun.

The third step of the clustering analysis was carried out using the CLUSTER procedure, which performed a hierarchical clustering on the clusters obtained by the previous step, using the average linkage method (UPGMA) for joining the clusters. The procedure displays a history of the clustering process, giving useful statistics for estimating the number of clusters in the population from which the data are sampled. In order to determine the final number of clusters, we studied the evolution along the clustering process of three statistics provided by CLUSTER: the pseudo t^2, the pseudo F and the cubic clustering criterion (CCC). We looked for certain kind of consensus among the three variables, to be precise 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. It must be noted that other methods can be used, but this one has the advantage of being simple and gives a good estimate of the clustering trend (Figure 8.1). The output data set of CLUSTER were passed to the TREE procedure, that drew a tree diagram (dendrogram) describing the clustering process, and produced the n-clusters solution in a final data set, which was utilized in the subsequent statistical analysis.

After clustering analysis, we compared treatments within subpopulations and subpopulations within treatments (we include clusters, PSP, RSP and the whole motile sperm population within the term “subpopulation”). We used the GLM (general linear models) procedure in order to carry out an ANOVA on the data. Viability and acrosomal status before and after cryopreservation were also compared with this procedure. Previously, percentages were subjected to arc sine transformation and absolute measures to log transformation. The Student-Newman-Keuls test was used for pairwise comparison when results were significant.In order to study the distributions of observations (individual motile spermatozoa) between subpopulations within treatments and between treatments within subpopulations, we used the χ^2 test, included in the FREQ procedure. When assumptions for χ^2 test were violated, the exact Fisher’s test was used instead.

In order to determine whether we could reduce the original number of variables and still obtain informative results, we carried out a stepwise discriminant analysis (STEPDISC procedure) in the eight variables and the three clusters (separately for each treatment). This kind of analysis indicates

Table 8.1 List of some acronyms frequently used in this work.

Acronym	Meaning
ST	Proportion of spermatozoa in a subpopulation respect to the total number of spermatozoa (either motile or inmotile) in the whole sample.
SM	Proportion of spermatozoa in a subpopulation respect to the number of motile spermatozoa in the whole sample.
PSP	Subpopulation comprising progressive sperm (VCL$>$10μm/s and STR$>$80 %).
RSP	Subpopulation comprising rapid sperm (VCL$>$75μm/s).
PCA	Principal components analysis.
CCC	Cubic clustering criterion.
PRIN1	First principal component extracted from a PCA.
PRIN2	Second principal component extracted from a PCA.
CL1, CL2 and CL3	Clusters obtained from the first clustering analysis.
CL1b, CL2b, CL3b, discriminant CL4b and CL5b	Clusters obtained from the second clustering analysis, after performing the analysis.

which of the original variables best reveal differences among the given groups. Thus, we selected those variables with high discriminatory power in the three treatments, and repeated the whole clustering analysis, entering only the selected variables. This time, we selected the number of principal components by the variance explained, rather than by their eigenvalues, since we expected only one principal component with an eigenvalue higher than one due of entering less variables in the PCA.

Finally, we studied some aspects of the “freezability” of the individual sperm samples. We defined “freezability” as the difference between post-thawed and pre-freezing sperm quality. Therefore, we used three variables for determining the “freezability” of each sample: total motility, sperm viability and acrosomal status, substracting their post-thawed value from the pre-freezing one. We called the new variables TMdiff, VIABdiff and ACRdiff, respectively. Then, we carried out a clustering analysis in order to classify the males according to the values of these variables. This cluster analysis was performed by carrying out a PCA and hierarchical clustering on “freezability” parameters in an identical manner as described above, but omitting the non-hierarchical clustering step (in this case there were few initial objects —males—, and this step was not necessary). Also, we carried out a correlation analysis between pre-freezing variables (including those derived from the subpopulation study) and “freezability” variables. This analysis was performed using the Spearman correlation coefficients (CORR procedure).

8.4 Results

8.4.1 General results and preliminary analysis

Samples acquired with CASA were analyzed and corrected. When a field could not be analyzed due to sampling errors (incorrect contrast or bright, causing most of the tracks to be incorrectly analyzed by the software), it was removed. After correcting the samples, we pooled all the data in a common database, with 893 observations for the initial analysis, 1526 observations for pre-freezing, and 919 observations for post-thawed analysis. Each observation was identified by three codes, the number of the deer, the treatment (initial, pre-freezing, or post-thawed), and the number of the sperm within each acquisition.

In general, motility parameters were good, even after thawing, considering the whole population, PSP and RSP subpopulations (Table 8.2). Comparison of the proportion of motile, progressive and rapid spermatozoa (χ^2 test) indicated significant differences amongst all treatments. Total motility had the lowest values initially, improved greatly in the pre-freezing, and decreased post-thawed, but were still higher than in the initial treatment. Conversely, PSP decreased pre-freezing and returned to initial values post-thawed, which also gave good numbers of RSP (in fact, sperm velocity was higher post-thawed). On the other hand, viability decreased significantly from a pre-freezing value of $84.5\pm1.5\,\%$ to a post-thawed value of $67\pm1.8\,\%$, whereas the percentage of spermatozoa with damaged acrosomes, as indicated by PNA-FITC staining, increased significantly from $3.2\pm0.7\,\%$ to $8.1\pm1.2\,\%$.

8.4.2 First cluster analysis

For each treatment, PCA rendered two principal components with eigenvalues above 1 (PRIN1 and PRIN2; Table 8.3), which accounted for more than 80 % of the variance. Considering the scores of CASA parameters, the first principal component was related to fast and linear movement, whereas the second principal component was related to fast erratic movement, including wide head lateral displacement.

The two principal components entered in the non-hierarchical clustering, and the resulting 15 clusters were grouped into three clusters, after applying the hierarchical procedure and studying the plots of CCC, pseudo F and pseudo t^2 vs. number of clusters (Figure 8.1). The dot plots of the two principal components for each treatment showed that the positions of the three clusters were similar in the multidimensional space defined by these principal components (Figure 8.2), thus we related each cluster in each treatment to its equivalent in the other two treatments and denominated them CL1, CL2 and CL3. CL1 and CL2 were well defined by their PRIN1 values (in general, CL1 with negative values and CL2 with positive ones). Therefore, accordingly to the eigenvalues of the motility descriptors for PRIN1, CL1 would include slow and non-linear spermatozoa, whereas CL2 would include fast and linear spermatozoa. In the same sense, CL3 has positive values for PRIN2, so

Table 8.2 Summary of selected motility parameters obtained in this study (SM, ST, VAP, LIN, ALH and BCF), considering subpopulations and treatments. Subpopulations include: the whole population of motile spermatozoa, PSP, RSP, and the three subpopulations defined by the first clustering analysis (CL1, CL2 and CL3). The treatments are I: initial, PF: pre-freezing and PT: post-thawed. Data are expressed as Mean±SD.

Subpopulation	Treatment	SM (%)[1]	ST (%)[1]	VAP (μm/s)	LIN (%)	ALH (μm)	BCF (Hz/s)
	I	100	68.6^{a}	68.4 ± 40.9^{aA}	64.3 ± 27.1^{aA}	2.8 ± 1.9^{aA}	7.8 ± 4.1^{A}
Motile[2]	PF	100	82.0^{b}	58.7 ± 37.8^{bA}	47.9 ± 24.1^{bA}	3.4 ± 2.1^{bA}	8.0 ± 3.5^{A}
	PT	100	77.6^{c}	74.6 ± 45.3^{aA}	47.9 ± 27.6^{bA}	3.4 ± 1.5^{bA}	8.0 ± 3.6^{A}
	I	69.5^{aA}	47.7^{aA}	81.4 ± 34.3^{aB}	78.4 ± 17.5^{aA}	2.6 ± 1.8^{aA}	8.1 ± 3.8^{A}
PSP	PF	42.8^{bA}	35.1^{bA}	69.0 ± 35.6^{bB}	69.8 ± 17.0^{bB}	2.9 ± 1.7^{aB}	9.3 ± 3.5^{B}
	PT	64.9^{cA}	50.4^{aA}	88.2 ± 39.8^{aB}	80.2 ± 16.2^{aB}	2.5 ± 1.2^{bA}	8.9 ± 3.5^{A}
	I	61.0^{aB}	41.9^{aB}	94.0 ± 29.0^{aC}	73.2 ± 22.3^{aC}	3.5 ± 2.0^{aB}	8.7 ± 4.0^{B}
RSP	PF	51.8^{bB}	42.5^{abB}	88.3 ± 27.0^{bC}	50.7 ± 23.7^{bA}	4.8 ± 2.0^{bC}	8.2 ± 3.5^{A}
	PT	58.6^{aB}	45.5^{bB}	106.2 ± 29.0^{cB}	73.1 ± 23.9^{aC}	3.3 ± 1.5^{aB}	8.6 ± 3.7^{A}
	I	27.9^{aC}	19.1^{aC}	20.3 ± 13.9^{aD}	31.7 ± 13.8^{aD}	1.9 ± 1.1^{aC}	4.9 ± 3.1^{aC}
CL1	PF	45.6^{bC}	37.4^{bAC}	26.8 ± 15.9^{bD}	32.9 ± 14.1^{aC}	2.4 ± 1.2^{bD}	7.2 ± 3.3^{bC}
	PT	33.0^{cC}	25.6^{cC}	28.2 ± 21.1^{bD}	35.8 ± 15.7^{bD}	2.2 ± 1.1^{bC}	7.0 ± 3.2^{bB}
	I	50.9^{aD}	34.9^{aD}	80.8 ± 28.8^{aB}	86.1 ± 11.2^{aE}	2.0 ± 0.8^{aD}	9.6 ± 3.8^{aB}
CL2	PF	45.9^{bC}	37.6^{aC}	82.6 ± 29.6^{aC}	67.3 ± 18.7^{bB}	3.6 ± 1.8^{bE}	9.0 ± 3.5^{bB}
	PT	60.2^{cB}	46.7^{bB}	96.1 ± 35.3^{bBC}	83.1 ± 13.0^{cB}	2.5 ± 1.3^{cA}	8.9 ± 3.6^{bA}
	I	21.2^{aE}	14.6^{aE}	101.8 ± 33.5^{C}	54.8 ± 17.4^{aF}	5.6 ± 1.8^{aE}	7.3 ± 3.6^{A}
CL3	PF	8.6^{bD}	7.0^{bD}	100.7 ± 20.9^{E}	23.9 ± 8.4^{bD}	7.7 ± 1.6^{bF}	7.1 ± 3.1^{C}
	PT	6.9^{bD}	5.3^{cD}	108.2 ± 37.3^{C}	30.3 ± 16.1^{cE}	5.9 ± 1.1^{cD}	6.9 ± 3.0^{B}

[1]: χ^2 on raw data (rest of comparisons by ANOVA+SNK test).

[2]: for the whole population of motile sperm, % (motile) is 100 % (not included in between-subpopulations comparison).

^{a,b,c}: rows (treatments within subpopulations) with different superscripts differ $P<0.05$.

^{A,B,C,D,E}: rows (subpopulations within treatments) with different superscripts differ $P<0.05$.

Table 8.3 Summary of the results of the PCA performed on the CASA data. The first two principal components obtained for each treatment (eigenvalues higher than 1) are showed. Variance explained is the proportion of the total variance explained by each principal component. The eigenvectors are a measure of association of the original parameters with the resulting principal components.

		Initial		Pre-freezing		post-thawed	
		PRIN1	PRIN2	PRIN1	PRIN2	PRIN1	PRIN2
Eigenvalues		4.57	2.05	3.82	2.63	4.47	2.02
Variance explained		0.57	0.26	0.48	0.33	0.56	0.25
Eigenvectors	VCL	0.35	0.46	0.35	0.44	0.37	0.44
	VSL	0.45	0.12	0.49	0.03	0.46	0.07
	VAP	0.43	0.24	0.45	0.27	0.43	0.26
	LIN	0.40	-0.34	0.38	-0.40	0.40	-0.35
	STR	0.35	-0.26	0.29	-0.41	0.33	-0.38
	WOB	0.37	-0.31	0.38	-0.27	0.40	-0.22
	ALH	0.12	0.64	0.20	0.54	0.11	0.64
	BCF	0.25	-0.18	0.16	-0.18	0.18	-0.09

this cluster would include fast, non-linear sperm, with high ALH. These characteristics are reflected in the mean values of the motility descriptors (Table 8.2). Observing the variation in the clouds of dots between treatments, we observed a higher dispersion of CL3 in the initial treatment, whereas CL2 was compact in the initial and post-thawed treatments, but more scattered in the pre-freezing treatment. These changes correspond with alterations in their motility descriptors, as can be observed in Table 8.2.

In general, the distribution of spermatozoa amongst the three clusters followed the trend found for the whole population, and PSP and RSP subpopulations. Interestingly, PSP included 93 % and 99 % of CL2 (rapid and linear) in the initial and post-thawed treatments, respectively, but in the pre-freezing one, only 74 % of CL2 was included into PSP, indicating a drop in the proportion of high-linear spermatozoa contained in CL2 (Figure 8.3). On the other hand, RSP (Figure 8.4) comprised the totality of CL3 (rapid and non linear) in the three treatments, plus a constant part of CL2 (around 75 %). Although spermatozoa in CL1 were considered slow and non-linear, 20–25 % were included in PSP, and around 11 % (initial) and 18 % (pre-freezing and post-thawed) were included in RSP.

8.4.3 Clustering analysis with a reduced set of variables

We selected VCL, VSL and LIN after carrying out the discriminant analysis. In the three treatments, these variables were among the four with the highest F and R^2 values (indicating good discriminant

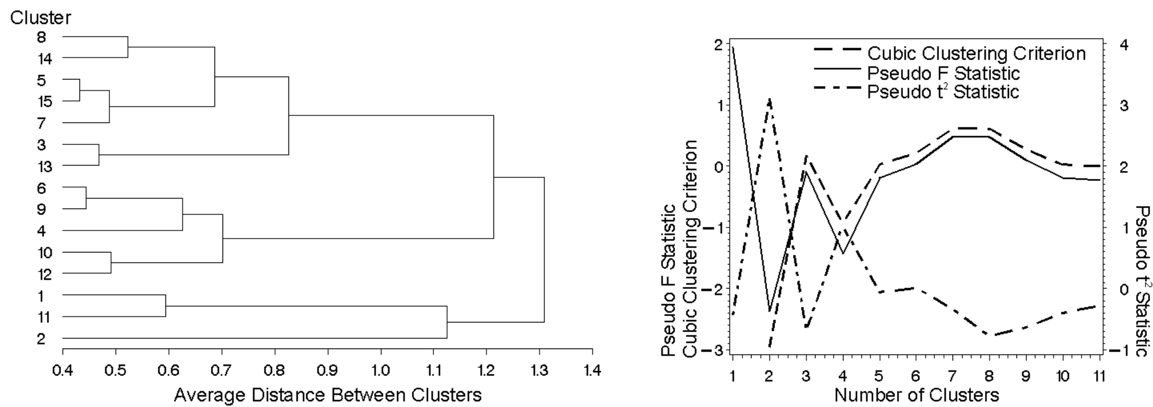


Figure 8.1 Example of the dendrogram derived from the hierarchical clustering analysis, and of the plot used for the determination of an adequate final number of clusters (data from the first clustering analysis, post-thawed treatment). The plot on the left shows the dendrogram resulting from the hierarchical clustering of the 15 clusters derived of the PCA and subsequent non-hierarchical clustering of post-thawed motility data. 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 the three 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 indicates 3 final clusters, although numbers of 5 or 8 may have been eligible for study.

Table 8.4 Summary of the second PCA, performed only with the variables selected after the discriminant analysis. Variance explained is the proportion of the total variance explained by each principal component. The eigenvectors are a measure of association of the original parameters with the resulting principal components.

		Initial		Pre-freezing		post-thawed	
		PRIN1	PRIN2	PRIN1	PRIN2	PRIN1	PRIN2
Eigenvalues		2.26	0.70	1.98	0.96	2.30	0.65
Variance explained		0.75	0.23	0.66	0.32	0.77	0.22
Eigenvectors	VSL	0.66	-0.04	0.70	0	0.65	-0.02
	VCL	0.55	-0.66	0.52	-0.69	0.55	-0.68
	LIN	0.52	0.75	0.49	0.72	0.52	0.73

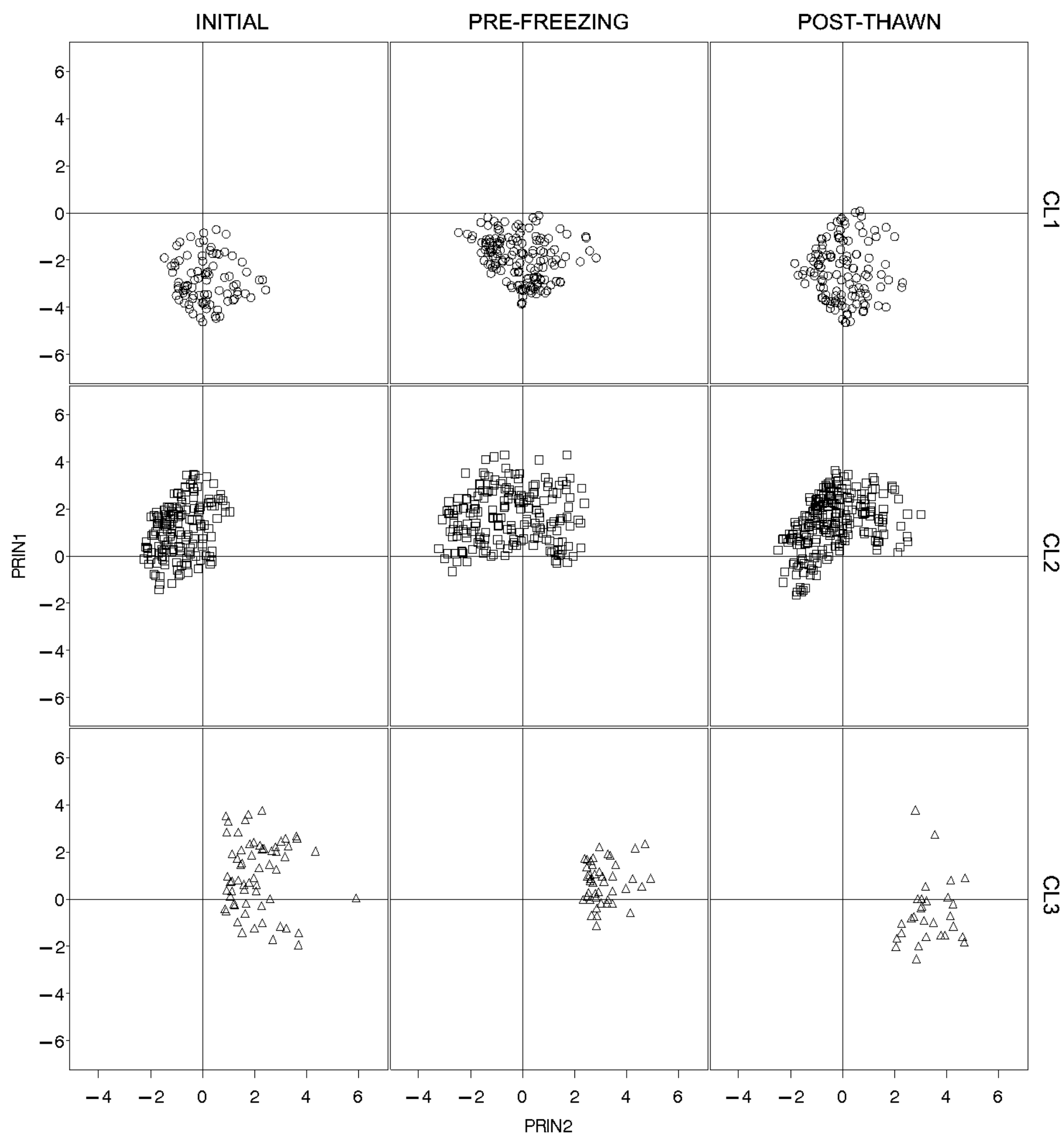


Figure 8.2 Dot plots corresponding to the clusters obtained from the first analysis, defined by the two first principal components (PRIN1 and PRIN2). In order to follow the evolution of each subpopulation through treatments, each original dot plot was decomposed in three, showing only one cluster each. Data from the initial, pre-freezing and post-thawed analysis are presented from left to right. Each event (only 20-40 % are showed, in order to avoid cluttering) represents one individual spermatozoa. The PCA allowed to represent the resulting eight-dimensional space in a two-dimensional plot, not only preparing the data for the clustering analysis, but also helping to its interpretation. Events are represented by different symbols in order to identify its membership to each cluster (CL1: ○; CL2: □; CL3: △).

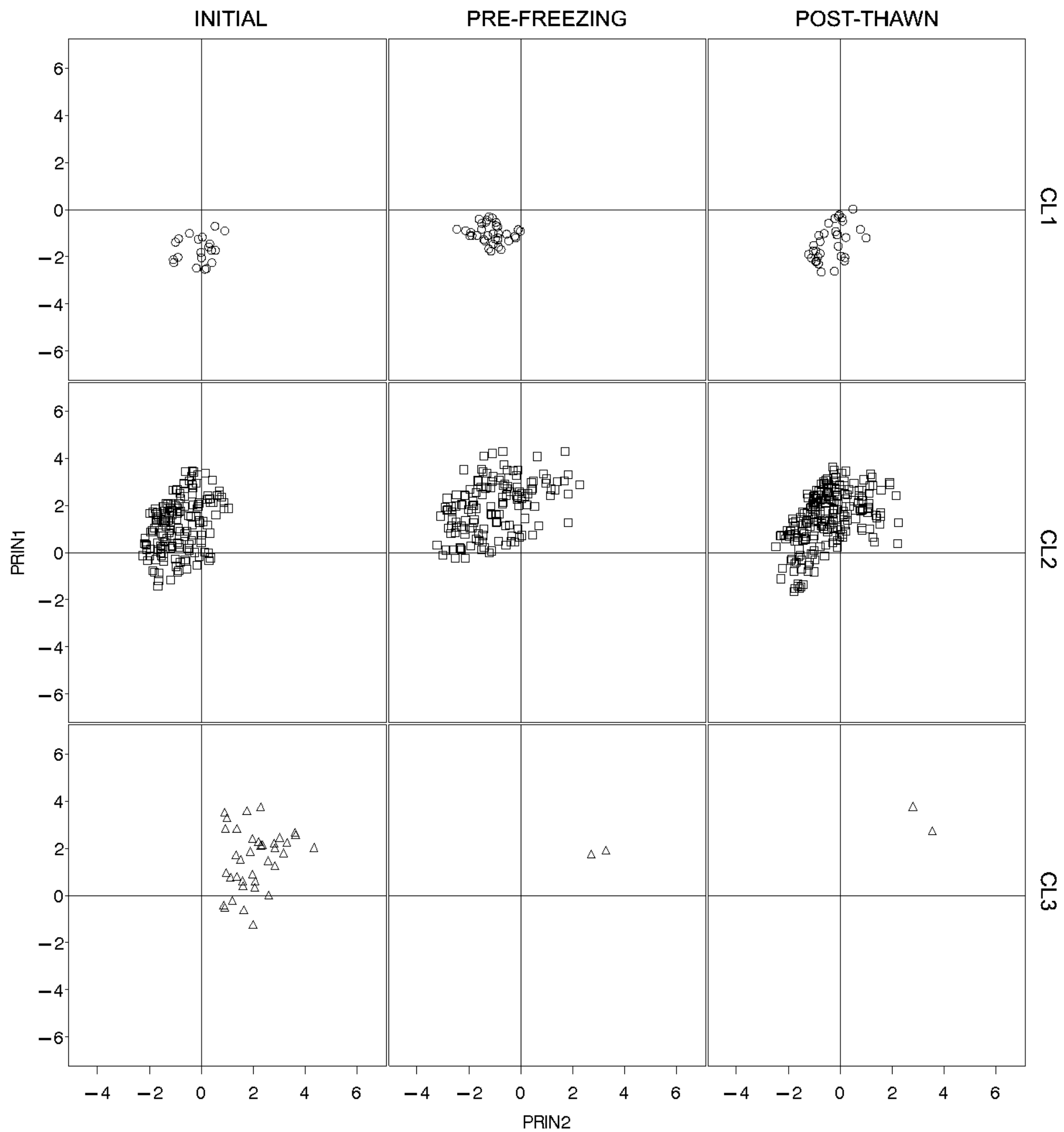


Figure 8.3 Representation of the "progressive" (PSP) subpopulation in the space defined by the two first principal components (PRIN1 and PRIN2, from the first clustering analysis). Data from the initial, pre-freezing and post-thawed analysis are presented from left to right. Each event (only 50 % are shown, in order to avoid cluttering) represents one individual spermatozoa. Events are represented by different symbols in order to identify its membership to each cluster (CL1: ○; CL2: □; CL3: △).

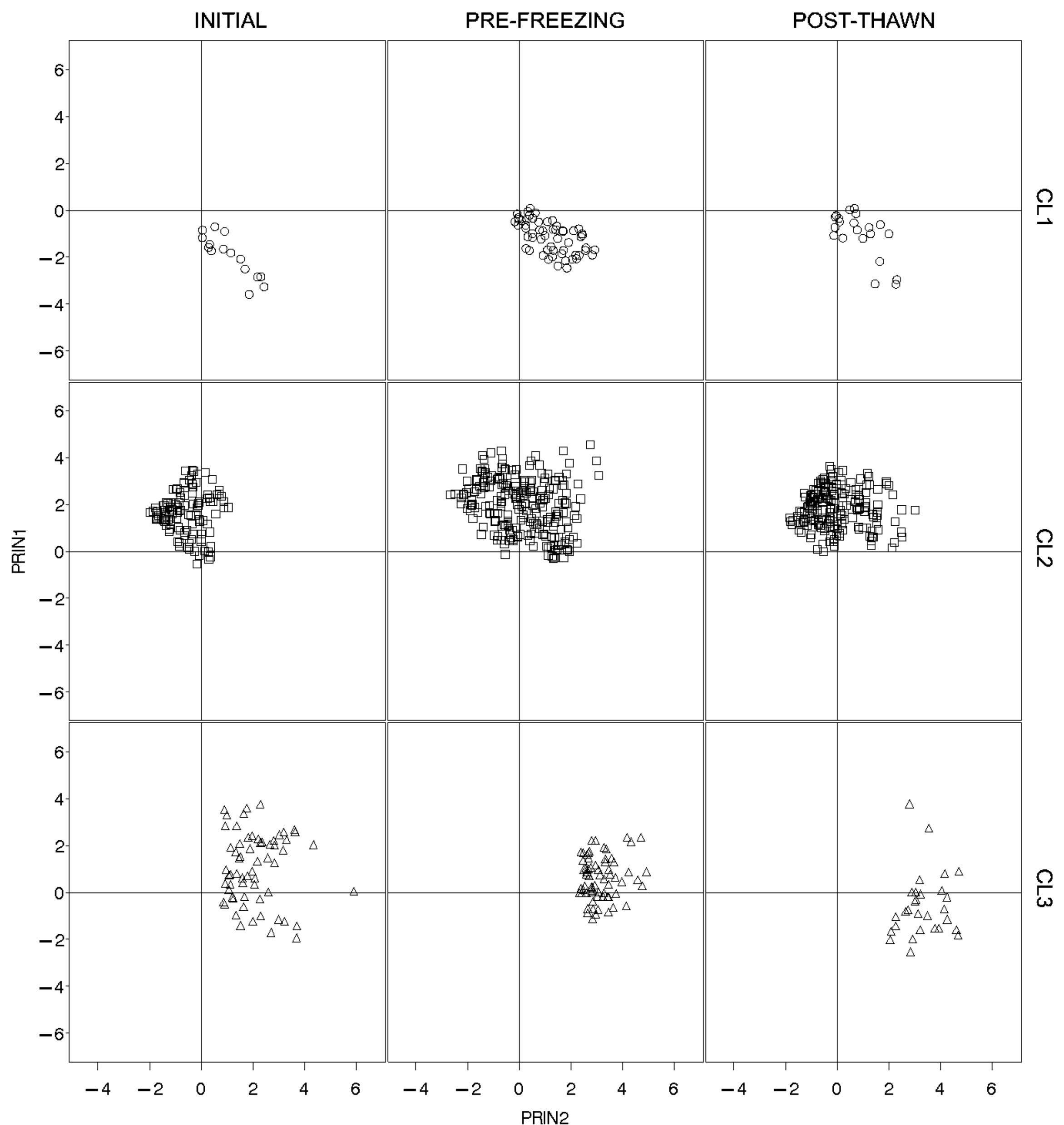


Figure 8.4 Representation of the "rapid" (RSP) subpopulation in the space defined by the two first principal components (PRIN1 and PRIN2, from the first clustering analysis). Data from the initial, pre-freezing and post-thawed analysis are presented from left to right. Each event (only 50 % are shown, in order to avoid cluttering) represents one individual spermatozoa. Events are represented by different symbols in order to identify its membership to each cluster (CL1: ○; CL2: □; CL3: △).

Table 8.5 Clustering obtained after the discriminant analysis. Summary of selected motility parameters obtained in this study (SM, ST, VAP, LIN, ALH and BCF), considering subpopulations and treatments. The treatments are I: initial, PF: pre-freezing and PT: post-thawed. Data are expressed as Mean±SD.

Subpopulation	Treatment	SM (%)[1]	ST (%)[1]	VAP (μm/s)	LIN (%)	ALH (μm)	BCF (Hz/s)
	I	32.2^{a}	22.1^{a}	22.8±14.9^{a}	35.9±17.2	2.0±1.1^{a}	5.5±3.4^{a}
CL1b	PF	48.8^{b}	40.0^{b}	27.6±15.7^{b}	36.4±16.3	2.3±1.1^{b}	7.5±3.5^{b}
	PT	33.7^{a}	26.1^{c}	26.0±17.6^{c}	36.9±16.5	2.1±1.0^{a}	7.2±3.3^{b}
	I	10.9^{a}	7.5^{a}	81.2±22.1^{a}	43.5±14.2^{a}	5.3±1.7^{a}	7.2±3.6
CL2b	PF	21.8^{b}	17.8^{b}	88.5±21.8^{b}	33.8±12.5^{b}	6.1±1.7^{b}	7.6±3.2
	PT	7.0^{c}	5.4^{c}	99.3±28.6^{c}	27.7±10.5^{c}	5.5±1.2^{a}	6.9±3.3
	I	20.8^{a}	14.2^{a}	123.9±18.8^{a}	80.1±14.2^{a}	4.0±2.2^{a}	8.8±3.8
CL3b	PF	16.6^{b}	13.6^{a}	110.8±17.3^{b}	71.6±12.9^{b}	4.3±1.6^{b}	9.1±3.3
	PT	40.7^{c}	31.6^{b}	114.4±20.2^{b}	83.4±13.3^{c}	2.9±1.2^{c}	8.9±3.9
	I	36.1^{a}	24.8^{a}	72.7±18.2^{a}	86.8±10.2	1.9±0.8^{a}	9.5±3.9^{a}
CL4b	PF	12.8^{b}	10.5^{b}	59.0±17.4^{b}	85.3±8.8	1.8±0.8^{b}	9.1±3.2^{a}
	PT	16.7^{c}	12.9^{c}	55.5±17.3^{b}	85.9±9.2	1.5±0.5^{c}	8.5±3.0^{b}
CL5b	PT	2.0	1.5	156.2±19.2	64.5±10.3	6.0±1.1	9.1±2.4

[1]: χ^2 on raw data (rest of comparisons by ANOVA+SNK test).

^{a,b,c}: rows (treatments within subpopulations) with different superscripts differ P<0.05.

power). BCF in the initial, and VAP in the post-thawed treatment had also good discriminant power, but were rejected because they did not get good values in all the three treatments. The PCA rendered three principal components, and the two with the highest eigenvalues passed to the clustering analysis (Table 8.4). Although PRIN2 was lower than 1, we included it in the analysis because it contributed with an appreciable proportion of the total variance. PRIN1 represented one variable related to both good velocity and linearity, whereas PRIN2 represented low velocity (VCL), but even better linearity than PRIN1.

We obtained four clusters in each treatment, which were called CL1b, CL2b, CL3b and CL4b, plus an extra cluster in the post-thawed treatment, CL5b. Considering their characteristics (Table 8.5), CL1b and CL2b were similar to CL1 and CL3, respectively. CL3b and CL4b were similar to CL2, sharing good linearity, but CL3b was faster and had higher ALH. Moreover, the evolution of the proportions of CL1 and CL2 compared with CL1b and CL3b+CL4b, respectively, were very similar. However, CL2b behaved differently than CL3, peaking in the pre-freezing treatment instead. The positions of each cluster in the space defined by the two principal components (Figure 8.5) coincided in the three treatments, and it was possible to carry out an interpretation similary to the one in the first analysis. CL1b and CL3b were mainly defined by PRIN1 (slow and non-linear vs. rapid and linear), and CL2b and CL4b by PRIN2 (rapid and non-linear vs. —comparatively— slow and linear). CL5b could be considered as a residual subpopulation, considering its low proportion and that its position coincides with part of CL3b in the other treatments. The proportion of each subpopulation included in PSP was similar to the numbers reported for the first clustering analysis, CL3b and CL4b being almost completely included. Although CL3b drop from above 90 % to 87 % in the pre-freezing treatment, this decrease was less important that the one undergone by CL2. Regarding RSP, it comprised totally both CL2b and CL3b totally. There were interesting variations between treatments considering CL4b, being its proportions in RSP being 71, 39 and 25 %, for the initial, pre-freezing and post-thawed treatments, respectively. This matched the decrease of the mean values of VAP throughout the treatments, such as showed in Table 8.5. CL1b also underwent also a consequent but less dramatic variation (12, 17 and 14 %, for each treatment).

8.4.4 Study of sperm "freezability"

Table 8.6 shows the values for "freezability" parameters and the proportions of the subpopulations obtained in the first part of this study (pre-freezing values) for each group of males. TMdiff, Viabdiff and ACRdiff were introduced in the PCA, which rendered two principal components (eigenvalues 1.38 and 1.06), taking account of 0.46 and 0.35 of total variance for each. The eigenvectors of the three "freezability" variables, for PRIN1 and PRIN2 respectively, were: TMdiff, 0.06 and 0.93; VIABdiff, 0.70 and -0.30; and ACRdiff, 0.72 and 0.20. In this case, PRIN1 was clearly related to viability and acrosomal status, whereas PRIN2 was mostly related to motility, and, to a lesser degree, to viability (inversely) and acrosomal status. The classification of the males depending on "freezability" parameters is shown in Figure 8.6. According to the interpretation of the principal

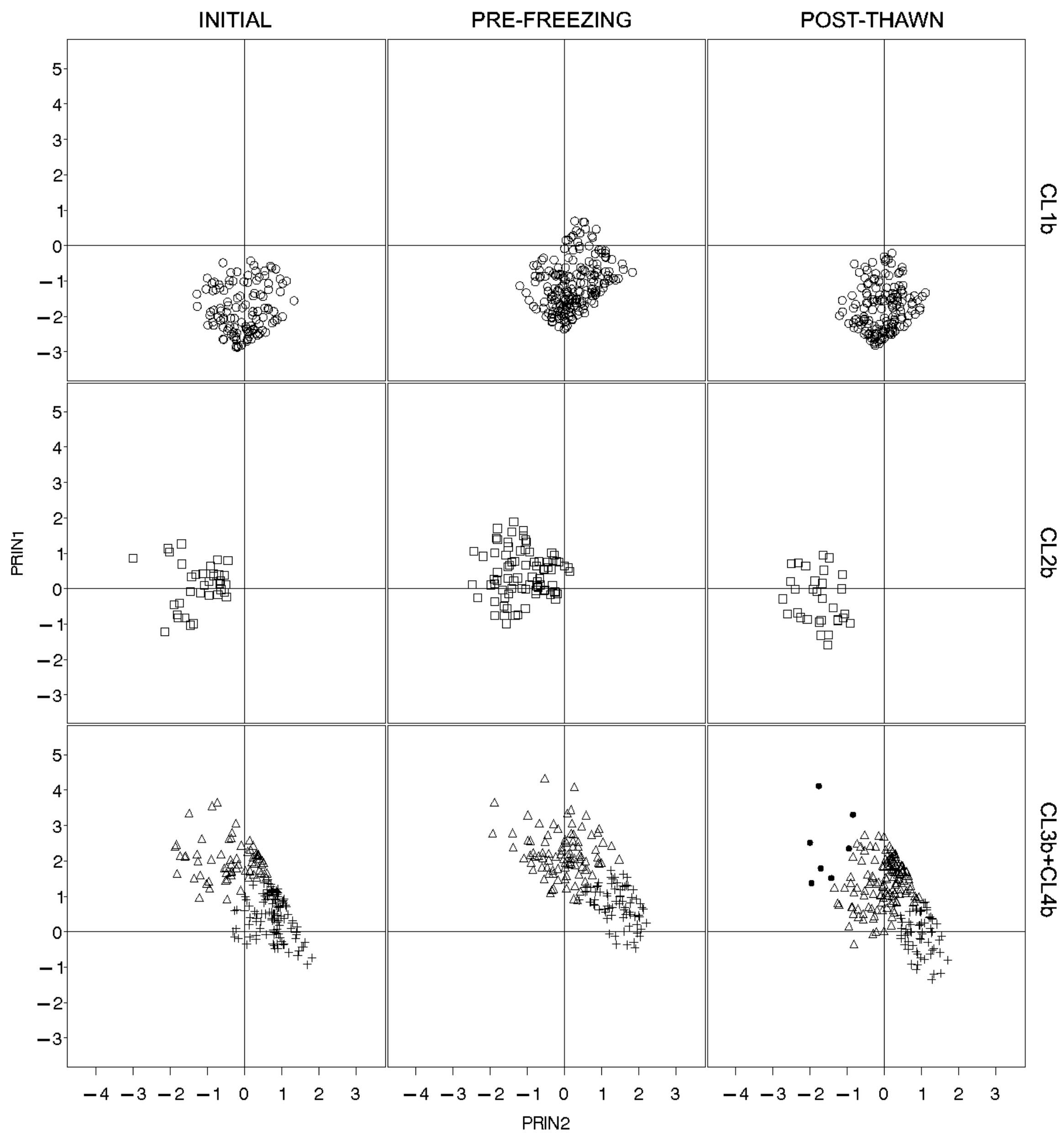
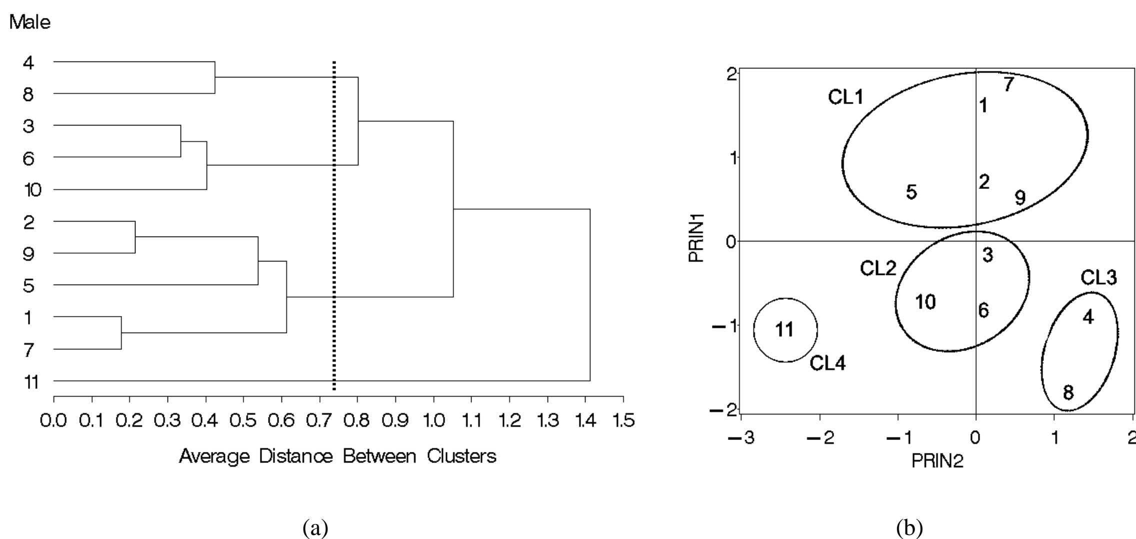


Figure 8.5 Clusters obtained after the discriminant analysis. Dot plots of the motility data defined by the two first principal components (PRIN1 and PRIN2). Data from the initial, pre-freezing and post-thawed analysis are presented from left to right. Each event (only 20–40 % are shown, in order to avoid cluttering) represents one individual spermatozoa. Events are represented by different symbols in order to identify its membership to each cluster (CL1b: ○; CL2b: □; CL3b: △; CL4b: +; CL5b: •).



(a) (b)

Figure 8.6 Clustering of the males depending on the "freezability" of their sperm samples. The males are represented by different numbers in the multidimensional space defined by the two first principal components extracted from PCA (PRIN1 and PRIN2). Ellipses indicate belonging to a concrete cluster.

components, cluster 1 would contain the samples that better resisted cryopreservation (its location, with positive PRIN1 values suggested better VIABdiff and ACRdiff), whereas cluster 2 represented samples with worse (lower) VIABdiff and ACRdiff. TMdiff seemed little affected in both cases, since these clusters have low absolute values for PRIN2. On the other hand, cluster 3 was characterized by bad VIABdiff and ACRdiff, but good motility recovery, because of PRIN2 positive values. Cluster 4 represented an interesting case, because its position indicated bad motility and acrosomal status recovery, but, because of VIABdiff negative eigenvector for PRIN2, viability recovery would be better than those of clusters 2 or 3. In fact, as shown in the dendrogram, male 11 may be an outlier, which would be removed from the analysis as more males were included in the study.

Interestingly, clusters 1 and 4 seemed to have a higher CL2 (rapid and linear) pre-freezing proportions, whereas clusters 2 and 3 had higher CL1 (slow and no linear) pre-freezing proportions (due to the low number of males, no significance test were performed). Cluster 4, with the poorest TMdiff and ACRdiff, had the highest CL3 (rapid and no linear) proportion. PSP and RSP proportions did not show any apparent relation with better or worse recovery of 'freezability' variables. The correlation analysis rendered a significant correlation between CL2 and VIABdiff (r=0.71, P=0.015). No significant correlations were found between the other parameters. However, the sum of CL3b (very rapid and linear) and CL4b (rapid and linear) (considered roughly equivalent to CL2) also correlated with VIABdiff (r=0.81, P=0.003).

Table 8.6 Description of the clusters obtained in the "freezability" clustering analysis (Figure 8.6). We have indicated the number of males included in each cluster, and the Mean±SD of the "freezability" parameters (TMdiff, VIABdiff and ACRdiff), total motility (TM) and the percentages of the previously studied subpopulations (CL1, CL2, CL3, PSP and RSP).

Cluster	Males	TMdiff	VIABdiff	ACRdiff	TM	CL1	CL2	CL3	PSP	RSP
1	5	-3.3±6.6	-16.3±3.5	-2.7±0.9	81.6±5.9	38.7±12.6	51.0±8.4	10.3±10.5	30.9±7.8	28.3±17.2
2	3	-6.1±5.5	-22.1±4.4	-5.5±1.0	80.4±3.8	46.3±7.7	45.8±3.7	7.8±4.8	21.4±2.9	48.1±16.4
3	2	6.9±1.2	-25.6±1.3	-6.9±2.9	76.5±0.0	65.1±19.6	25.9±6.9	9.0±12.7	25.3±7.1	34.2±4.2
4	1	-22.3	-17.4	-9.1	87.2	24.6	53.8	21.5	20.4	46.4
Total	11	-4.0±9.1	-19.7±4.9	-4.8±2.5	80.8±5.0	44.3±16	45.3±11.6	10.4±8.9	26.3±7.3	36.4±16.1

8.5 Discussion

8.5.1 Considerations on the statistical procedure

Multivariate clustering methods for the study of CASA data are not of general usage in sperm work. However, a review on articles that apply this kind of analysis show a number of interesting findings from this kind of analysis. We can also appreciate that different authors have chosen a great variety of combinations of different statistical test. For instance, Davis et al. [27] carried out a multi-step iterative procedure, combining the k-means model with multivariate discriminate analysis. On the other hand, Holt [6] compared a non-hierachical clustering method (k-means model) with two hierarchical ones (UPGMA and WPGMC). However, these authors compared these methods, but did not use them sequentially, such as in the present work. Abaigar et al. [22] utilized a different approach, using the PATN software. This software package provides many statistical tools which allow to extract and displaying patterns in multivariate data. These authors performed a non-hierarchical clustering carried out using non-parametric algorithms, its output was redirected to a hierarchical clustering analysis (UPGMA), and the final number of clusters was selected with the help of the FUSE module of the statistical package. To facilitate interpretation of data, they completed the analysis performing principal coordinates analysis and principal axis correlation, which allowed them to present multivariate data in a simpler format. This statistical package and a similar statistical procedure has been followed in other articles [24, 25, 34].

Other authors have followed simpler methods, which have provided useful information, though. Quintero-Moreno et al. [7, 28] selected the motility descriptors that should pass to the clustering step by means of applying the VARCLUS procedure of the SAS package, which divides a set of numeric variables into either disjoint or hierarchical clusters. This classification, enables one to select the most representative parameters for the data to be studied. After selecting the most representative parameters, they performed a non-hierarchical cluster analysis using the k-means model (FASTCLUS procedure), obtaining the corresponding clusters.

The main objective of this work was to combine several statistical procedures in order to extract several clusters (sperm subpopulations) from CASA data. We based it on the cited studies, specially the one by Abaigar et al. [22]. However, we intended to develop a protocol that would not depend on one specific type of software, but could be easily implemented elsewhere (SPSS, Statistica, S/STAT, R, etc). Thus we have given a detailed description of the SAS procedures that we utilized. Since information on this statistical package is widely available [33], anybody with some knowledge of statistics should be able to apply the described analysis to their own statistical software. Firstly, we had to decide which clustering analysis to use. Taking into account the huge amount of data that a CASA analysis can produce, we decided to perform the clustering in two steps: a previous non-hierarchical clustering, which would reduce the data to a relative small number of clusters, followed by a hierarchical clustering step. Holt [6] recommended hierarchical clustering methods to the non-hierarchical k-means model, because the former provide more information and allows the clustering process history to be thoroughly studied (in order to select the final number of clusters, for instance). Moreover, the use of the k-means model implies a previous knowledge of the clustering structure, since the final number of clusters must be provided before performing the analysis (although some statistics can be used to test the efficacy of the clustering procedure). A good reason for the use of a non-hierarchical method as an intermediate step is the detection of outliers, as explained in Material and Methods.

On the other hand, an important issue is the selection of the motility descriptors that may enter in the clustering analysis. Quintero-Moreno et al. [28] pointed out that most motility descriptors are often highly correlated, and that the relative importance of these parameters may vary between species. In fact, these authors used the VARCLUS procedure to select the variables that would enter in the clustering analysis. Another problem is the need of transforming motility parameters prior to the clustering analysis, at least by performing a standardization step. Otherwise some descriptors would outweigh other because of different scales. It is important to note that Abaigar et al. [24] overcame this problem without having to of transforming their data because they used non-parametric algorithms in their non-hierarchical clustering method. Our proposal for resolving these issues was to perform a principal component analysis before carrying out any clustering analysis. We thus converted a number of unstandardized, highly correlated parameters into few variables, representing linear combinations of the former parameters, standardized and uncorrelated. This analysis not only serves to simplify the interpretation of our data, because of the parameter reduction, but also provides abundant information that can be useful for the interpretation and representation of the results of the clustering analysis. We also carried out a discriminant analysis, from which we selected a subset of variables that were used successfully in the second clustering analysis. In fact, results obtained with that subset even provided more detailed information on some aspects, indicating the suitability of this kind of analysis when performing in-depth studies with CASA data.

Another fundamental issue in the clustering analysis is the selection of the final number of clusters obtained from the analysis. The study of the cluster distances and the dendrograms produced

by hierarchical cluster analysis can greatly help in this step, especially when we have a previous knowledge of the internal structure of our data. The method used in this work, based on the evolution of pseudo F, CCC and pseudo t^2 statistics in the clustering process, has many limitations (it works better with compact or slightly elongated clusters [33]), but provides an objective and flexible way to determine the final number of clusters. Although many studies analyze significant differences amongst the mean values of clusters, these results should be carefully considered, especially if the analyzed variables were those that entered in the clustering analysis. The reason is that clustering algorithms intend to maximize the variability between clusters, thus significant differences are not surprising, but rather expected. Significant differences amongst clusters may be used as an aid to explain clusters characteristics or, such as in this study, to showing the differences between the clusters and other groups of motile sperm obtained by different means.

8.5.2 Subpopulation structure and cryopreservation effects

The clustering analysis allowed us to obtain three distinct subpopulations. It may be interesting to test whether CL3 or CL2b (rapid and no linear spermatozoa) contained spermatozoa with some kind of hiperactivation, but unfortunately the acquisition conditions (chamber only 10 μm deep) were not adequate to perform such an analysis. It is important to point out that we obtained the same number of subpopulations in the three treatments, in both analyses, and, although mean values were different for some parameters, their general characteristics remained similar. This indicates that the internal structure of the CASA data was mostly preserved, undergoing few changes through the processing and freezing-thawing. Interestingly, a similar pattern was obtained by Holt [6] in boar sperm after applying a hierarchical clustering method. However, other reports indicate different characteristics, especially considering sperm progressivity. This may have happened because of different clustering techniques, different species, and different sources of sperm. However, there is a general coincidence on the number of subpopulations (three or four, in general). Quintero-Moreno et al. [28] highlighted this coincidence between studies, and suggested that this pattern could be a widespread fact.

On the other hand, the source of spermatozoa could be a very important factor regarding differences between works, since epididymal and ejaculated spermatozoa are different in many senses. Although sperm from the cauda epididymis is almost analogous to ejaculated semen, it has not had contact with seminal plasma and its diverse factors, which are known to alter many characteristics of sperm [35, 36]. For instance, Holt [6] and Abaigar et al. [22] represented the location of the different clusters in plots in a multidimensional space defined by canonical or factor variables, where the clusters appeared mostly as clearly separated groups, whereas in our study it was difficult to visually separate the cloud of points defined by PRIN1 and PRIN2 in well-defined groups. The lack of separation between clusters may be a characteristic of epididymal sperm, although we need to compare with ejaculated deer sperm in order to confirm this.

One interesting fact in our data is the higher proportion of motile sperm in thawed samples when compared with the initial sampling. This may seem surprising if we do not analyze the pre-freezing

sampling. In order to explain the variations in the motility characteristics in the three treatments, we have to consider many factors, the most important being the source of sperm. The samples (still in the cauda epididymis) arrived at our laboratory after being stored many hours at 5 °C, and the first evaluation of motility took place in a relative simple medium. Our experience with epididymal sperm has showed us that spermatozoa recently salvaged from the cauda epididymis generally are slower, compared with those from ejaculates, and frequently many of them just present a weak the tail beat, with very slow displacement or none at all, and are therefore not detected as motile by CASA. Only after warming the sample for some time (generally from 20 to 40 min) did we obtain a stable and representative motility pattern that could be reliably measured with the CASA equipment. The situation was very different for the pre-freezing samples, possibly because the sperm had been diluted with the extender. This is a more complex medium including egg yolk, which is known to alter sperm motility [37–39]. Epididymal sperm may undergo some changes when in contact with the extender, activating the motility of some sperm [40], and therefore altering the characteristics and proportions of the subpopulations, as we have observed. In fact, the increasing in the proportion of CL1 (slow, non-linear sperm) may be due mostly to the activation of spermatozoa that were not detected as motile in the initial sampling, rather than the conversion of CL2 (rapid and linear) or CL3 (rapid and no linear) spermatozoa to CL1 spermatozoa. Furthermore, after being in contact with the extender, long warming times were not necessary anymore, and motility could be acquired at 5 or 10 min of warming time with no difference. Nevertheless, we kept 20 min as warming time, in order to respect the initial protocol, and considering that there was no alteration with regard to shorter times.

The discriminant analysis and the subsequent clustering analysis showed that it is possible to reduce the number of initial variables and still obtain a good subpopulation study. Still, the similarity of the subpopulation structure of both analysis (with the exception of the different number of subpopulations) suggest that most of the motility descriptors were redundant and may be removed from the analysis without problems. However, the use of more complex descriptors, such as those based on hyperactivation, angular and oscillation parameters, may improve this kind of study. However, the correct analysis of these parameters implies certain conditions that we were unable to achieve, such as sufficient chamber depth to allow the free movement of spermatozoa [41]. In the second part of the study we obtained even more extensive information. For instance, assuming that CL3b+CL4b (very rapid and linear, and rapid and linear) were equivalent to CL2 (rapid and linear), that means that there were some variations in the internal structure of CL2 between different treatments. That is, CL3b was stable considering its motility parameters, but the mean velocity of CL4b dropped in the pre-freezing and post-thawed treatments when compared to the initial one, which was not evident in the mean values of CL2. The reason was that, in the pre-freezing treatment, a considerable part of CL2b (rapid and non linear) was included in CL2, and, in the post-thawed treatment, CL3b prevailed over CL4b. Although we considered CL2b equivalent to CL3 (rapid and non linear), it had a more complex nature, since in the initial treatment many CL3 members (the most linear ones) were included in CL3b, and in the pre-freezing treatment, as noted before, CL2b included not only CL3,

but also part of CL2.

It is noteworthy that, without considering sperm subpopulations, all this interpretation would have not been possible. In fact, the inclusion of PSP (progressive) and RSP (rapid), although not as informative as the subpopulations derived from cluster analysis, provided some useful information that is not obtained when only the mean values of motility descriptors are considered. These subgroups have the advantage that they can be readily extracted from the CASA data. However, clusters provided more extensive results, and there was not a clear identity between them and PSP or RSP. On the other hand, the definition of "progressive" or "rapid" spermatozoa allowed to deepen the internal structure of the clusters.

Considering the "freezability" analysis, both sperm viability and acrosomal status were clearly affected by the cryopreservation process, since these parameters are highly dependent on the status of sperm membranes. The clustering of the males according to our "freezability" parameters grouped effectively those samples with similar ability for maintaining these parameters after thawing. On the other hand, the correlation between the fre-freezing proportion of CL2 and VIABdiff suggested some kind of relationship between the predominance of a sperm subpopulation and the outcome of the cryopreservation process. This seemed to be further supported by the correlation found between VIABdiff and the sum of the pre-freezing proportions of CL3b and CL4b. The lack of correlation with CL3b or CL4b separately, but with CL3b+CL4b, may indicate that the presence of a subpopulation with linear spermatozoa, irrespective of its velocity, may be related to good post-thawed recovery of sperm viability. Unfortunately, our limited data did not allow us to present a thorough analysis on this issue, including the relationship of subpopulations with fertility. Anyway, this was not one of the objectives of this study, which were aimed at applying the studied statistical methods and describing the subpopulations obtained. However, further studies should be carried out to test the real meaning of these subpopulations in relation to fertility, as suggested by other authors [27]. In this sense, Quintero-Moreno et al. [28], working with stallion sperm, found that ejaculates with high fertility shared a special subpopulation pattern. Nevertheless, the same authors [7] did not find such a relationship when studying boar semen. The exact nature of sperm subpopulations and their influence on the fertility of a sample may be a complex issue, and the use of different CASA protocols and statistical analysis may complicate the comparison between different studies.

8.5.3 Conclusions

In conclusion, we have applied a statistical method that enabled us to find sperm subpopulations defined by motility parameters. By examining the obtained subpopulations, we were also able to determine some characteristics of red deer epididymal sperm, and allowed us to study the variations it underwent through a cryopreservation protocol. One interesting fact was the conservation of the subpopulation pattern between the different treatments. We could relate one of these subpopulations, characterized by rapid and linear spermatozoa, to good post-thawed viability recovery. The study of two motility subgroups defined by us independently of the clustering analysis, one of rapid and

other of progressive sperm, helped to obtain useful information on the internal composition of the clusters. This study was necessarily limited, including the lack of fertility data, but the clustering analysis gave interesting information not available using conventional motility analysis. Due to the possibilities of this kind of analysis, we consider that the study of sperm subpopulations defined by motility descriptors should be widely considered, especially when including fertility results.

8.6 Acknowledgements

This study has been supported by Junta de Castilla y León (AB29). The authors thank Carlos Bernabé (Cáceres, Spain) for their collaboration in the collection of the samples used in this work; and Ana Rocío Díaz, Enrique Anel, Juan Daniel Muro, and Patri Martínez for their help in the processing of the samples.

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Capítulo 9

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

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Enviado a Biology of Reproduction el 22 de septiembre de 2004

9.1 Abstract

We have studied the effect of postmortem time and season in 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 postmortem (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. Postmortem time caused an important decrease of motility quality, and a general deterioration of subpopulation characteristics. We found three subpopulations the first day, and the one indicating good aperm quality decreased with postmortem time until it dissapeared on the fourth day. This may indicate important impairment of the samples after 72 h postmortem, which could compromise its use in AI programs. Considering 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 postmortem sperm recovery.

Keywords: postmortem changes, seasonal variations, epididymis, sperm subpopulations, cluster analysis, red deer

9.2 Introduction

The use of germplasm banks has allowed to preserve the genetic wealth of many domestic and wild species, and nowadays there are both economical and conservational reasons to create and develop them [1]. Therefore, the set-up of such banks must be carried out with great care, specially when it is aimed to keep material from wild species, because there are many factors that complicate the acquisition and use of the germplasm. In this case, sperm recovered postmortem from the cauda epididymis has become a widely used source [2], since collection by other means is often very difficult or unaffordable. Furthermore, 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, postmortem time becomes a factor which must be taken into account. Many studies have considered this issue, concluding that epididymal sperm is clearly affected by the time elapsed from the death of the animal to its cryopreservation (for instance, [3–6]). There is a general agreement that few hours are enough to impair epididymal sperm, although its quality can be acceptable for several days. Thus, postmortem 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 to domestic species. This phenomenon is due to complex hormonal interactions, ultimately based on daylenght, which differentiate 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 in the characteristics of epididymal sperm must be assessed in order to determine if samples of a concrete season are adequate for storage and future use.

At the moment of the writing of this paper, we have carried out two studies on epididymal sperm from red deer, roe deer and chamois [14, 15], regarding the effect of postmortem time and seasonality, respectively. The present study was aimed to complement 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 within our samples, in function of sperm motility.

It is widely accepted that sperm samples are not homogeneous, and that it is possible to identify sperm subpopulations depending of different parameters [16–20]. The use of motility descriptors for performing such studies has rendered interesting information, not evident if using only the mean values of the whole motile population [21–24]. In fact, motility subpopulations may act as markers for good or bad quality of the sample [25], and some authors have found relationships between the presence of determinate subpopulations and the fertility of the sample [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 performing other kind of analyses.

Another motivation for carrying out this work is the interest for the species we studied. Iberian red deer is a valuable trophy in Spain, and there are plans to establish germplasm banks for this and other wild ruminant species. These species are highly seasonal (including a period of complete reproductive arrest), and the period of time from hunting to sample processing (postmortem time) can be highly variable. Thus, we considered to perform this kind of study in order to provide some information that might be useful for the management of any future germplasm banks of these or related species.

9.3 Material and methods

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

9.3.1 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 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, 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 postmortem.

Sample manipulation was carried out in a walk-in fridge (5 °C). Testicles with epididymes and vas deferens attached were isolated from scrotum and other tissues. Epididymes were dissected free from the testicles, and cleaned of connective tissue. To avoid blood contamination, superficial blood vessels were previously cut, wiping its content. Season and postmortem time were attached to each sample. Sperm was 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.

9.3.2 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 minutes. Then, a pre-warmed Makler counting chamber (10 μm depth) was loaded with 5 μL of sample. The CASA system consisted on an optical phase contrast microscope (Nikon Labophot-2, equipped with negative phase contrast objectives and a warming stage at 37 °C), a Sony XC-75CE camera, and a PC with the Sperm Class Analyzer software (SCA2002, Microptic, Barcelona, Spain). The magnification was $\times 10$. All samples were analyzed at least twice, in order to discard errors due to incorrect sampling. At least 5 fields per sample were acquired, summing at least 100 motile sperm. Image sequences were saved and analyzed afterwards. CASA acquisition parameters were: 25 images acquired, at an acquisition rate of 25 images per second. For each sperm analyzed, the SCA2002 rendered the following data: VCL (velocity according to the actual path; μm/s), VSL (velocity according to the straight path; μm/s), VAP (velocity according to the average —smoothed— path; μm/s), LIN (linearity; %), STR (straightness; %), WOB (wobble; %), ALH (amplitude of the lateral displacement of the sperm head; μm), and BCF (frequency of the flagellar

beat; Hz). Detailed explanation of these descriptors of sperm movement is provided elsewhere [25, 27–29].

9.3.3 Data preparation and statistical analysis

Firstly, CASA data was pooled in a common database. Each observation (spermatozoa) was identified by three fields (male, postmortem time and season). After determining the total motility of the samples (TM: % of spermatozoa with VCL>10μm/s), only samples with TM>10 % were used in the rest of the analyses. This was decided in order to avoid bias due to the presence of a lot of samples from different males which contributed with very few motile sperm to the database each. After that, the database was duplicated, and each copy was utilized either for postmortem time analysis or for season analysis, and modified accordingly.

In order to study both factors, we had to decide previously the periods we were to divide postmortem time and season. Since we knew that both postmortem time and seasonality affected sperm quality, we had to filter each database, in order to prevent interfereces. First, we determined the seasonality periods. We only could obtain samples during the hunting season, which in our case was limited to the autumn. Therefore, we divided the atumn in several periods, depending on the breeding activity of Iberian red deer. According to wardens' records, rut comprised from the end of summer to mid-autumn, although mating could still be observed until winter, but with decreasing frequency. Thus, and also taking into account other previous observations [15], we decided to consider the second fortnight of September and the first fortnight of October as the rut season, the second fortnight of October and November as the transition period, and the first fortnight of December as the post-rut period.

Taking this division into account, for studying the effect of postmortem time we used data only from the rut period. This data was divided in one-day intervals (we could not get any sample below 19 h postmortem, thus the first interval included only 19–24 h postmortem), resulting in 4 intervals of 24 h each. Samples with higher postmortem times (>96 h) were discarded because of very bad quality (generally almost no motility).

On the other hand, only samples with postmortem times from 19 to 30 h were accepted for the study of seasonality. We determined that this period would be adequate, because it included a fairly large number of samples, and variation due to postmortem time would be acceptable, according to a previous study [14]. Then, samples were assigned to the three periods defined as rut, transition and post-rut.

All statistical analyses were carried out using the SAS/STAT^{TM} package V. 8 (SAS Institute, Cary, NC) [30]. Where applicable, P<0.05 was considered as statistical significance, unless otherwise stated. The clustering analysis we followed has been extensively described in a previous study [31], thus we will summarize it here (SAS^{TM} 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)

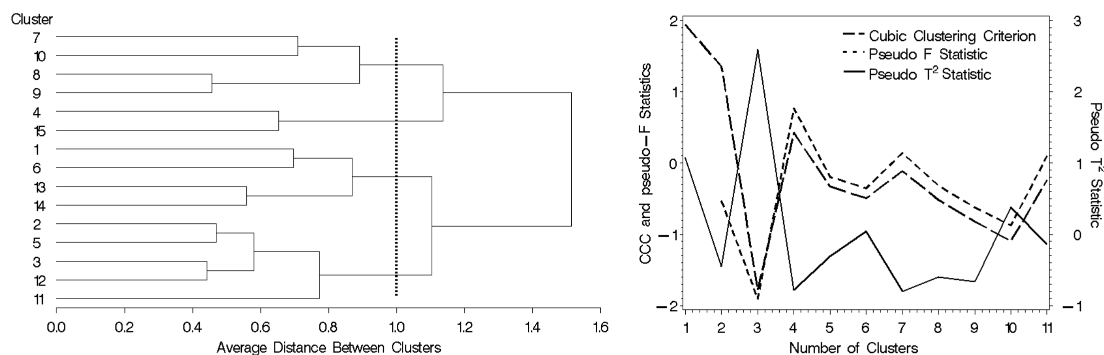


Figure 9.1 Example of a dendrogram derived from the hierarchical clustering analysis, and of the plot used for the determination of an adequate final number of clusters. The plot on the left shows the dendrogram resulting from the hierarchical clustering of the 15 clusters derived of the PCA and subsequent non-hierarchical clustering of motility data (this example corresponds to the]48–72] h interval of the postmortem 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 4 clusters (indicated in the dendrogram), although 2 or 7 would be also eligible.

were used in the subsequent analysis in the place 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 a 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. 9.1 shows an example of dendrogram and the corresponding plot used for choosing the final number of clusters.

A descriptive statistic of each cluster in each postmortem period or season, and the analysis of PCA allowed us to carry out a preliminary characterization. Therefore, clusters were compared between postmortem periods or seasons. For this comparison, we used the general linear models procedures (GLM). In the case of postmortem time, the factors included in the model were the postmortem period (days 1 to 7) and male nested within the postmortem period. In the case of season,

Table 9.1 Values of some motility descriptors depending on postmortem time and season. Data are expressed as adjusted least-squares means±SEM.

	Period	TM (%)[1]	VAP (μm/s)	LIN (%)	ALH (μm)	BCF (Hz)
Postmortem	[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}
time (hours)	]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}

[1] proportion of motile sperm (VCL>10 μm/s).

[a,b,c,d]: rows (within experiment, postmortem or season) with different superscripts differ P<0.05.

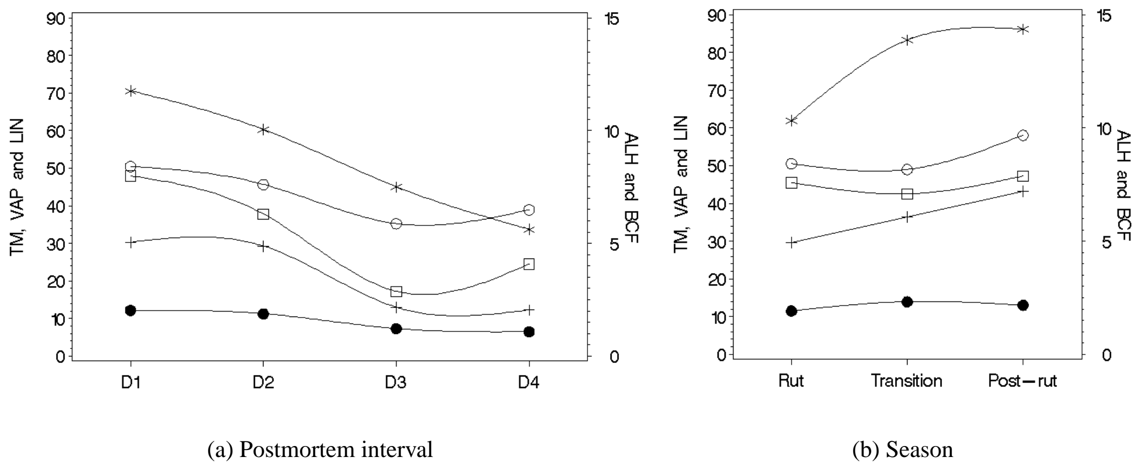
factors were the season (rut, transition and post-rut) and male nested within the season. When the model was significant for the postmortem period or the season, the respective classes were compared using adjusted least-squares means (LSMEANS statement). Previously, variables were transformed for normality (log: VCL, VSL, VAP, ALH, BCF; arc sine: LIN, STR, WOB). Comparisons of cluster proportions between postmortem 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.

9.4 Results

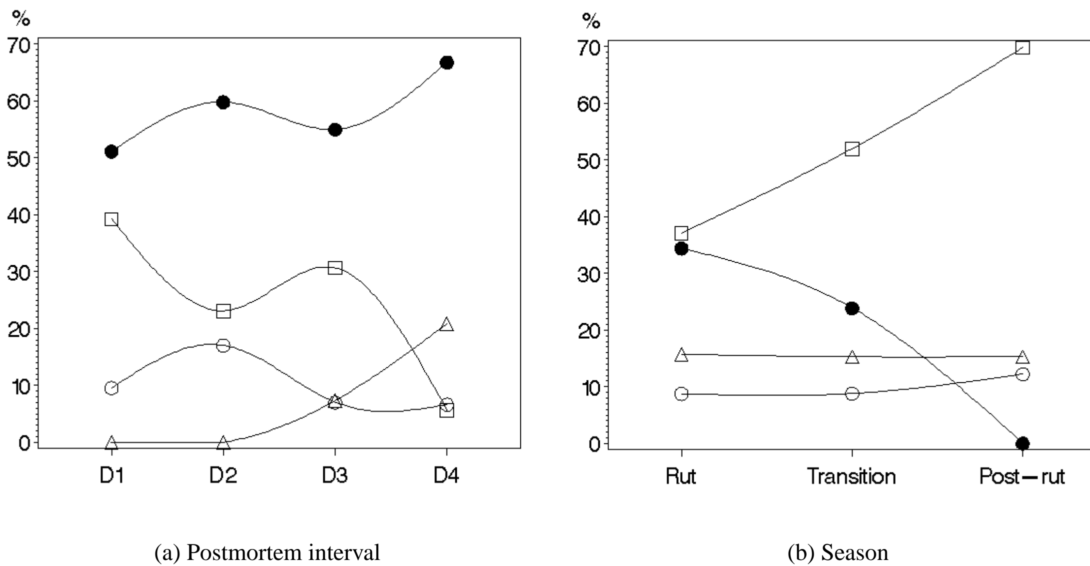
9.4.1 Sperm motility (general results)

Twenty-one samples complied the condition for enter the postmortem time study (collected during the rut season and no more than 96 h postmortem), and 39 samples (19 to 30 h postmortem) were used for the season study. As Table 9.1 and Figure 9.2 show, there were important differences between postmortem intervals and between seasons. The percentage of motile spermatozoa decreased clearly with postmortem time, being the fourth day only one half of the value of the first day. Considering motility descriptors, they underwent a similar evolution, although they seemed to drop more quickly just after 48 h postmortem and remain more stable afterwards. Regarding seasonality, transition and, specially, post-rut periods rendered higher results than rut, either considering the proportion of motile sperm or the motility descriptors.



(a) Postmortem interval (b) Season

Figure 9.2 Evolution of several motility parameters depending on postmortem time and season. For postmortem 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: $\ast$; VAP: $+$; LIN: $\bigcirc$; ALH: ●; BCF: $\square$.



(a) Postmortem interval (b) Season

Figure 9.3 Changes of the proportions of motility subpopulations depending on postmortem interval or season. For postmortem intervals, D1: [0–24] h; D2:]24–48] h; D3:]48–72] h; D4:]72–96] h. CL1: ●; CL2: $\square$; CL3: $\bigcirc$; CL4: $\triangle$.

Table 9.2 Values of some motility descriptors corresponding to the clusters obtained from the postmortem time analysis (CL1p–CL4p). Postmortem time is given in hours. Data are expressed as adjusted least-squares means±SEM.

Cluster	Postmortem time	%[1]	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^{ab}	8.32±0.53^{ab}
	]72–96]	5.7^{d}	30.55±5.59^{abc}	71.18±5.96^{abc}	1.66±0.25^{ab}	7.82±1.38^{ab}
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^{ab}
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

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

Data are expressed as percentages, and comparisons were performed using the χ^2 test on raw data.

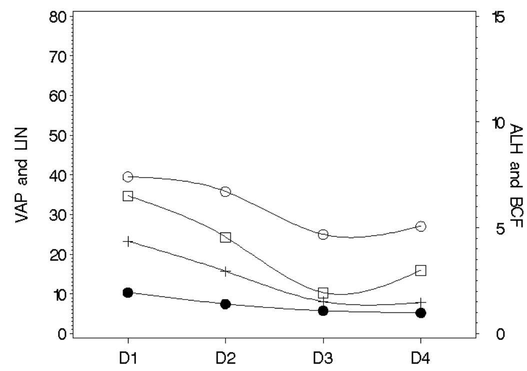
^{a,b,c,d}: rows (postmortem intervals within clusters) with different superscripts differ P<0.05.

9.4.2 Effect of postmortem time on sperm subpopulations

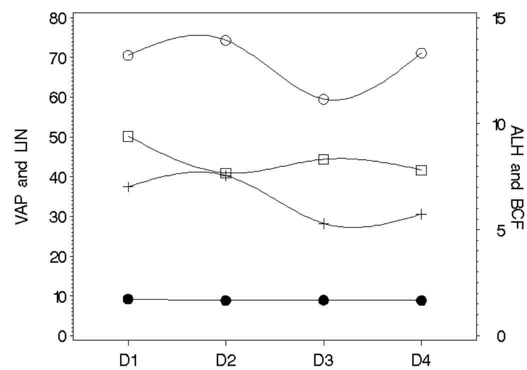
Cluster analysis rendered three clusters per postmortem interval, excepting for the fourth day, with four clusters. Considering their motility descriptors and the results of PCA, we concluded that there were four different kinds of clusters, which were called CL1p, CL2p, CL3p and CL4p. In general, cluster distribution varyed pronouncedly between postmortem intervals (fig. 9.3(a)). Statistics of the clusters are showed in Table 9.2 and Figure 9.4.

Subpopulation 1 (CL1p)

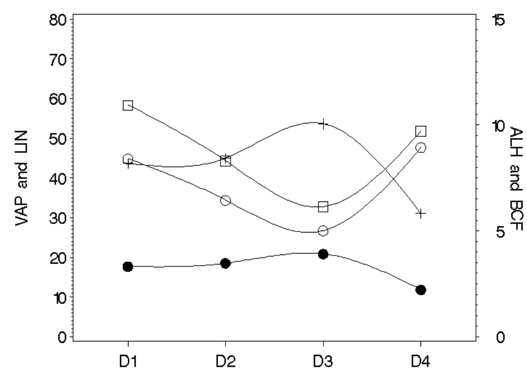
CL1p comprised slow and non-linear sperm, with low ALH and BCF values. All the motility descriptors were affected by postmortem time, although they stabilized with low values after 48 h postmortem (except for the increase of BCF). Considering its relative proportion, it increased from 51 % in the first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



(a) CL1p



(b) CL2p



(c) CL3p

Figure 9.4 Motility parameters of CL1p, CL2p and CL3p, depending on postmortem time. For postmortem 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: □.

Subpopulation 2 (CL2p)

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

Subpopulation 3 (CL3p)

CL3p showed the higher velocity, ALH and BCF values, but it was not as linear as CL2p. VAP and ALH were little affected by postmortem time (decreasing only in the last interval), but LIN and BCF decreased greatly in the]48–72] h interval, although they recovered their initial values in the last interval.

Subpopulation 4 (CL4p)

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

9.4.3 Effect of season on sperm subpopulations

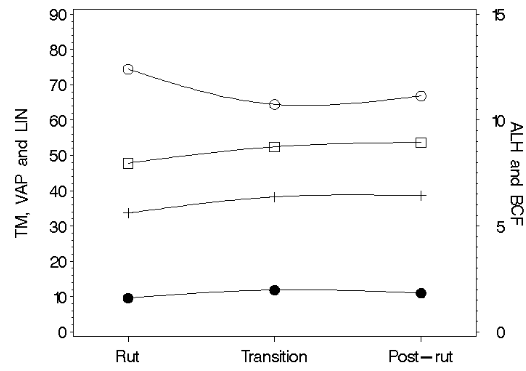
In this case, we found four different kind of clusters (CL1s, CL2s, CL3s, CL4s), unevenly distributed between seasons (Figure 9.3(b)). We obtained 6 clusters in the rut and post-rut periods, but two of them were removed, because we considered them residual (see below). Statistics of the clusters are showed in Table 9.3, and Figure. 9.5. The variation of the proportions of the different clusters between seasons can be appreciated in Figure 9.3(b).

Subpopulation 1 (CL1s)

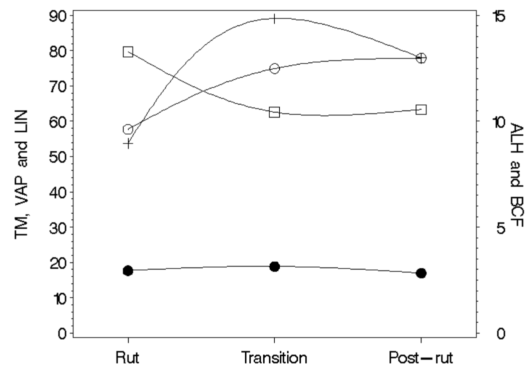
CL1s presented low velocity and linearity. It comprised one third of the spermatozoa in the rut, decreased to less than 25 % in the transition period, and it did not appear in the post-rut. In this case, VAP and ALH were higher in the transition period, whereas LIN and BCF were higher in the rut.

Subpopulation 2 (CL2s)

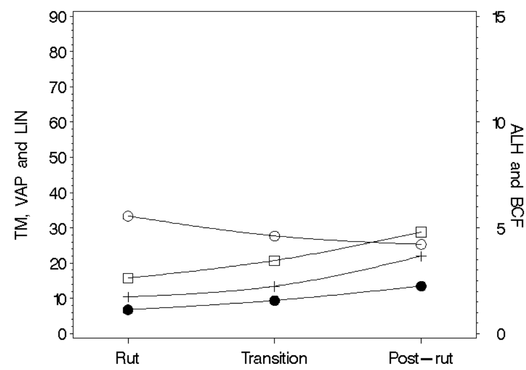
CL2s was characterized by medium velocity and high linearity, accompanied by low ALH and high BCF. Its presence was important in all the three seasons, increasing from more than one third of the motile sperm in the rut, to more than two thirds in the post-rut. Its parameters were also similar in the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



(a) CL2s



(b) CL3s



(c) CL4s

Figure 9.5 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: □.

Table 9.3 Values of some motility descriptors corresponding to the clusters obtained from the season analysis (CL1s–CL4s). Data are expressed as adjusted least-squares means±SEM.

Cluster	Season	%[1]	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

[1] proportion of each subpopulation respect to the total number of motile sperm (VCL>10 μm/s).
Data are expressed as percentages, and comparisons were performed using the χ^2 test on raw data.
^{a,b,c}: rows (seasons within clusters) with different superscripts differ P<0.05.

Subpopulation 3 (CL3s)

CL3s was similar to CL2s, fast and linear, with lower ALH and higher BCF. Thus, it contained mainly spermatozoa with very linear trajectories. It 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.

Subpopulation 4 (CL4s)

CL4s included slow and non-linear sperm, with low ALH and BCF. However, its VAP, ALH and BCF improved greatly in the post-rut. This subpopulation included above 15 % of spermatozoa in all the three seasons.

Other subpopulations

We found also other two subpopulations both in the rut and pos-rut intervals. Two of them shared some characteristics in the rut and post-rut (medium velocity, non-linear), but the other two were very different in the two periods (slow and non-linear in the rut and fast and linear in the post-rut). They also appeared in very low proportions (below 3 %). This suggested that these subpopulations were

in fact "outlier" clusters, due to grouping of spermatozoa with extreme values, which segregated in the clustering process as independent clusters. Therefore, we removed them from further analysis.

9.5 Discussion

It is well known that motility of epididymal sperm is deeply affected by storage time. Many authors [3, 5, 6, 32], working with different species, have reported that several motility parameters are altered as soon as few hours postmortem, and these changes were noticed before other characteristics, such as morphology or viability, seemed to be affected. In a prior study on the effect of postmortem time on epididymal sperm from Iberian red deer and roe deer [14], we found that the percentages of motile and progressive spermatozoa dropped just after the first 24 h postmortem (although we did not found significant differences before 48 h). In this work we have been able to confirm the decrease of motility with postmortem time, not only of percentage of motile sperm, but also in its quality (subpopulation pattern). We have to point out that our earliest sample was 19 h postmortem, and, ideally, we should recover and process the sperm as soon as possible, since some authors reported loss of quality and fertilizing ability within few hours [33, 34]. However, Kaabi et al. [6] did not find significant differences between the cleavage rates of oocytes fertilized with ram sperm, which had been salvaged from epididymis either <2 h or 24 h postmortem, although it was clearly lower when sperm was recovered at 48 h postmortem.

The subpopulation pattern that we have found in the first interval of the postmortem time study, could be explained considering sperm maturation. Many authors have focused on this subject [35–39], reporting that motility increases during the transit of spermatozoa through the different parts of the epididymes, gaining velocity and linearity. Furthermore, between the proximal and distal cauda there is a increase of linearity, but not of other parameters, due to biochemical changes related to epididymal maturation [37, 40]. Regarding the first interval, CL2p (medium velocity and linear) would represent a subpopulation containing mature spermatozoa, whereas CL1p (slow and non-linear) would be formed mainly by spermatozoa which had not complete their epididymal maturation. Moreover, CL3p (rather rapid, but little linear) would contain mature spermatozoa undergoing some kind of activation, considering its high VAP, BCF and ALH, and its lower LIN. In fact, Yeung et al. [40] found that and increase of cAMP was the cause of the maturation of epididymal sperm, but it also induced hyperactivation in already matured spermatozoa.

The increase of CL1p with postmortem time, and its decreasing quality, reflects the effect of postmortem time on spermatozoa. Not only the general proportion of motile spermatozoa and its quality decreased, 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 of CL2p and increase of CL3p in the]24–48] h effect seemed to be a consequence of individual variation, rather than an actual consequence of postmortem time. This may also be the cause of the alteration of VAP and LIN of CL2p and CL3p in]48–72] h. On the other hand, the drop of CL2p in 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 that drop may suggest that such a long postmortem time may impair the samples so notably that salvaging would not be worthy. However, it would depend on the sample (non-endangered vs. 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 apparition 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. It is possible that a noticeable presence of this subpopulation indicates bad quality in a sample, in the same sense that CL2p might indicate good quality.

Abaigar et al. [23] carried out an experiment on Mohor gazelle (*Gazella dama mhorr*), based also on the analysis of sperm subpopulations. Part of it consisted in the study of the variation of the subpopulations pattern, depending on storage time (from 0 to 96 h). It is difficult to compare our results with those of these authors, since they worked with different species, and used ejaculated semen (with very high velocities and low linearity, comparing with our samples). However, they also found that some populations underwent rises and decreases on their proportions through the storage period. These authors connected the variations in the proportions of the subpopulations to functional changes associated to prolonged storage, which is in agreement to 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 on 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 referrences 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. Rodríguez and Bustos Obregon [43] carried out a study on stallion semen, obtained both during 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 on hypermaturation, which possibly caused faulty male pronuclear formation. In our case, hypermaturation would reveal 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 estrus during the rut season, but this event is less frequent thereafter, because most of

the females are already pregnant. Therefore, a male has less opportunities to mate after the rut. Rapid renewal of epididymal sperm during the rut occur also to non-dominant males, not only because they might mate, but also because deers ejaculate during the rut 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 in "fresh" spermatozoa, which would complete their maturation at the moment of contact 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, therefore, would be composed mostly by long-stored spermatozoa, which would have been exposed to the epididymal environment for a long time. Hypermaturation may become apparent in the form of more motile sperm, with higher motility descriptors than during the rut, but this increase of motility quality may not translate into 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 with little changes. In addition, CL1s (slow and non-linear) may comprise spermatozoa that have still not achieved mature motility. In fact, not only CL2s increased after the rut, but it did at almost at the same pace 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, translating into 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 along 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 in motility data greatly improved the interpretation of motility changes between postmortem intervals and seasons. Our results showed that the subpopulation pattern was deeply altered with postmortem time, and the quality of the samples was severely compromised after several days postmortem (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 of the fertility of the samples, and could even indicate worse sperm quality. The results of this work are based in 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.

9.6 Acknowledgements

This study has been supported by Junta de Castilla y León (AB29). The authors thank Juan José Martínez, César Gómez, Juan Carlos Peral, the Territorial Service of Environmental Affairs of León, and the gamekeepers of the hunting reserves of Picos de Europa, Mampodre and Ancares (León, Spain), and Carlos Bernabé (Cáceres, Spain) for their collaboration in the collection of the samples used in this work; and Vanesa García, Mercedes Álvarez, Juan Daniel Muro, and Patri Martínez for their help in the processing of the samples.

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Capítulo 10

Comparison of two methods for obtaining spermatozoa from the cauda epididymis of Iberian red deer

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Enviado a Theriogenology el 4 de octubre de 2004

10.1 Abstract

We have compared two methods for salvaging epididymal sperm from postmortem samples of Iberian red deer. Of each pair of testicles, one cauda epididymis was processed by means of cuts (sperm was immediately diluted with extender) and the other was detached from the corpus and flushed from the vas deferens with 1 ml of extender. Sperm was processed for cryopreservation, and analyzed just after recovery, pre-freezing and post-thawed. Total spermatozoa recovered, contamination (concentration of epididymal cells and red blood cells-RBCs) and quality (motility by CASA, and acrosomal status, viability and mitochondrial status by flow cytometry) were used to compare both methods. The number of recovered spermatozoa was similar for both methods. Contamination was higher for the cuts method, but when considering the final dilution before freezing, only RBCs concentration was significantly higher. Motility was similar just after extraction, but higher for flushed sperm both pre-freezing and post-thawed. Pre-freezing acrosomal status ($P<0.05$) and viability ($P<0.1$) were better for flushing; however post-thawing results were similar for the two methods. A clustering analysis using CASA data showed that the subpopulation pattern of motile sperm was different depending on the method, being better for flushing. Considering yield, lower contamination (especially RBCs) and, in general, better quality results, flushing seems to be a more recommendable method for postmortem sperm recovery. Cuts method may be more practical in some occasions, but care must be taken in order to achieve quick extension of the sample and avoiding contamination in order to improve sample condition.

Keywords: red deer, post-mortem recovery, contamination, epididymal sperm, method comparison

10.2 Introduction

Germplasm banks have arisen as one of the most interesting means to achieve conservation of biodiversity, endangered due to habitat destruction and human activity [1]. However, gamete collection has still to overcome many issues related to the characteristics of the species involved, thus the techniques available in domestic species are frequently inappropriated. Considering the nature of wild species and the advisability of minimizing disturbance, collection of epididymal sperm from harvested or accidentally dead males appears as a suitable and convenient option, since it is known that spermatozoa from the cauda epididymis have an adequate degree of maturity and fertility [2, 3].

One of the first steps in a protocol aimed to the cryopreservation of epididymal sperm is the salvaging of that sperm from the cauda epididymis. There are many methods of recovery described in the literature, and they vary depending on authors and species. In the case of small animals, due to the size of the epididymis, the preferred method (flotation method) consists in mincing or slicing the cauda epididymis in a buffered medium, allowing it to rest for some minutes. In this manner, the spermatozoa will swim into the medium and can be recovered by filtration of careful collection of

the medium [4–7]. This technique has also being used with samples from large animals [8, 9].

A similar technique is based in performing many cuts on the cauda with a blade, squeezing gently the cauda and collecting the spermatic fluid emerging from the cut tubules [8, 10, 11]. Another possibility is to use a needle to puncture the tubules [12]. Furthermore, Kishikawa et al. [13] utilized forceps to squeeze the cauda epididymis of mice in order to recover the spermatozoa.

Another different method widely used consists in the retrograde flushing of the cauda epididymis, applying pressure from the vas deferens until the contents of the cauda emerge from a cut performed near the junction with the corpus. The pressure is generated from a syringe, which usually also injects some media, thus the sample is already slightly diluted when recovered. This technique has been extensively used in many species, domestic [14–16] and wild [17–20] . Other authors use no medium, but only air [21–23].

However, there is a lack of information on these techniques. It is necessary to evaluate their impact in sperm characteristics and their suitability for inclusion in germplasm banking protocols, determining which techniques are more adequate for each situation. To our knowledge, only Cary et al. [24] have carried out such a kind of study, comparing flushing and flotation methods. In this context, we have compared two methods for recovery of sperm from the cauda epididymis of red deer: by means of cuts or by flushing. Our group has been studying many reproductive aspects of Iberian red deer and other wild mammals, aiming to improve protocols in order to establish germplasm banks for wild species [25–29]. In this study, we have analyzed the production (spermatozoa obtained), and the quality of the spermatozoa, not only after obtaining the sample, but also before and after freezing/thawing following a standard cryopreservation protocol. We also evaluated the contamination of the sample (epididymal or blood cells) with each method, since the presence of strange elements may influence negatively the quality of sperm. Furthermore, we have carried out a subpopulation study in the basis of motility pattern analysis, in order to determine if the method of extraction modified the internal structure of the samples.

10.3 Material and Methods

All reagents were obtained from Sigma (Madrid, Spain). Media were prepared in our laboratory, not purchased as such.

10.3.1 Genitalia collection

Genitalia were collected from 29 Iberian red deer (*Cervus elaphus hispanicus*), in the game reserves of Ancares, Mampodre and Picos de Europa (Cantabrian mountains in León, Spain), and in several private game reserves of Cáceres, (Spain). All the animals were adults and lived in a free-ranging regime. Harvesting was carried out during the breeding season (autumn). 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).

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, 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.

10.3.2 Sperm recovery

The processing of the samples was carried out in a walk-in fridge (5 °C). Genitalia were dissected, isolating the epididymis and vas deferens. For each sample, it was decided randomly which epididymis was to be processed by cuts and which by flushing.

Recovery by means of cuts

One of the epididymis was thoroughly cleaned, and the superficial blood vessels of the cauda were punctured, so most of the blood could be wiped off. Then, we extracted the sperm from the cauda by means of cuts performed with a scalpel, removing the white fluid coming out from the cut tubules with the aid of the blade. Immediately after the extraction, the sample was diluted 1:1 with extender (Tes-Tris-Fructose, 10 % egg yolk and 4 % glycerol [10]).

Recovery by means of flushing

The other epididymis was cleaned, and the cauda+vas deferens were isolated from the rest of the epididymis performing a cut near the junction of the corpus and the proximal cauda. Then, we carried out a retrograde washing of the vas deferens and cauda epididymis. We used a syringe loaded with 1 mL of the same extender used in the cutting method, and canulated the vas deferens using a blunted 21G needle. The vas defereds and cauda were perfused with the extender, injecting air afterwards, until all the content was flushed out of the cauda epididymis. The sample was collected in a plastic tube. It was sometimes necessary to perform some extra cuts in the cauda epididymis, in order to allow the fluid to emerge.

10.3.3 Determination of the number of recovered spermatozoa and contamination by blood or epididymal cells

Sperm concentration was estimated diluting using a Bürker chamber and a phase-contrast microscope (×400). Then, the total number of sperm recovered with each method was estimated by means

of multiplying the corresponding concentration by the total volume of sample.

Using the same method, we estimated the concentration of contaminant elements in the samples. We differentiated between red blood cells (RBCs, easily recognizable because of their refringency and shape) and epididymal cells. Moreover, we subjectively assigned three levels of "dirtiness" to each sample, depending on the aspect of the background (A-almost clean, B-many contaminants and some background, C-lots of contaminants and dirty background).

The concentration of epididymal cells and RBCs was estimated also in the final dilution (after adding extender to reach 100×10^6 spermatozoa/mL, see below) before freezing.

10.3.4 Quality assessment of the collected sperm

Quality was estimated by assessing motility, membrane functionality and organelle functionality. Only those samples with a proportion of motile sperm higher than 10 % (see below) were analyzed for quality.

Motility assessment was performed using a CASA system. Sperm were diluted ($10–20\times10^6$ cells/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 minutes. Then, a pre-warmed Makler counting chamber (10 μm depth) was loaded with 5 μL of sample. The CASA system consisted on an optical phase contrast microscope (Nikon Labophot-2, equipped with negative phase contrast objectives and a warming stage at 37 °C), a Sony XC-75CE camera, and a PC with the Sperm Class Analyzer software (SCA2002, Microptic, Barcelona, Spain). The magnification was $\times10$. All samples were analyzed at least twice, in order to discard errors due to incorrect sampling. At least 5 fields per sample were acquired, summing at least 100 motile sperm. Image sequences were saved and analyzed afterwards. CASA acquisition parameters were: 25 images acquired, at an acquisition rate of 25 images per second. For each sperm analyzed, the SCA2002 rendered the following data: VCL (velocity according to the actual path; μm/s), VSL (velocity according to the straight path; μm/s), VAP (velocity according to the average —smoothed— path; μm/s), LIN (linearity; %), STR (straightness; %), WOB (wobble; %), ALH (amplitude of the lateral displacement of the sperm head; μm), and BCF (frequency of the flagellar beat; Hz). From each sample, we also obtained the percentage of motile spermatozoa (TM; VCL$>$10 μm/s). Detailed explanation of these descriptors of sperm movement is provided elsewhere [30–33].

Organelle functionality was evaluated using three different assays, using fluorescent probes and flow cytometry. For each assay, we diluted each sample 1:100 in the hepes buffered solution described for the motility analysis. For viability (plasma membrane intactness), we used the LIVE/DEAD kit (Molecular Probes, The Netherlands), consisting in propidium ioide (PI; red fluorescence; 1.2 μmol/L) and SYBR-14 (green fluorescence; 0.1 μmol/L). We determined the percentage of non-viable (red), viable (green) and moribund (green and red) spermatozoa [34]. For mitochondrial status, we used the JC-1 mitochondrial probe (1.4 μmol/L), which stains mitochondria orange, if they are active (high membrane potential), or green, if they are not active [35]. And for

acrosomal status, we utilized PNA-FITC (peanut agglutinin, FITC conjugated; 1 mg/L), which stains the acrosome green if it is damaged or reacted, and PI (37 nmol/L). Thus, we obtained four different subpopulations: red (non-viable sperm; intact acrosome), green (viable sperm, damaged acrosome), red and green (non-viable sperm, damaged acrosome), or non-stained (viable sperm, intact acrosome) [36]. Samples were read with the flow cytometer after 30 min at 37 °C (LIVE/DEAD kit and JC-1) or 10 min at ambient temperature (PI/PNA-FITC). The samples were read using a FACScalibur flow cytometer (Becton Dickinson, CA). For further analysis, we used the percentage of moribund sperm (MOR; LIVE/DEAD staining), the percentage of viable sperm (VIAB; LIVE/DEAD staining), the percentage of non-viable sperm with damaged acrosomes (ARNV; PI/PNA-FITC staining), the percentage of viable sperm with damaged acrosomes (ARV; PI/PNA-FITC staining) and the percentage of sperm with active mitochondria (MIT; JC-1 staining).

10.3.5 Cryopreservation of the spermatozoa

The sample (sperm diluted with extender, see above) was kept for 2 h at 5 °C. Then, the samples were further diluted down to 100×10^6 sperm/mL, using the same extender, and packaged in 0.25 mL French straws. Freezing was carried out using a programmable biofreezer (Planner MRII®), at -20 °C/min down to -100 °C, and then transferred to liquid nitrogen containers. Thawing was performed dropping the straws in water at 65 °C for 6 s. Thawed samples were analyzed for quality as described before, and we determined recuperation of each parameter by means of subtracting its post-thawed value to its pre-freezing value.

10.3.6 Statistical analysis

Statistical analysis were carried out using the SAS™ package v. 8 (SAS Institute, Cary, NC), and $P<0.05$ was used in all tests for statistical significance. For production, contamination and fluorescence data, in order to determine if there were differences between the cuts and flushing method, results obtained for each method were subtracted, and the Wilcoxon signed rank test was used to test $H_0=0$. Level of “dirtiness” was analyzed using the χ^2 test, or the Fisher’s exact test when assumptions for the later were violated. CASA motility data was analyzed using the general linear models procedure, analyzing the differences between methods of extraction within treatment (initial, pre-freezing and post-thawing) and between treatments within method of extraction, including male as factor in both cases. Classes were compared using adjusted least-squares means. Previously, variables were transformed for normality (log transformation, except for proportions, where arc sine transformation was used).

We also studied the subpopulations defined from the clustering analysis of CASA motility data. The clustering analysis we followed has been presented more extensively in another study [37], thus we will summarize it here (SAS™ procedure names are given just for reproducibility, since documentation and equivalences to other statistical packages are available elsewhere). Firstly, we

reduced the number of descriptors to 2 principal components (principal component analysis using the PRINCOM procedure), which were used to carried out a non-hierarchical cluster analysis (k-means model, FASTCLUS procedure). We obtained 15 clusters, which were passed to the CLUSTER and TREE procedures, in order to perform a hierarchical clustering on them (average linkage method, UPGMA). The final number of clusters was estimated 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). Clusters were compared between methods of extraction within treatment (initial, pre-freezing and post-thawing) and between treatments within method of extraction, using their motility descriptors as comparison variables and taking into account the males (male nested within the corresponding factor). We used the general linear models procedure and, when the model was significant, the respective classes were compared using adjusted least-squares means. Previously, variables were transformed for normality (log transformation, except for proportions, where arc sine transformation was used). Comparisons of cluster proportions (relative to motile spermatozoa or to the total number —motile+immotile— of spermatozoa) between extraction methods or treatments were carried out by means of the χ^2 test.

10.4 Results

10.4.1 Sperm production and evaluation of contamination

The number of spermatozoa obtained was not significantly different using either the cuts or the flushing method. We obtained medians of 493.7×10^6 and 648.7×10^6 spermatozoa for the cuts method and flushing method, respectively (Figure 10.1), with very wide interquartile ranges (more than 600×10^6 in both methods). In our study we did not find statistical differences between the two methods.

We found epididymal cells in all the samples, but 34 % (10 of 29) samples obtained by flushing were completely free of RBCs. Table 10.1 shows the concentrations (medians) of epididymal cells and the scores of “dirtiness” of each extraction method. Figure 10.2 shows the distribution of the samples for the four contaminant evaluations and the two extraction methods. The samples obtained by means of cuts were much more contaminated than those obtained by means of flushing. After diluting the samples to its final concentration of 100×10^6 sperm/mL before freezing, the concentration of epididymal cells was not significantly different between methods, but the concentration of RBCs was still higher in the case of the cuts method. Considering the “dirtiness” classification, most samples obtained by flushing had an A score (almost clean), whereas, in the case of cuts, not only there were as many A samples as B samples, but also C samples supposed an important proportion.

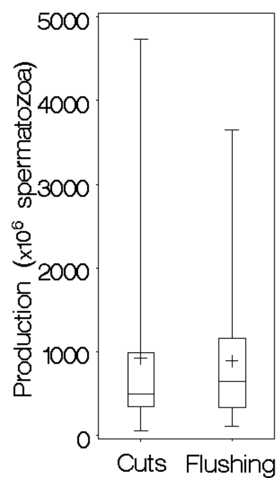


Figure 10.1 Production results for the cuts method and the flushing method. Differences between methods were not significant. In the box plots, the box comprises from the 1st to the and 3rd quartile, the whiskers indicate the most extreme values, and the horizontal line indicate the median. The cross indicates the mean.

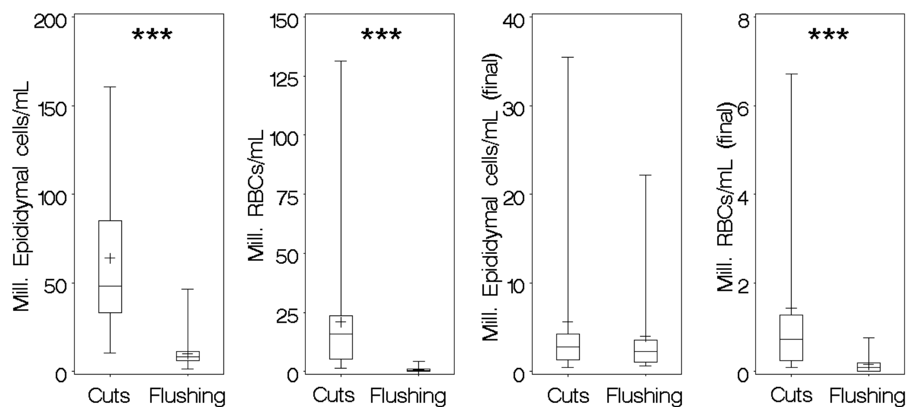


Figure 10.2 Contamination results for the two salvaging methods. Results for epidydimal cells and RBCs are showed, considering its concentration in the sample just after extraction (initial) and just before freezing, after diluting it down to 100×10^6 spermatozoa/mL (final). Significances (Wilcoxon signed rank test) are indicated as: + P<0.1; * P<0.05; ** P<0.01; *** P<0.001. In the box plots, the box comprises from the 1st to the and 3rd quartile, the whiskers indicate the most extreme values, and the horizontal line indicate the median. The cross indicates the mean.

Table 10.1 Contamination assessment for each method of extraction. Data are expressed as medians (concentration of epididymal cells —E. Cells— and RBCs, $\times 10^6$ particles/mL), except for the "dirtiness" classification, where data are given as percentages, with the corresponding number of samples within parentheses. Rows with different letters differ significantly (Wilcoxon signed rank test, and χ^2 or Fisher's exact test for the "dirtinessçlassification).

			E. Cells	RBCs	"Dirtiness" classification[2]		
Extraction	E. Cells	RBCs	(final)[1]	(final)[1]	A	B	C
Cuts	48.04[a]	15.70[a]	2.76	0.73[a]	43.3 (13)[a]	40 (12)[a]	16.7 (5)[a]
Flushing	8.42[b]	0.54[b]	2.24	0.09[b]	75.9 (22)[b]	20.7 (6)[b]	3.4 (1)[b]

[1] concentration after the final dilution of the sample (down to 100×10^6 sperm/mL).

[2] A: almost clean; B: many contaminants and some background; C: lots of contaminants and dirty background.

10.4.2 Sperm quality

Samples from 8 animals were rejected because of poor motility after extraction (TM<10 %). Results of analyzed samples are shown in Table 10.2 and Figure 10.3. Average motility results (Table 10.2) did not significantly differ between the two extraction methods when analyzed just after extraction. However, in the pre-freezing and post-thawing analysis, flushed samples had better motility than the corresponding samples obtained by cuts, showing that flushed samples not only had higher proportion of motile sperm, but also better condition (especially considering VAP and LIN values).

Fluorescence-based analysis showed little differences between extraction methods (Figure 10.3). Before freezing, samples obtained by means of cuts had a significantly higher proportion of ARNV sperm (non-viable sperm with damaged acrosomes). Nevertheless, we detected a trend towards signification (P<0.1) in the pre-freezing analysis of MOR and VIAB (less moribunds and more viable sperm for flushing), and in the post-thawing analysis of ARV (more viable sperm with damaged acrosomes sperm for cuts).

10.4.3 Sperm subpopulations

Clustering analysis rendered four different clusters, named CL1, CL2, CL3 and CL4. Characteristics and proportions of these clusters for each extraction method and treatment (initial, pre-freezing and post-thawing) are showed in Table 10.3. CL1 was composed of slow, non-linear and little active sperm. CL2 spermatozoa were rapid and very linear, with high BCF. CL3 consisted in very fast but not very linear sperm. CL4 consisted in fast and non-linear sperm.

The characteristics of the clusters were affected by treatment and method of extraction, but were very similar in most cases. However, the proportions of the clusters varied greatly. Samples obtained by means of cuts had a higher proportion of CL2 (42.22 %), followed by CL1 (34.08 %) and CL3 (23.70 %) in the initial analysis, but, in the pre-freezing analysis, CL1 increased to more

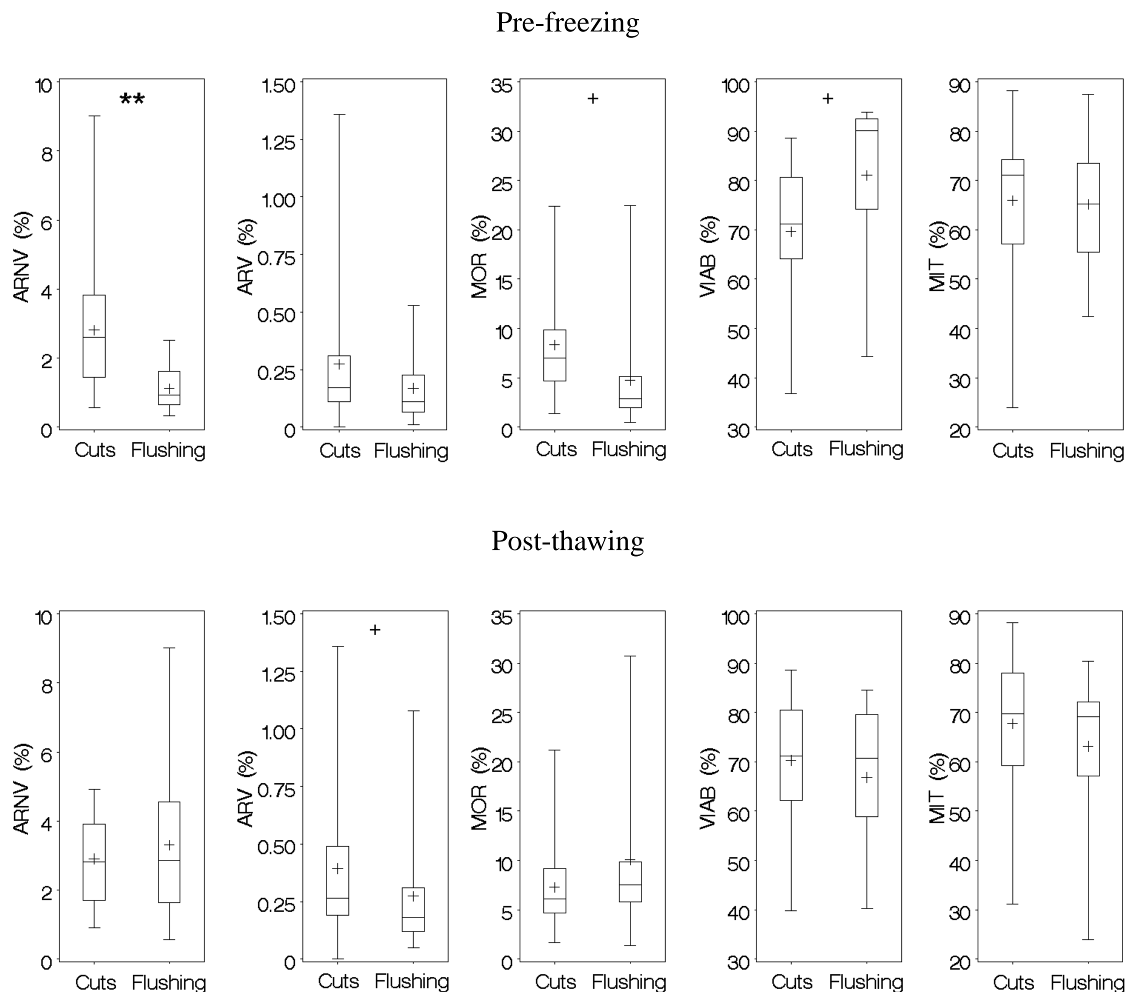


Figure 10.3 Comparison of the results obtained analyzing the samples with fluorescent probes and flow cytometry, for the two methods of extraction (pre-freezing —top— and post-thawing —bottom— analysis). ARNV: % acrosome reacted, non-viable spermatozoa; ARV: % acrosome-reacted, viable spermatozoa; MOR: % moribund spermatozoa; VIAB: % viable spermatozoa; MIT: % spermatozoa with active mitochondria. Significances (Wilcoxon signed rank test) are indicated as: + $P<0.1$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$. In the box plots, the box comprises from the 1st to the 3rd quartile, the whiskers indicate the most extreme values, and the horizontal line indicate the median. The cross indicates the mean.

Table 10.2 Values of some motility descriptors depending on extraction method and treatment. Data are expressed as mean±SEM.

Treatment	Extraction	TM (%)	VAP (μm/s)	LIN (%)	ALH (μm)	BCF (Hz)
Initial	Cuts	51.38 ± 5.61^{A}	38.08 ± 7.27^{A}	48.17 ± 4.31^{AB}	2.02 ± 0.24^{A}	5.95 ± 0.55^{A}
	Flushing	49.17 ± 5.94^{A}	46.21 ± 6.40^{A}	50.99 ± 3.54^{A}	2.30 ± 0.21^{A}	6.71 ± 0.46^{A}
Pre-freezing	Cuts	72.49 ± 5.98^{aB}	50.98 ± 6.11^{aB}	42.11 ± 2.47^{aA}	3.14 ± 0.32^{B}	7.20 ± 0.40^{B}
	Flushing	84.48 ± 6.23^{bB}	67.63 ± 7.24^{bB}	50.73 ± 3.94^{bA}	3.39 ± 0.35^{A}	7.25 ± 0.55^{AB}
Post-thawed	Cuts	69.61 ± 4.30^{aB}	60.79 ± 8.21^{B}	52.58 ± 5.18^{aB}	2.46 ± 0.18^{B}	7.19 ± 0.44^{aB}
	Flushing	80.20 ± 4.25^{bB}	69.68 ± 8.30^{B}	59.00 ± 5.01^{bB}	2.47 ± 0.22^{B}	7.76 ± 0.43^{bB}

^{a,b}: rows (extraction method within treatment) with different superscripts differ P<0.05.

^{A,B,C}: rows (treatments within extraction method) with different superscripts differ P<0.05.

than 50 %, with a similar decrease of CL2. However, in the post-thawing analysis, the proportions of the subpopulations returned to values similar to the initial ones, but with a higher CL1 proportion and lower CL3. We found CL4 only in the post-thawing treatment, and in a very low proportion.

On the other hand, samples obtained by flushing had a very different subpopulation pattern and evolution. Initially, CL2 was clearly the predominant subpopulation, accounting for almost 2/3 of motile sperm, and with CL3 below 10 %. Before freezing, the pattern changed radically, since CL3 rose to almost 45 %, whereas CL2 fell below 20 %, but CL1 decreased slightly. Remarkably, we found CL4 in the pre-freezing treatment, and with an appreciable proportion. In the post-thawing analysis, the subpopulation pattern changed radically again, with a low proportion of CL1 (11 %), CL2 increasing to more than 50 % and CL3 dropping below 30 %. CL4 decreased to 9 %

10.5 Discussion

The purpose of this work was the comparison of two methods for obtaining sperm samples from the cauda epididymis of red deer. Our research group has been working in epididymal sperm for many years, mainly from wild ruminants, and with special interest in Iberian red deer [28, 29, 37–40]. Most of our work has been aimed to the adaptation and improvement of protocols for setting up germplasm banks based in the storage of doses prepared from epididymal sperm collected post-mortem. However, we have found very little information concerning evaluation of recovery methods, especially in terms of contamination and yield.

Although most studies on epididymal sperm describe the method used to salvage the spermatozoa, its evaluation is not considered as one of the objectives. Recently, Cary et al. [24] compared flushing and flotation methods for salvaging sperm from stallion epididymes. Flotation method was preferred because of being more easy to prepare and rendering sperm of better quality. However,

Table 10.3 Values of some motility descriptors depending on extraction method and treatment, by cluster. Data are expressed as adjusted least-squares means±SEM.

Cluster	Tr.[1]	E.M.[2]	ST[3,5]	SM[4,5]	VAP (μm/s)	LIN (%)	ALH (μm)	BCF (Hz)
CL1	I	C	21.74^{aA}	34.08^{aA}	17.77±0.88^{A}	30.53±1.02^{aA}	1.77±0.06^{A}	4.80±0.24^{aA}
		F	17.12^{bA}	26.04^{bA}	13.63±1.34^{A}	27.42±1.55^{bA}	1.49±0.09^{A}	5.01±0.37^{bA}
	PF	C	42.50^{aB}	53.13^{aB}	28.12±0.77^{aB}	36.24±0.80^{aB}	2.27±0.04^{aB}	6.88±0.16^{aB}
		F	17.49^{bA}	19.19^{bB}	32.51±1.01^{bB}	31.82±1.05^{bB}	2.49±0.06^{bB}	6.22±0.21^{bB}
	PT	C	29.27^{aC}	39.29^{aC}	25.60±1.11^{C}	35.74±1.04^{aB}	2.03±0.07^{aC}	6.28±0.21^{C}
		F	9.66^{bB}	11.24^{bC}	28.64±2.04^{C}	28.77±1.93^{bAB}	2.30±0.13^{bB}	6.79±0.39^{B}
CL2	I	C	26.92^{aA}	42.22^{aA}	53.86±3.73^{A}	77.01±1.70^{aA}	1.81±0.11^{A}	9.84±0.47^{a}
		F	43.62^{bA}	66.34^{bA}	54.06±2.39^{A}	70.14±1.08^{bA}	2.24±0.07^{A}	9.47±0.30^{b}
	PF	C	14.83^{aB}	18.54^{aB}	87.73±2.97^{aB}	83.07±0.94^{B}	2.64±0.10^{aB}	10.13±0.37^{a}
		F	18.20^{bB}	19.98^{bB}	76.10±1.93^{bB}	83.32±0.61^{A}	2.04±0.06^{bA}	9.15±0.24^{b}
	PT	C	30.02^{aC}	40.30^{aA}	88.93±1.93^{aB}	87.40±0.85^{aC}	1.98±0.04^{aC}	9.35±0.26
		F	44.15^{bA}	51.35^{bC}	74.55±1.69^{bB}	82.62±0.75^{bB}	1.81±0.04^{bB}	9.27±0.23
CL3	I	C	15.12^{aA}	23.70^{aA}	79.57±4.73^{aA}	52.65±2.94^{A}	4.89±0.27^{aA}	7.18±0.59
		F	5.01^{bA}	7.62^{bA}	135.10±8.85^{bA}	50.14±5.51^{A}	6.17±0.51^{Ab}	8.71±1.11
	PF	C	22.66^{aB}	28.33^{aB}	94.58±2.10^{aB}	43.02±1.16^{aB}	5.65±0.12^{aB}	7.88±0.28^{a}
		F	40.79^{bB}	44.77^{bB}	95.45±1.35^{bB}	59.43±0.75^{bB}	4.36±0.08^{bB}	8.49±0.18^{b}
	PT	C	24.35^{aC}	17.38^{aC}	112.99±2.25^{aC}	62.74±1.37^{aC}	4.44±0.09^{aA}	8.25±0.34
		F	12.95^{bC}	28.31^{bC}	119.41±1.64^{bA}	75.90±1.00^{bC}	3.50±0.07^{bC}	8.41±0.25
CL4	PF	F	14.64^{A}	16.06^{A}	87.79±2.74^{B}	23.24±1.12^{A}	6.48±0.18^{B}	6.64±0.40
	PT	C	2.25^{a}	3.02^{a}	105.41±5.75	20.37±3.26^{a}	6.65±0.24^{a}	6.77±0.73
		F	7.83^{bB}	9.10^{bB}	115.22±4.22^{B}	33.23±2.39^{bB}	5.73±0.18^{bB}	7.00±0.53

[1] Treatment. I: initial; PF: pre-freezing; PT: post-thawing.

[2] Extraction method. C: cuts; F: flushing.

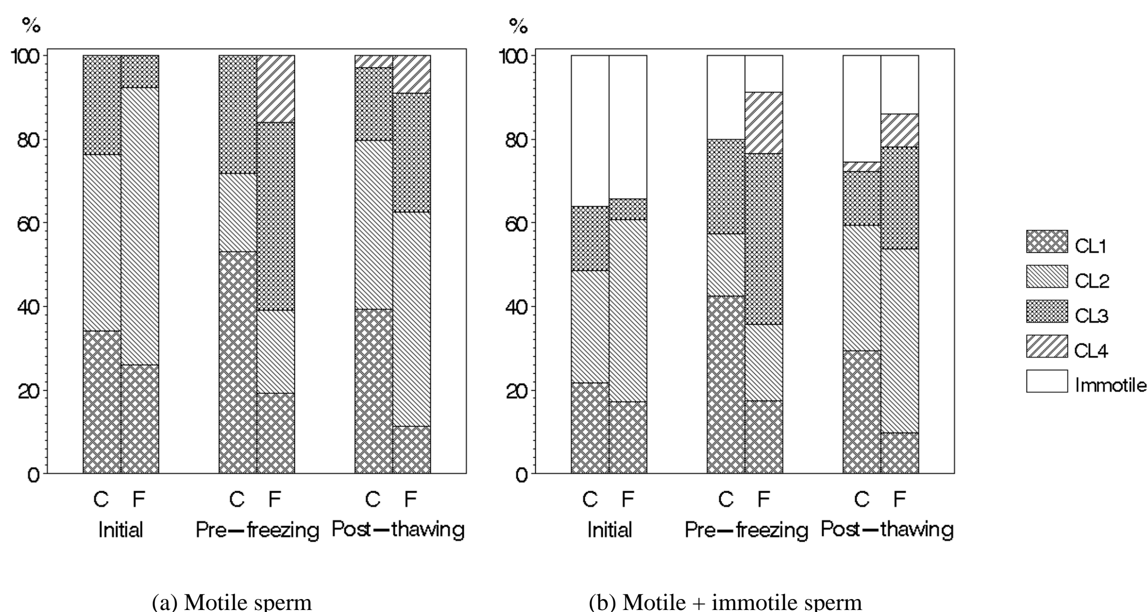
[3] proportion considering all the spermatozoa in the sample (motile and immotile).

[4] proportion considering only motile spermatozoa in the sample.

[5] χ^2 on raw data.

^{a,b}: rows (extraction methods within treatment) with different superscripts differ P<0.05.

^{A,B,C}: rows (treatments within extraction method) with different superscripts differ P<0.05.



(a) Motile sperm (b) Motile + immotile sperm

Figure 10.4 Representation of the proportions of each cluster (as defined after the clustering analysis of CASA data) depending on method of extraction (C: cuts; F: flushing) and treatment (initial, pre-freezing and thawing). Pattern codes for each cluster are given on the right (CL1: slow and non-linear; CL2: rapid and linear; CL3: very rapid and non-linear; CL4: rapid and non-linear). Table 10.3 shows detailed data and significant differences.

the two methods did not differ significatively, thus the authors concluded that either method may be acceptable for terminal sperm collection.

In this study, we carried out three different comparisons between cuts and flushing: yield, contamination and quality. Yield —the number of spermatozoa recovered— have a practical direct consequence in the number of doses produced from each postmortem sample. The significance of this in germplasm banking is evident, especially considering that there is only one opportunity to obtain sperm doses from each male, and that this kind of protocols may be applicable to endangered species or valuable individuals. Although we did not find differences between the two methods, flushing may render better results, because, at least in theory, it would allow us to wipe all the content of the whole cauda epididymis, including great part of the vas deferens. However, this must be performed carefully, since we have observed that the cuts performed for detaching the cauda from the rest of the epididymis, or for helping the sperm to come out, may cut tubules near to the junction epididymis-vas deferens, thus the technique would bypass a great part of the cauda.

However, contamination was much evident using the cuts method. Instead of performing one or two cuts, as in the case of the flushing method, it is necessary to cut the cauda several times in order to assure that most of the tubules are cut and their contents come out. This, combined with the squeezing and the collection of sperm with the blade, explain the elevate quantity of extraneous

elements in the samples obtained by means of cuts. The effect of these particles in the sample is unclear, since most articles related to contamination of sperm samples have dealt on leukocytic or bacterial contamination instead [41, 42]. Nevertheless, Rijsselaere et al. [43] related erithrocyte presence with lower post-thawing quality. These authors did not find negative effects of adding blood or serum to extended dog semen, which was cooled and stored for several days, but observed a negative effect if samples treated with whole blood were frozen-thawed. These authors interpreted this as an effect of the hemolysis and free hemoglobin. Furthermore, Verberckmoes et al. [44] found a negative effect of blood and serum in an IVF experiment using bovine semen, which could not be noted using other analysis of sperm quality.

Therefore, techniques that minimize this kind of contamination would be preferable. Extracting the sperm by means of cuts expose spermatozoa to the blood and interstitial fluid released when cutting. Although this contact can be minimized mixing quickly the sperm with medium or extender, there is a variable interval of time (many seconds at least) in which an appreciable quantity of sperm is exposed to this hazardous environment. Apart from the possible effect of RBCs described in the referred studies, blood and interstitial fluid may alter the composition, pH and osmolality of the spermatic fluid, therefore subjecting the spermatozoa to deleterious conditions. Flushed samples are better protected, not only because the sperm is pull by extender, but also because the contact with blood and other fluids is limited due to the few cuts performed. This could be also the reason why Cary et al. [24] did not find significant differences between flushing and flotation methods, since in both cases sperm passed directly from the epididymis to the extender. Here, we have found that in the final dilution, previously to packaging and freezing the samples, there were no differences between cuts and flushing considering the concentration of epididymal cells, therefore this kind of contamination may not be worsened using the apparently more destructive cuts method.

However, we still have to consider blood contamination, which was marginal in the case of flushing, but it was an appreciable problem when using cuts, and very important in many samples. Although we have not related this to lower quality, and RBCs concentrations were lower than the ones used by Rijsselaere et al. [43] and Verberckmoes et al. [44], there is a risk to impair the fertility of this kind of samples. Flotation method may release even more blood and epididymal cells to the sperm solution that the method used in this work. Furthermore, the effect of these contaminants within the genital tract of inseminated females is unknown, considering the use of these samples for AI programmes. Therefore, this kind of techniques may be complemented with filtration or purification steps, in order to remove these contaminants.

Flushing seemed also to be better in terms of sperm quality, especially considering motility parameters. It is interesting to note that, for both methods, motility was worse initially (just after recovering the samples) than pre-freezing or post-thawing. We had already noted this in another study [37], where we considered that it may be related to the activation of epididymal sperm due to the dilution in the extender [19], and to the effect of egg yolk on sperm motility [45]. In fact, differences between the two methods were evident in the pre-freezing and post-thawing analysis.

Thus the alteration in motility characteristics due to dilution could have help to disclose these differences, due to different responses of sperm recovered by different methods. The effect of sperm dilution in extender (timing, dilution rate, extender composition) must not be disregarded, and deserves further work.

Interestingly, although we did not found significant differences considering moribund and viable sperm, viability seemed to be better for flushed samples before freezing, but results were almost equal after thawing. This could reflect that many sperm are susceptible to undergo damage when subjected to environmental changes. Thus, in the samples obtained by means of cuts, this subpopulation may have been affected from the extraction, causing the apparently higher proportion of moribund sperm and lower viable sperm. On the other hand, this subpopulation would not have been much affected when using the flushing method, because of the more immediate contact with the extender and the lesser level of contamination. However, freezing/thawing would have impaired this subpopulation anyway. Consequently, after freezing, both methods showed very similar viability results.

Sperm subpopulation analysis gave us some valuable information that support the idea of deep differences provoked by the extraction method. The analysis indicated that samples obtained by different methods had different subpopulation pattern. Furthermore, flushed samples may have a better quality, because of the higher proportion of CL2 (rapid and linear sperm), and lower proportion of CL3 (rapid and non-linear sperm), both in the initial and post-thawing analysis. In a previous study on cryopreserved epididymal sperm from red deer, we found that a subpopulation similar to CL2 was possibly related to better post-thawing condition [37]. Moreover, the presence of CL4 in the pre-freezing analysis of flushed samples, and not in samples obtained by means of cuts, suggests that there are more differences, maybe related to the presence of a sensitive subpopulation, as we proposed above. Furthermore, the decrease of CL4 in the post-thawing analysis coincided roughly with the decrease of TM, which may indicate a relationship of CL4 (although not necessarily a coincidence) with that sensitive subpopulation.

In conclusion, flushing would be a more recommendable method for postmortem salvaging of sperm from the cauda epididymis. Flushed samples were less contaminated and had higher quality, and, if done properly, the yield of this method should be similar or even higher than others. Moreover, it can be used in the field without problems [19]. Although other authors have described similar methods based in injecting only air [21–23], injecting some medium seem to be recommendable, because of its protective effect. Furthermore, from our experience, injecting some medium previously to air eases the extraction of sperm and reduces the risk of breaking the epididymal tubules because of pressure.

On the other hand, flushing may not be as easy to apply in small animals as in big ones, because of the lack of needles of adequate gauge and the low diameter of epididymal tubules, which would made flushing very slow or impossible. Furthermore, cuts were a quicker and easier method, and may be more practical in some cases. Thus, techniques based in cuts cannot not be discarded, but

it is advisable to carry out an immediate dilution of the sperm with protective diluents, and limiting contamination (especially by blood) as possible. Finally, further research in these techniques is necessary to improve this step on the protocols for postmortem obtaining and cryopreservation of spermatozoa.

10.6 Acknowledgements

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

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Capítulo 11

Discusión general

Como se menciona en la introducción, la creación de un banco de recursos genéticos para especies silvestres debe estar sustentada sobre una base de conocimiento suficiente. De esta manera, los protocolos y la estrategia general pueden ser optimizados, consiguiendo un ahorro de espacio y recursos. Esto supone no sólo que la recogida de muestras, transporte, procesado, criopreservación y aplicación sean adecuados, sino también discriminar entre aquellas muestras aptas para entrar en el banco y aquellas cuya conservación no merece el gasto de recursos [1].

Dentro de la estrategia general de un banco de germoplasma, podemos definir varios puntos de decisión para admitir o descartar una muestra. El objeto de estudio en esta Tesis ha consistido en muestras espermáticas epididimarias, obtenidas de ciervo rojo ibérico en actividades cinegéticas. La naturaleza de la muestra nos proporciona un primer criterio, dependiendo de la época del año en que transcurra la cacería (estacionalidad). Otro punto de control se establece al llegar la muestra al laboratorio, dependiendo del tiempo post-mortem hasta el procesado de las muestras. Por supuesto, hay otro punto de decisión importante tras el análisis de la muestra, en el cual se debería establecer la potencialidad de la muestra en cuanto a fertilidad y capacidad de resistir el proceso de criopreservación.

Los experimentos presentados en este trabajo fueron realizados teniendo presente la aplicación de sus resultados a la gestión de en bancos de germoplasma. Aunque el objeto de estudio hayan sido muestras procedentes de rumiantes silvestres, y, concretamente, de ciervo rojo ibérico, buena parte de las conclusiones podrían extrapolarse a otras especies silvestres si una opción para la preservación de su germoplasma fuese la obtención post-mortem.

11.1 Tiempo post-mortem

El efecto del tiempo post-mortem sobre la calidad de muestras recogidas de cadáveres ha sido objeto de atención en diversos estudios. La importancia de este factor es muy importante, ya que muestras de gran calidad pueden deteriorarse rápidamente, sobre todo si se mantienen en condiciones inadecuadas. En este trabajo, seguimos unas condiciones típicas de recogida en el campo durante la

temporada de caza, siendo los propios celadores los que se encargaron de recoger los testículos y refrigerarlos lo antes posible tras la muerte del animal. Por otra parte, el transporte hasta la Clínica Veterinaria de la Universidad de León se llevó a cabo cuando las circunstancias lo permitían, no siempre que se recogieron muestras. La metodología, consistente en dejar los testículos y epidídimos protegidos simplemente por el escroto y refrigerados a 5 °C, fue elegida tras revisar los trabajos de otros autores. Por ejemplo, Sankai et al. [2] ensayaron varias condiciones de almacenamiento de epidídimos, intentando preservar la calidad de los espermatozoides epididimarios, y encontraron que mantener los testículos y epidídimos dentro de la bolsa escrotal era un método tan eficaz como otros más complicados. Sobre la refrigeración, hay autores [3] que indican que la fertilidad sería similar a medio plazo (ovino; comparación a 24 y 48 h), se refrigeren los testículos o no, aunque otros trabajos en ratón [2] han indicado que la refrigeración es beneficiosa a más largo plazo (4 días), aunque sus resultados indican que deberían analizarse varias temperaturas de refrigeración (de hecho, recomiendan refrigerar a 10 °C, en vez de 5 °C).

La importancia del estudio recogido en el capítulo 6 reside en la identificación de puntos críticos en el proceso de degeneración de los espermatozoides epididimarios a medida que avanza el tiempo post-mortem. La definición de estos puntos críticos nos indicaría si una muestra procesada a un determinado tiempo post-mortem sigue conservando gran parte de su calidad original o si las posibilidades de obtener una muestra aceptable son mínimas. Hay que resaltar que los espermatozoides epididimarios resisten mejor que los eyaculados el período de refrigeración. La Figura 6.5 plasma de una manera gráfica esta idea: una persona encargada de la admisión de muestras en un banco de germoplasma puntuaría negativamente aquellas muestras que llegasen con un tiempo post-mortem superior a 2 días, y rechazaría aquellas con más de 5 días post-mortem.

Este fenómeno parece afectar de manera bastante similar a distintas especies, y, por lo general, otros autores han encontrado que la movilidad es severamente afectada, mucho más que otros parámetros [3–8], posiblemente porque depende de múltiples factores, sensibles a los diferentes tipos de estrés a los que se encuentran sometidos los espermatozoides (refrigeración, cambios en pH y osmolaridad, etc.). De hecho, la variación del pH y la osmolaridad tiene gran importancia, y podría deberse en gran parte al deterioro de los túbulos epididimarios indicado por Hishinuma et al. [8]. Es posible, como sugiere un estudio reciente Gerber et al. [9], que la calidad de la muestra pudiese prolongarse si, en vez de refrigerar los testículos y epidídimos, la extracción se llevase a cabo en el mismo lugar de la caza y las muestras, diluidas en medio protector, se conservasen refrigeradas hasta el momento de la criopreservación. Sin embargo, este paso requeriría entrenar a las personas encargadas de la recogida de genital, complicando la estrategia de gestión del banco de germoplasma.

Acerca de la movilidad espermática, en el capítulo 9, se puede ver que estos cambios tuvieron un efecto profundo en el patrón de subpoblaciones espermáticas. Si conseguimos distinguir una subpoblación como la responsable de la buena calidad de una muestra, entonces podremos tratar de procesar esa muestra, intentando recuperar aquellos espermatozoides con características adecuadas [10–12]. No obstante, de acuerdo con nuestro análisis, después de 72 h post-mortem, las subpobla-

ciones de mayor calidad tendieron a desaparecer, y fueron sustituidas por subpoblaciones con malas características. Un efecto similar debido al tiempo post-mortem ha sido observado también en semen eyaculado de gacela Mohor (*Gazella dama mhorr*) [13]. Por lo tanto, este es un argumento a favor de desechar automáticamente muestras con varios días post-mortem (5 días en nuestro estudio), para su inclusión en un banco de recursos genéticos.

11.2 Estacionalidad

El estudio del efecto de la estacionalidad, en los capítulos 7 y 9, proporcionó algunos resultados notables. Los cambios estacionales en los cérvidos han sido bien estudiados, y los resultados obtenidos en este trabajo coinciden con los de otros autores en cuanto a la superioridad de los parámetros morfométricos de testículos y epidídimo y de la producción espermática en la berrea, respecto al resto de la época reproductiva y a la época no reproductiva [14–16]. Dada la fuerte estacionalidad del ciervo rojo ibérico, sería de esperar que las muestras recogidas durante la berrea fuesen las de mejor calidad, teniendo en cuenta que la testosterona plasmática alcanza su máxima concentración justo al comenzar la berrea y luego disminuye rápidamente [15, 17]. Sin embargo, la calidad resultó ser mejor durante el período posterior a la berrea.

El estudio de subpoblaciones espermáticas basado en el análisis de los datos de movilidad proporcionados por CASA (capítulo 9), proporcionó información nueva de gran utilidad para comprender estos resultados aparentemente contradictorios. En primer lugar, los datos generales indicaron también que la movilidad mejoraba después de la berrea, lo cual no fue evidente en el estudio descrito en el capítulo 7 (posiblemente porque en ese estudio la determinación de la movilidad se hizo subjetivamente, y no con un sistema CASA). En segundo lugar, el análisis de subpoblaciones descubrió una subpoblación con movilidad no linear, que decreció a lo largo de la estación reproductiva a la vez que una subpoblación linear aumentó equivalentemente. Esto nos indicaría que las muestras serían de mayor calidad después de la berrea, sin llegar a salir de la época reproductiva.

Sin embargo, Rodríguez and Bustos Obregon [18], trabajando con caballos, indicaron que el semen recogido fuera de la estación reproductiva tenía alteraciones en la cromatina —empaquetamiento excesivo— no presentes en las muestras recogidas en estación, que asociaron a procesos de hipermaduración en la cola del epidídimo. La evolución de subpoblaciones detectada en este trabajo podía explicarse de manera similar, por lo que propusimos la hipótesis planteada en el capítulo 9: la subpoblación CL1s (no linear) contendría espermatozoides con capacidad para madurar en la cola del epidídimo (posiblemente espermatozoides que habían migrado recientemente desde el cuerpo), y que la subpoblación CL2s (linear) estaría compuesta por espermatozoides que habían terminado su maduración en la cola. Según avanzaba la estación, y teniendo en cuenta la disminución progresiva de actividad sexual, se podría esperar que los espermatozoides hubiesen pasado más tiempo en la cola del epidídimo que los de las muestras obtenidas durante la berrea, con lo cual se podía establecer una relación con el citado fenómeno de hipermaduración.

Sin embargo, esta hipótesis debe ser confirmada, especialmente mediante estudios bioquímicos y análisis de la cromatina. De ser cierta, habría que cuestionarse la inclusión en bancos de recursos genéticos de las muestras recogidas después de la berrea, aunque estuviesen aún dentro de la estación reproductiva, ya que las alteraciones generalizadas en la cromatina o en las rutas bioquímicas de señalización podrían disminuir dramáticamente la fertilidad de las muestras [18].

Por otra parte, los resultados del capítulo 7 ponen en duda la conveniencia de aprovechar las muestras recogidas fuera de la época reproductiva, tanto debido al escaso número de espermatozoides recogidos, que sólo permitirían preparar un reducido número de dosis seminales, como por la baja calidad de las muestras.

11.3 Subpoblaciones espermáticas

El estudio de subpoblaciones espermáticas a partir de datos de CASA es un enfoque relativamente nuevo del análisis espermático. En el capítulo 8 se propone un método de análisis estadístico, el cual se utilizó también en los estudios descritos en los capítulos 9 y 10. Esta propuesta se basa en los trabajos de otros autores [10, 19–21], intentando conseguir dos objetivos: portabilidad, es decir, que el método pudiese implementarse fácilmente en distintos sistemas sin tener que recurrir a un software estadístico concreto; y flexibilidad, para que se pudiese adaptar a las peculiaridades de distintas muestras. De ahí que se incluyesen pasos de estandarización, análisis de componentes principales, y dos tipos de análisis de clústers, así como un método objetivo para seleccionar el número final de clústers. Además, el diseño del método permite también detectar valores aberrantes e irregularidades en el conjunto de los datos.

De hecho, en el mismo capítulo 8 se incluye un análisis de subpoblaciones a lo largo un protocolo estándar de criopreservación de muestras epididimarias de ciervo rojo ibérico. Comparando la información proporcionada utilizando únicamente las medias de cada descriptor de movilidad, y la información obtenida tras efectuar el análisis de subpoblaciones, se puede apreciar las ventajas de este tipo de análisis. De hecho, diversos autores han advertido sobre la pérdida de información que se produce al utilizar datos medios para intentar describir una población altamente heterogénea [19, 20, 22]. Como se puede comprobar en ese capítulo, se puede obtener información de interés diferenciando subpoblaciones en base a criterios simples, como es el caso de la progresividad y velocidad. Esta aproximación sencilla ha dado buenos resultados en estudios de otros autores [23].

Uno de los hallazgos más importantes del estudio es el incremento de movilidad de las muestras epididimarias al comparar el primer análisis, justo después de la extracción, y el efectuado antes de la congelación (lo cual se resalta también en el capítulo 10). Hay que tener en cuenta que los espermatozoides epididimarios utilizados estuvieron sometidos a un entorno inhibidor de la movilidad y fueron mantenidos en refrigeración muchas horas. Aunque se diluyeron en un medio favorable y se templaron a 37 °C durante un tiempo razonable, es posible que este tratamiento no fuese suficiente para estimular la movilidad espermática. En cambio, en el análisis pre-congelación,

los espermatozoides habían sido mantenidos en el diluyente de congelación durante 2 horas. Este contacto más prolongado con un medio no inhibidor, y el hecho de que el diluyente contuviese yema de huevo, que puede modificar la movilidad espermática [24–26], podría explicar el mayor porcentaje de espermatozoides móviles y la disminución de progresividad.

Efectivamente, el análisis de subpoblaciones del capítulo 8 reveló que tanto la adición de diluyente de congelación como la criopreservación alteraron la movilidad espermática, lo cual se reflejó en patrón de subpoblaciones, que sufrió variaciones apreciables. Por otra parte, el segundo estudio de subpoblaciones, modificado para utilizar sólo aquellos descriptores que resultaron ser más importantes en el primero, indicó que, para realizar un buen análisis, no haría falta incluir todos los descriptores que proporciona el CASA. Sin embargo, el elegir los descriptores adecuados a priori puede ser un proceso complicado o equívoco. El método presentado incluye un análisis de componentes principales como paso inicial, precisamente para poder comenzar con un número grande de variables sin tener que recurrir a una etapa de selección. Estas variables son reducidas a unos pocos componentes principales con los que se puede trabajar cómodamente.

Otro punto a destacar del capítulo 8 es el estudio para discriminar entre machos utilizando otro análisis de clústers. Aunque el interés práctico de este análisis en el contexto de recogida post-mortem es limitado, estudios similares con semen eyaculado han mostrado que se puede discriminar eficazmente entre machos dependiendo de la calidad de su semen [27]. De mayor interés es la relación entre una de las subpoblaciones encontradas (rápida y linear) y la recuperación de viabilidad espermática tras la descongelación. Encontrar relaciones entre una subpoblación determinada y la calidad espermática es uno de los objetivos de los grupos de investigación que utilizan este tipo de análisis, aunque los resultados no son concluyentes aún [10, 28, 29].

La aplicación de este tipo de técnicas a un banco de germoplasma sería posible a partir de la caracterización de una serie de subpoblaciones, cuya presencia o ausencia en una muestra indicasen buena fertilidad o resistencia a la criopreservación. La salida de datos de un sistema CASA podría ser encauzada fácilmente a un software estadístico que realizase automáticamente el análisis de subpoblaciones, calificando la muestra espermática en consecuencia.

11.4 Metodología de extracción

El primer paso del procesamiento propiamente dicho de las muestras post-mortem es la recuperación de los espermatozoides de la cola del epidídimo. A pesar de la importancia de este paso, no se ha investigado a fondo, y apenas hay trabajos comparativos de las diversas técnicas disponibles. Cary et al. [30], en un estudio en semen de caballo, compararon el método de flotación (disección de la cola del epidídimo en el seno de un medio) y el de perfusión, sin encontrar diferencias significativas en cuanto a la calidad espermática. Posiblemente, esto se debió a que en ambas técnicas los espermatozoides quedaron inmersos en medio inmediatamente, el cual ejerce un efecto protector.

En el experimento presentado en el capítulo 10, el método de perfusión proporcionó mejores

resultados cualitativos que el de cortes, mientras que el rendimiento (espermatozoides recuperados) resultó ser similar. Aunque la muestra extraída por medio de cortes fue diluida rápidamente en el mismo medio de congelación con el que se realizó la perfusión, el cambio de entorno resultante de cortar la cola del epidídimo afectó claramente a los espermatozoides. Otros trabajos han descrito el efecto negativo de la sangre y sus componentes sobre el semen [31, 32], por lo que parece razonable utilizar métodos que minimicen la manipulación de la cola del epidídimo. Sin embargo, el trabajo de Cary et al. [30] sugiere que la presencia inmediata de medio protector (cubriendo la cola en el momento de efectuar los cortes) es suficiente para evitar estos efectos negativos. Por otra parte, Rijsselaere et al. [32] encontró que los efectos negativos de la presencia de eritrocitos en el semen se expresarían sólo después de la criopreservación; y Verberckmoes et al. [31] detectaron un descenso en la fertilidad de semen cuando se añadía sangre o suero, pero el efecto negativo no fue evidente para las pruebas de evaluación de la calidad espermática.

Además, las pruebas de viabilidad y del estado acrosomal sugieren una calidad pre-congelación superior para las muestras obtenidas mediante perfusión, pero tras la descongelación apenas hubo diferencias. Todo esto indica que las condiciones del método de cortes afectarían sobre todo a una subpoblación de espermatozoides sensibles, los cuales apenas serían perjudicados en las muestras obtenidas por perfusión. Sin embargo, estos espermatozoides sensibles sufrirían más daños durante la criopreservación, explicando el igualamiento de las muestras descongeladas. Sin embargo, el análisis de subpoblaciones mostró que el patrón subpoblacional era más favorable en las muestras obtenidas mediante perfusión, tanto inicialmente como antes y después de la criopreservación. Esto indica que los cambios de medio tuvieron un impacto mucho más profundo que el reflejado en la alteración de la viabilidad de un determinado porcentaje de espermatozoides.

Parece evidente que el método de perfusión debe ser preferido al de cortes en cualquier protocolo para la obtención de muestras epididimarias para bancos de germoplasma. No obstante, debemos investigar a fondo este problema, comparando y mejorando los métodos de extracción. En todo caso, tenemos que tener en cuenta que el método de perfusión es más complejo que el de cortes, para el cual se necesita simplemente una hoja de bisturí, un recipiente limpio y un poco de medio protector. Si, como indicamos más arriba, se confirmase que las muestras extraídas a pie de animal y refrigeradas tras ser diluidas en medio protector soportan mejor el período post-mortem que aquellas que se dejan en la cola del epidídimo, el método de cortes podría considerarse como una buena alternativa en el caso de que hubiese que improvisar la recogida de la muestra (por ejemplo, en caso de muerte inesperada de un animal valioso lejos del laboratorio).

11.5 Consideraciones finales

Los estudios realizados han proporcionado información útil y potencialmente aplicable al diseño y gestión de bancos de germoplasma de rumiantes silvestres. Los resultados de los estudios sobre el efecto del tiempo post-mortem y la estacionalidad han sugerido unos períodos en los que el

procesado de las muestras sería más recomendable: antes de 48 h post-mortem y durante la berrea. La conveniencia de recoger muestras durante el resto de la época reproductiva es una opción a considerar, pero requiere de estudios más detallados. El método de perfusión es más recomendable que el de cortes para la obtención de muestras, aunque éste último, por su sencillez, no sería descartable en determinadas situaciones. Por último, el método de análisis de subpoblaciones propuesto funcionó adecuadamente y pudo ser utilizado para completar los otros estudios, siendo especialmente útil para determinar el alcance de las diferencias entre los dos métodos de recogida.

Las recomendaciones sugeridas en este trabajo han de encuadrarse en el contexto de un banco de germoplasma para una especie que no esté especialmente amenazada, y de la que podemos obtener un número importante de muestras anualmente debido a actividades cinegéticas. Este banco de germoplasma estaría, por lo tanto, orientado a la mejora genética (trofeos) y a prevenir riesgos futuros (endogamia por aislamiento de poblaciones, reducción acusada de machos de buena calidad, epidemias, etc.), presentes ya en algunas poblaciones semicerradas de ciervo ibérico. Por lo tanto, este tipo de banco podría permitirse el descartar un buen número de muestras que presuntamente no tendrían buena calidad (excesivo tiempo post-mortem o fuera de la berrea, por ejemplo). Por supuesto, la estrategia variaría con el propósito del banco de germoplasma, o dependiendo del valor de las muestras. Así, en el caso de animales especialmente valiosos, o de bancos destinados a la preservación de especies amenazadas, se podría optar por la preservación de muestras que no reuniesen los criterios recomendados. En este caso serían especialmente interesantes los criterios de valoración descritos, ya que sirven para etiquetar correctamente las diferentes partidas de semen almacenadas en un banco de germoplasma. Actualmente disponemos de técnicas que nos permitirían utilizar muestras de baja calidad (FIV, ICSI) o seleccionar subpoblaciones espermáticas especialmente aptas (swim-up, filtración, fraccionamiento), de manera que incluso muestras seminales con un diagnóstico de calidad dudoso podrían ser utilizadas en programas de recuperación.

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Capítulo 12

Conclusiones

A continuación se enumeran las conclusiones derivadas de los resultados obtenidos en los trabajos presentados en esta Tesis.

1. El tiempo post-mortem influyó negativamente en los parámetros cualitativos de las muestras espermáticas, aunque de forma desigual; la calidad resultó ser aceptable hasta 48 h post-mortem, pero fue mejor a tiempos post-mortem menores, por lo que es recomendable procesar las muestras lo antes posible.

2. Las muestras epididimarias recogidas durante la berrea dieron los mejores resultados cuantitativos, y los cualitativos fueron aceptables; en cambio, las muestras recogidas durante el resto de la estación reproductiva (después de la berrea) fueron las de mejor calidad. No obstante, los resultados de estas muestras están condicionados por otros factores, referidos en la conclusión número 6. Las muestras recogidas fuera de la estación reproductiva (después de la época de monta) dieron resultados muy pobres.

3. El modelo estadístico propuesto para el estudio de subpoblaciones espermáticas ha demostrado su utilidad y flexibilidad, proporcionando información no disponible con las técnicas convencionales.

4. En el estudio de la movilidad durante el proceso de dilución-refrigeración-criopreservación, se encontró una subpoblación espermática de alta calidad (espermatozoides rápidos y progresivos), que se relacionó con una mejor recuperación de la viabilidad espermática tras la criopreservación.

5. El análisis de subpoblaciones espermáticas a distintos tiempos post-mortem confirmó los resultados del primer estudio sobre el efecto del tiempo post-mortem, indicando severos cambios en el patrón de subpoblaciones tras 48–72 h post-mortem, señalando un punto crítico para la calidad de las muestras.

6. El patrón de subpoblaciones varió a lo largo de la época reproductiva, con el predominio creciente de una subpoblación con buena movilidad (espermatozoides rápidos y progresivos), después de la berrea. Sin embargo, podría estar relacionado con procesos de hipermaduración en la cola del epidídimo, que comprometerían la calidad espermática, por lo que las muestras recogidas después de la berrea podrían no ser adecuadas para su preservación en bancos de germoplasma.

7. El rendimiento productivo (cantidad de espermatozoides recuperados de la cola del epidídimo) de las técnicas de cortes o perfusión fue muy similar; sin embargo, la técnica de perfusión produjo muestras de mejor calidad y menor contaminación, por lo que debe ser la técnica preferente en la obtención de muestras epididimarias post-mortem. No obstante, la técnica de cortes puede ser una alternativa útil en casos especiales.

Capítulo 13

Conclusions

Here follow the conclusions derived from the results obtained in the studies presented in this Thesis.

1. Post-mortem time affected negatively to the qualitative parameters of sperm samples, although some of them were more resilient than others; quality was acceptable before 48 h post-mortem, but it was better when post-mortem time was shorter, thus it is recommendable to process the samples as early as possible.

2. The samples collected during the rut rendered the better quantitative results, and their quality was acceptable; however, the samples collected after the rut rendered the best quality. Nevertheless, their results are conditioned by other factors, referred in the conclusion number 6. Epididymal samples collected out of the mating season rendered poor results.

3. The statistical model proposed for the study of sperm subpopulations demonstrated its utility and flexibility, providing information non available using conventional techniques.

4. A high-quality sperm subpopulation (rapid and linear sperm), found in the analysis of epididymal samples through the process of extension-refrigeration-cryopreservation, was related to better recovery of sperm viability after cryopreservation.

5. The analysis of sperm subpopulations at different times post-mortem confirmed the results of the first study on the effect of post-mortem time, showing drastic changes in the subpopulation pattern after 48–72 h post-mortem, and indicating a critical point regarding the quality of the samples.

6. The subpopulation pattern varied through the mating season, indicating the predominance of a subpopulation with good motility (rapid and linear sperm), after the rut. Nonetheless, this could be related to hypermaturation in the cauda epididymis, which may compromise sperm quality; thus, the samples collected after the rut might not be accepted in germplasm banks in a routine basis.

7. The yield (spermatozoa recovered from the cauda epididymis) of the cuts and flushing methods were very similar; however, the flushing method yielded samples of better motility (subpopulational patterns) and lower contamination, thus, it is a preferential method for post-mortem sperm recovery from the epididymis. Nevertheless, the cuts method might be used as an useful alternative in especial cases.

Agradecimientos

Agradezco la ayuda y amistad de todas aquellas personas que de una manera u otra han participado en este trabajo y me han acompañado durante los últimos años. Quiero concretar mis agradecimientos a mis directores, Paz, Luis y Paulino, por la confianza depositada en mí desde el primer momento. A todos mis compañeros del Departamento, sobre todo a Camino y Kaabi, sin los cuales no habría podido realizar muchos de los estudios aquí presentados. A Tere y Pepín, por enseñarme la técnica del swim-up en Zaragoza. A los Drs. Heriberto Rodríguez, Anders Johanisson y Paul Bartels, por su ayuda y consejos durante mis estancias en Uppsala (Suecia) y Pelindaba (Sudáfrica), y a todos aquellos que conocí allá y que tanto me ayudaron.

Muy especialmente doy las gracias a mi familia su apoyo incondicional, su cariño y su paciencia. Y a todos mis amigos, por su amistad.

Además, este estudio no podría haberse realizado sin la colaboración de mucha gente que me proporcionó las muestras procedentes de los animales cazados, por lo que doy las gracias a los miembros del Servicio Territorial de Caza y Pesca de la Junta de Castilla y León, a los celadores de las reservas de caza de Picos de Europa, Mampodre y Ancares, y a Carlos Bernabé (Cáceres).

Finalmente, hago extensivo mi agradecimiento a todos mis compañeros investigadores en precario, de la FJI (Federación de Jóvenes Investigadores-Precarios) y de Precarios-León (Asociación de Jóvenes Investigadores de León), por la gran labor realizada para dignificar la labor de los investigadores y su contribución a un diseño racional y coherente de la carrera investigadora.

Apéndice I: anexo gráfico

Figura 1 Localización de las zonas de caza donde se obtuvieron las muestras utilizadas en este trabajo. En el norte de la península, reservas de caza de Ancares (zona Oeste) y Mampodre y Picos de Europa (zona Este); hacia el centro, diversas reservas cinegéticas particulares (Cáceres).

Figura 2 El ciervo rojo ibérico (*Cervus elaphus hispanicus*) fue la especie objeto de atención en este trabajo. Su temporarada reproductiva, que ocupa todo el otoño, se inicia con un período de intensa competición entre machos, denominada berrea.

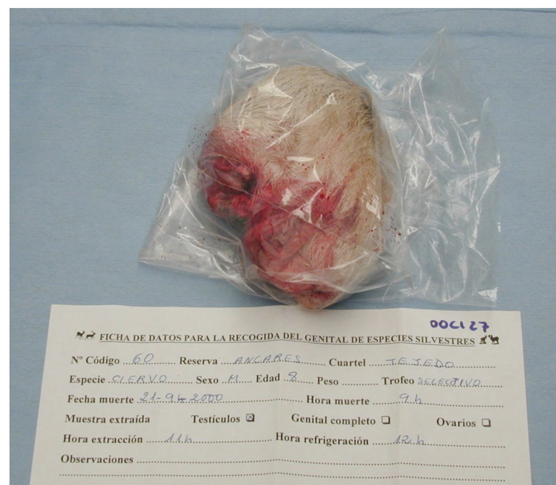


Figura 3 Los testículos obtenidos en actividades cinegéticas fueron aislados en bolsas de plástico e identificados por fichas en las que se recogieron los datos básicos de la muestra. Los testículos y epidídimos permanecieron en todo momento protegidos por el escroto, y a 5 °C.

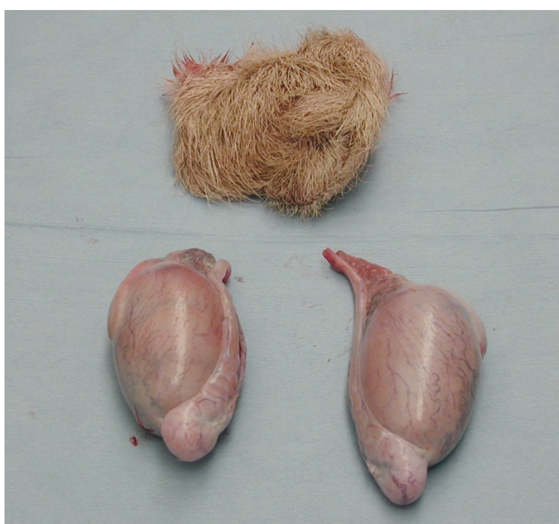


Figura 4 Las muestras fueron disecadas, separando los testículos con los epidídimos y los vasos deferentes del resto de los tejidos, limpiando bien los restos de sangre y otros fluídos que hubiesen podido acumularse entre la túnica vaginal y los testículos.

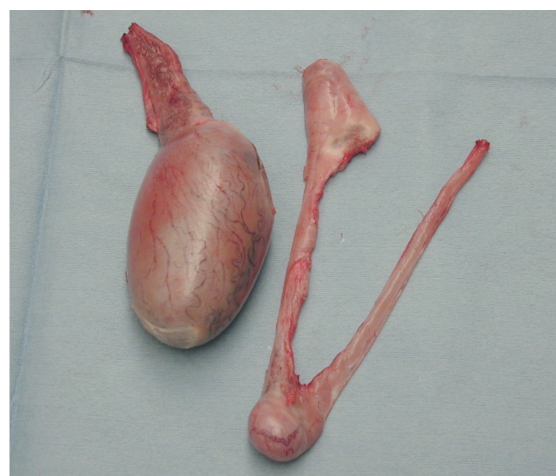
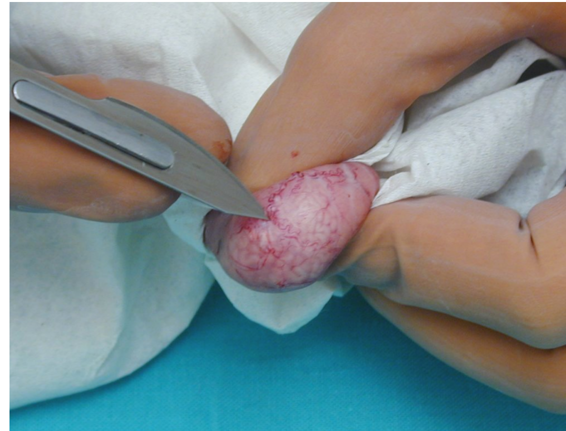


Figura 5 La disección se completó separando con cuidado el epidídimo y el vaso deferente de cada testículo. En la imagen se puede observar un testículo con el ligamento pampiniforme aún adherido, y el epidídimo con el vaso deferente. La diferenciación morfológica del epidídimo es evidente, distinguiéndose, de la parte superior de la fotografía a la inferior, cabeza, cuerpo y cola.

Figura 6 Para extraer la muestra seminal de la cola del epidídimo por medio de cortes, primeramente se limpió bien su superficie. Los vasos sanguíneos superficiales fueron puncionados y vaciados. La sangre se retiró absorbiéndola con papel toalla.



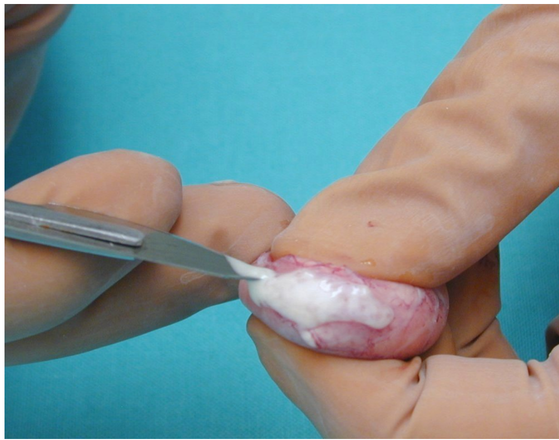


Figura 7 Después de eliminar la sangre de los vasos superficiales, se realizaron uno o dos cortes longitudinales con una hoja de bisturí nueva. En la fotografía se observa el líquido blanquecino rico en espermatozoides emergiendo de los túbulos cortados.

Figura 8 La papilla seminal se recogió con la hoja de bisturí, depositándola en un cono de plástico. Para asegurar la recogida de la mayor parte del contenido de la cola del epidídimo, se efectuaron más cortes en los túbulos expuestos, a la vez que se realizaba una ligera presión.

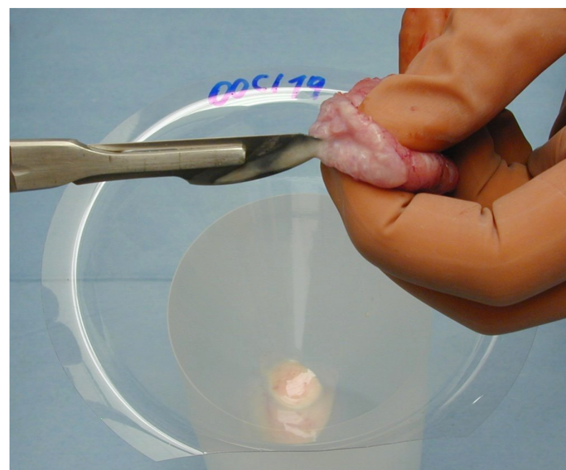
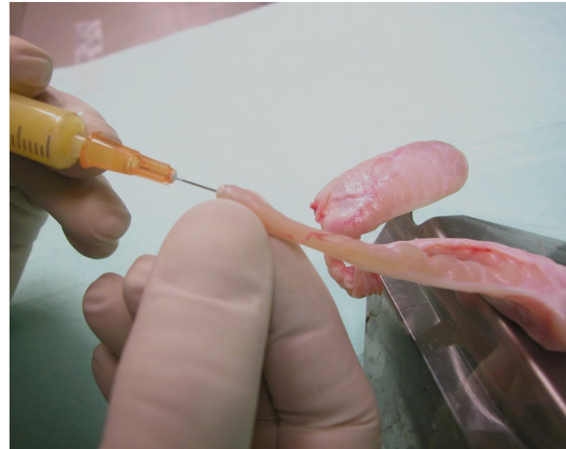


Figura 9 La recogida seminal mediante perfusión del epidídimo se realizó canulando el vaso deferente con una aguja hipodérmica despuntada e inyectando medio de congelación con una jeringa.



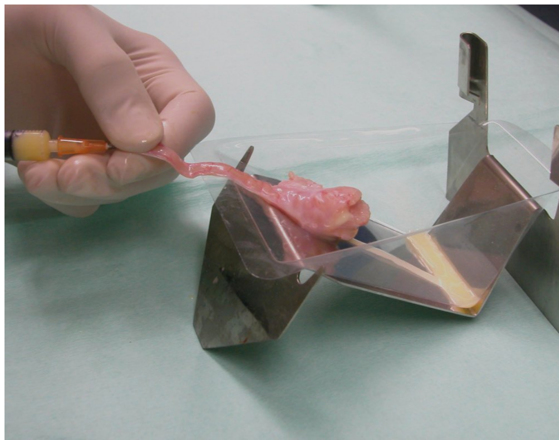


Figura 10 El contenido de la cola del epidídimo y el medio inyectado emergieron por la zona de corte efectuada al separar la cola del cuerpo del epidídimo.

Figura 11 Para asegurar la recuperación de todo el contenido epididimario, tras inyectar el medio de congelación se inyectó aire con la jeringa, para empujar el líquido remanente del túbulo epididimario.

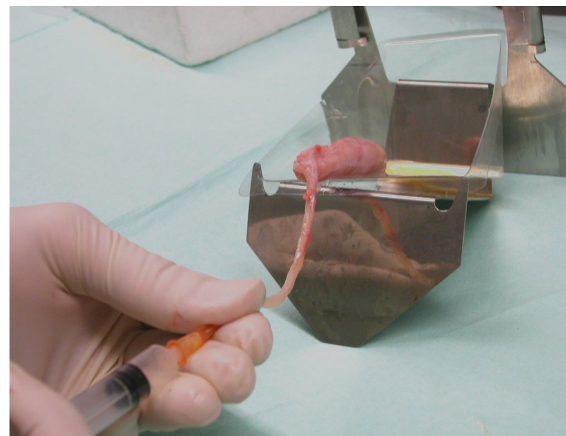
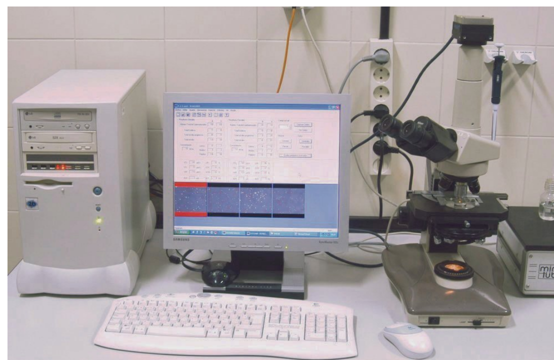


Figura 12 Equipo CASA de análisis de movilidad espermática utilizado en este trabajo. El equipo se compone de un microscopio de contraste de fases Nikon Labophot-2, equipado con un objetivo $\times 10$ de contraste negativo, y complementado con una pletina calentable y una cámara para captura de imagen (en este caso, una cámara digital Basler). La muestra se monta en una cámara de recuento Makler (10 μm de profundidad) precalentada. La señal de la cámara es procesada en un ordenador con el software CASA SCA2002 (Microptic, Barcelona).



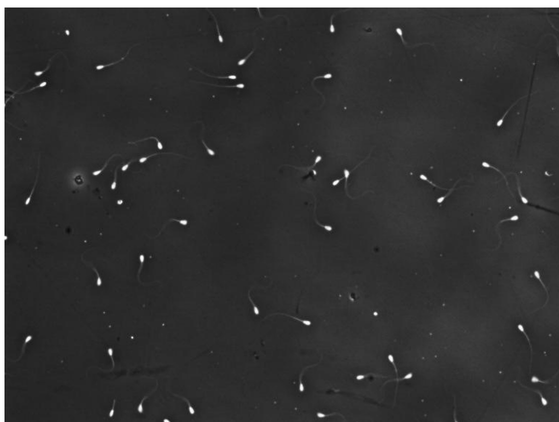
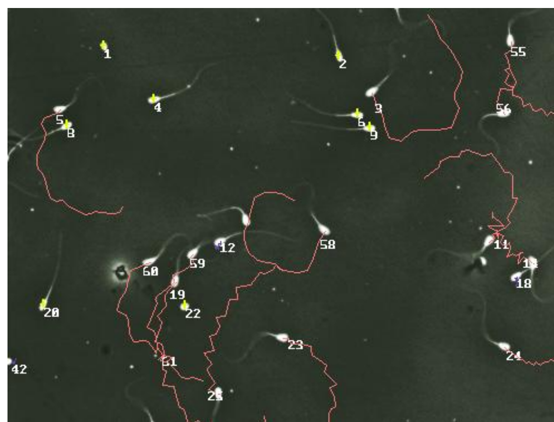


Figura 13 Imagen extraída de una secuencia capturada para analizar la movilidad espermática. El fondo oscuro y las células claras es una característica definitoria del contraste de fases negativo.

Figura 14 Resultado del análisis CASA de la secuencia representada por la imagen 13. Puede verse que los elementos detectados como espermatozoides han sido numerados y se han sobreimpresionado sus trayectorias (los eventos inmóviles son identificados por cruces amarillas). El análisis de cada secuencia puede ser editado parcialmente para eliminar trayectorias o eventos incorrectos. Después de analizar una muestra, la información de cada espermatozoide (descriptores de movilidad) puede ser volcada a una base de datos para el análisis estadístico.



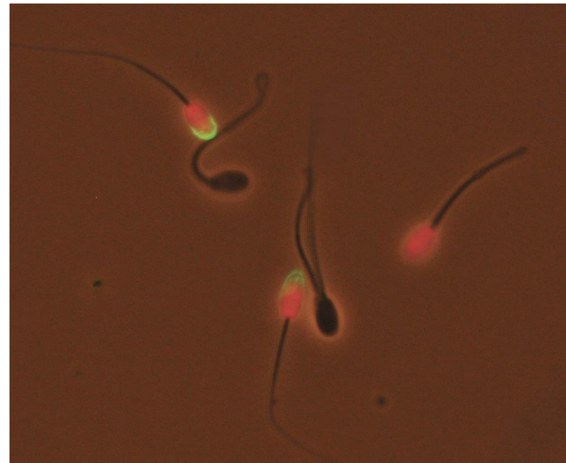


Figura 15 Tinción con los colorantes fluorescentes ioduro de propidio (IP) y PNA-FITC (aglutinina de cacahuete conjugada con fluoresceína). El IP tiene gran afinidad por el ADN, y penetra únicamente en los espermatozoides con la membrana plasmática dañada (no viables), ya que no puede atravesar membranas fosfolipídicas intactas. En cambio, la PNA es una lectina con afinidad por ciertas glicoproteínas de la membrana acrosomal, por lo que tiñe de verde aquellos acrosomas reaccionados o dañados. En la imagen se pueden observar dos espermatozoides sin tinción (viables con el acrosoma intacto), uno teñido de rojo (no viable con el acrosoma intacto) y dos con doble tinción (no viables y con acrosoma dañado).

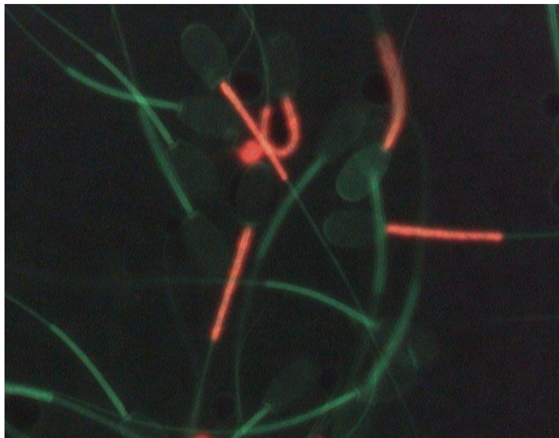


Figura 16 Tinción con el colorante fluorescente JC-1. El JC-1 se acumula en las mitocondrias, formando agregados (fluorescencia naranja) si el potencial de membrana es alto, mientras que permanece en forma monomérica (fluorescencia verde) si el potencial de membrana es bajo. De esta manera, se puede discriminar entre espermatozoides con mitocondrias activas (tracto intermedio naranja) o inactivas (tracto intermedio verde).

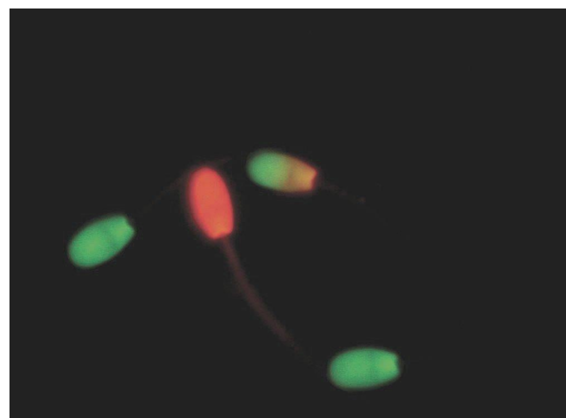


Figura 17 Tinción con el LIVE/DEAD Sperm Kit. Este kit está compuesto por dos colorantes fluorescentes, ioduro de propidio (IP) y SYBR-14. Los espermatozoides viables son teñidos únicamente por SYBR-14, que tiene (verde). Este colorante puede atravesar membranas fosfolipídicas intactas, uniéndose al DNA nuclear. Si las membranas están dañadas, el IP (fluorescencia roja) puede unirse al DNA, desplazando al SYBR-14 y marcando diferencialmente a los espermatozoides no viables. Cuando el daño de membrana no es muy considerable se observa una mezcla de ambas coloraciones (espermatozoides ≪moribundos≫).

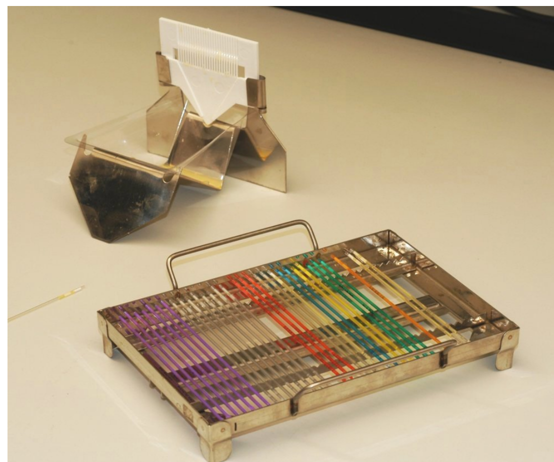


Figura 18 Las muestras seminales criopreservadas durante las experiencias descritas en este trabajo se empaquetaron previamente en pajuelas de 0,25 mL de capacidad, que fueron selladas y colocadas en soportes metálicos para su congelación.

Figura 19 Las dosis seminales fueron congeladas en un biocongelador Planer, siguiendo una rampa de -20 °C/min. Los soportes con las pajuelas fueron introducidos en el biocongelador a una temperatura inicial de 5 °C, y fueron extraídos cuando se alcanzaron -100 °C. Las pajuelas se pasaron directamente a nitrógeno líquido, y toda la manipulación posterior se realizó siempre con las pajuelas sumergidas.

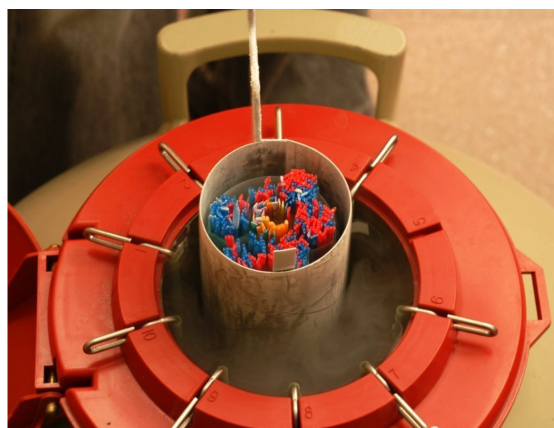


Figura 20 Las dosis congeladas se colocaron en gobelets identificados, los cuales se almacenaron en tanques isotermos llenos de nitrógeno líquido. Este tipo de tanques son los componentes más importantes de los bancos de germoplasma, ya que las muestras pueden permanecer inalteradas por tiempo casi indefinido, debido a las bajas temperaturas de almacenamiento (-150 °C en almacenamiento ≪seco≫ —vapores de nitrógeno líquido— y -196 °C si permanecen sumergidas en nitrógeno líquido).

Apéndice II: código del programa utilizado para el análisis de subpoblaciones

En esta sección se muestra el código del programa utilizado para analizar los datos de movilidad y extraer las correspondientes subpoblaciones. El lenguage de programación es el propio de SAS/STAT™ (SAS Institute, Cary, CA). Sin embargo, se podría reproducir en cualquier paquete estadístico con capacidad para realizar análisis de agrupamientos y de componentes principales. El texto encerrado entre los comandos /* y */ corresponde a comentarios y es ignorado por el intérprete de SAS.

Para la entrada se utilizó un archivo CSV que contenía los datos de los espermatozoides, con cada registro identificado según el tipo de experimento a realizar. Los primeros procedimientos del programa deberían ser modificados de acuerdo con la estructura de este archivo de entrada y las variables que contenga.

```
/*
PROGRAMA PARA REALIZAR UN ANÁLISIS DE SUBPOBLACIONES ESPERMÁTICAS
A PARTIR DE DATOS DE MOVILIDAD.

Las palabras clave de SAS se transcriben en mayúscula. Las
variables se transcriben en minúscula.

Este programa es software libre; puede ser redistribuido y/o
modificado bajo los términos de la GNU General Public License,
tal y como es publicada por the Free Software Foundation; se
aplica la versión 2 de la licencia o (a su criterio) cualquier
versión posterior.

Los términos de la GNU General Public License pueden encontrarse
en http://www.gnu.org/licenses/gpl.html. La versión digital de este
programa debería estar acompañada por una copia de la GNU General
Public License; en caso contrario, contacte con the Free Software
Foundation, Inc., 59 Temple Place, Suite 330, Boston, MA 02111-1307
USA.

Probado en SAS v. 8 sobre plataforma WinXP.
```

```
León, 31 de agosto de 2004
*/

/*
Entrada de datos
*/

PROC IMPORT OUT=work.data DATAFILE="D:\entrada\datos-CASA.csv"
     DBMS=CSV REPLACE ;
     GETNAMES=YES ;
RUN ;

/*
Primer filtro de datos. Las condiciones dentro de este
procedimiento se pueden modificar según el diseño de nuestra base
de datos y los factores que se deseen tener en cuenta.
*/

DATA datos ;
     SET DATA ;
     WHERE expt='p' AND puntos>4 AND outlier='n' ;
RUN ;

/*
Análisis de componentes principales (ACP) y estandarización
de los componentes principales producidos.
*/

PROC PRINCOMP STD OUT=prin ;
     VAR vcl vsl vap lin str wob alh bcf ;
RUN ;

/*
Primer análisis de clústers (no jerárquico).
El número de clústers de salida está fijado a 15, pero puede
ser modificado.
Las variables prin1 y prin2 son los dos primeros componentes
principales producidos por el ACP. Dependiendo del criterio de
selección, pueden incluirse más o menos componentes principales.
*/

PROC FASTCLUS MAXCLUSTERS=15 MAXITER=1000 DATA=prin MEAN=clus
OUT=fileout ;
     VAR prin1 prin2 ;
RUN ;
```

```
/*
Se exporta la matriz de datos a un archivo Excel 2000.
Cada registro (espermatozoide) está identificado con un
número del 1 al 15, dependiendo del clúster en el que haya
sido incluido.
*/

PROC EXPORT DATA=fileout OUTFILE="D:\salida\clusters.xls"
     DBMS=EXCEL2000 ;
RUN ;

/*
Segundo análisis de clústers (jerárquico).
*/

PROC CLUSTER DATA=clus OUTTREE=treedata METHOD=AVERAGE CCC
     PSEUDO ;
     VAR prin1 prin2 ;
     ID cluster ;
RUN ;

/*
Pasos previos para dibujar el gráfico de CCC, pseudo-F
y pseudo-t2 vs. no. de clústers.
La estandarización de las tres variables a media=0 y SD=0 es
necesaria para poder representarlas simultáneamente en el mismo
gráfico.
*/

PROC STANDARD MEAN=0 STD=1 ;
     VAR _ccc_ _psf_ _pst2_ ;
RUN ;

PROC SORT ;
     BY _ncl_ ;
RUN ;

/*
Parámetros del gráfico de CCC, pseudo-F y pseudo-t2 vs. no. de
clústers.
*/

LEGEND1 LABEL=NONE SHAPE=SYMBOL(4,1) POSITION=(TOP CENTER INSIDE)
     MODE=SHARE ;
SYMBOL1 COLOR=RED INTERPOL=JOIN VALUE=DOT HEIGHT=1 ;
SYMBOL2 COLOR=BLUE INTERPOL=JOIN VALUE=DOT HEIGHT=1 ;
```

```
SYMBOL3 COLOR=BLACK INTERPOL=JOIN VALUE=DOT HEIGHT=1 ;

/*
Dibujo del gráfico de la evolución de CCC, pseudo-F y
pseudo-t2 vs. no. de clústers.
Las tres variables aparecen en el mismo gráfico, para
poder señalar aquellos puntos en los que su evolución
indica un posible punto de corte.
*/

PROC GPLOT ;
     WHERE _ncl_<12 ;
     PLOT _ccc_*_ncl_ _psf_*_ncl_ /OVERLAY LEGEND=LEGEND1 ;
     PLOT2 _pst2_*_ncl_ ;
RUN ;

/*
Este paso dibuja el dendrograma y produce un conjunto de datos
con las correspondencias entre los clústers del procedimiento
jerárquico y los del procedimiento no jerárquico.
En este paso hay que fijar en número final de clústers,
dependiendo de la decisión tomada por el análisis del gráfico
de CCC, pseudo-F y pseudo-t2 vs. no. de clústers.
*/

PROC TREE HORIZONTAL SPACES=2 DATA=treedata NCLUSTERS=3
     OUT=treeout ;
     ID cluster ;
     COPY prin1 prin2 ;
     HEIGHT _rsq_ ;
RUN ;

/*
Se exportan las correspondencias a un archivo Excel 2000.
*/

PROC EXPORT DATA=treeout OUTFILE="D:\salida\clusters-finales.xls"
     DBMS=EXCEL2000 ;
RUN ;

QUIT ;

********** Fin del programa **********
```

La salida de este programa produce dos archivos, uno con los datos originales, pero con cada espermatozoide asignado a un clúster producido en el procedimiento no jerárquico. El segundo

archivo contiene sólamente las correspondencias de los clústers obtenidos en el procedimiento no jerárquico, con aquellos obtenidos en el procedimiento jerárquico. Estas nuevas asignaciones hay que incluirlas en el archivo de datos, manualmente o mediante macros o scripts.

Generalmente, habrá que realizar dos o tres pasadas al archivo de datos inicial con este programa. Por lo general, antes de la primera pasada no se tiene información sobre la estructura de los datos. Esta pasada indica el número adecuado de componentes principales que se deben tomar, si existen outliers (en cuyo caso habría que incluir un procedimiento que actuase de filtro, o editar el archivo de entrada), y cuál es el número final de clústers. Sólo después de ajustar todos estos parámetros se puede realizar el análisis definitivo.

Apéndice III: justificantes de aceptación de artículos

En las páginas siguientes se incluyen los justificantes de aceptación de los artículos presentados en esta Tesis y que aún no han sido publicados, expedidos por las revistas correspondientes.

THERIOGENOLOGY
European Editorial Office
Istituto di Anatomia degli Animali Domestici
via Celoria, 10 – 20133 Milano ITALY

Dr. Felipe Martinez
Reproduction and Obstetrics,
Veterinary Clinic Hospital,
University of León,
24071, León,
Spain.

Milano, 01 October 2004

Manuscripts accepted

Dear Dr Martinez

this is to certify that the manuscripts entitled "Decay of sperm obtained from epididymes of wild ruminants depending on postmortem time" and "Season effect on genitalia and epididymal sperm from Iberian red deer, roe deer and chamois" have been accepted and are being processed for publication

Best regards

Prof. Fulvio Gandolfi
Co-Editor in Chief

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