UNIVERSIDAD DE CÓRDOBA

FACULTAD DE VETERINARIA

Departamento de Medicina y Cirugía Animal

"OPTIMIZACIÓN DE LA METODOLOGÍA DE PRESERVACIÓN DEL ESPERMA DE ASNO ANDALUZ (EQUUS ASINUS)"

Memoria para optar al grado de Doctor presentada por

Daniel Acha Valls

Bajo la dirección de los Doctores

Jesús M. Dorado Martín
Manuel Hidalgo Prieto

Córdoba, 2015.

TITULO: *Optimización de la metodología de preservación del esperma del asno andaluz (Equus Asinus)*

AUTOR: *Daniel Acha Valls*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

www.uco.es/publicaciones
publicaciones@uco.es

Este trabajo ha sido financiado por el Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (RZ2009-00006-00-00), Ministerio de Economía y Competitividad.

Tesis Doctoral como compendio de publicaciones

1. Sperm motility patterns in Andalusian donkey (*Equus asinus*) semen: Effects of body weight, age, and semen quality. J. Dorado, **D. Acha**, M.J. Gálvez, I. Ortiz, J.J. Carrasco, B. Díaz, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo. *Theriogenology* 2013, 79: 1100-1109.

2. Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (*Equus asinus*). J. Dorado, **D. Acha**, I. Ortiz, M.J. Gálvez, J.J. Carrasco, B. Díaz, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo. *Animal Reproduction Science* 2013, 143: 64-71.

3. Effect of extender and amino acid supplementation on sperm quality of cooled-preserved Andalusian donkey (*Equus asinus*) spermatozoa. J. Dorado, **D. Acha**, I. Ortiz, M.J. Gálvez, J.J. Carrasco, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo. *Animal Reproduction Science* 2014, 146: 79-88.

4. Freezability of Andalusian donkey (*Equus asinus*) spermatozoa: effect of extenders and permeating cryoprotectants. **D. Acha**, M. Hidalgo, M.J. Gálvez, I. Ortiz, J.J. Carrasco, V. Gómez-Arrones, J. Dorado. *Reproduction, Fertility and Development* 2015, accepted 4 June 2015.

TÍTULO DE LA TESIS: Optimización de la metodología de preservación del esperma de asno Andaluz (*Equus asinus*)

DOCTORANDO: ***Daniel Acha Valls***

(i) INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

D. *Jesús M. Dorado Martín* y **D. *Manuel Hidalgo Prieto***, Profesores del Departamento de Medicina y Cirugía Animal de la Facultad de Veterinaria de la Universidad de Córdoba,

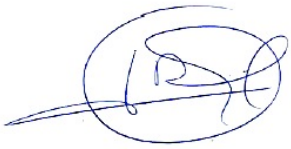
INFORMAN:

Que el trabajo de tesis presentado por **D. Daniel Acha Valls**, titulado "***Optimización de la metodología de preservación del esperma de asno Andaluz (Equus asinus)"*** ha sido realizado bajo nuestra dirección y cumple con los artículos 24 y 35 de la norma reguladora de los Estudios de Doctorado de la Universidad de Córdoba para su presentación como compendio de publicaciones así como para obtener la mención internacional.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 24 de junio de 2015

Firma de los directores



Fdo: Jesús M. Dorado Martín

Manuel Hidalgo Prieto

Agradecimientos

Agradecimientos

- Al Dr. Jesús M. Dorado Martín. Por haber confiado en mí para llevar a cabo este proyecto, por enseñarme que con esfuerzo y dedicación, las cosas salen adelante. Por su trato diario, siempre educado y sin un mal gesto en todos estos años. Por tantas horas de trabajo. Por su apoyo y amistad.
- Al Dr. Manuel Hidalgo Prieto, Director de esta Tesis Doctoral, por su humildad y por todos sus consejos. Por su apoyo, amistad, y todos esos cafés que nos hemos tomado en su despacho.
- A mis compañeros Isabel Ortiz, María José Gálvez, Lucrecia Alcaraz, Sebastián Demyda y Maite Urbano Luque, porque, gracias a ellos, he ido al departamento, día tras día, con ganas e ilusión. Por ayudarme siempre que lo he necesitado.
- Al Centro de Selección y Reproducción Animal (CENSYRA) que cedió sus instalaciones y animales.
- Al personal del CENSYRA, D. Juan Jesús Carrasco y Dña. Vanesa Gómez-Arrones, por su ayuda y colaboración.
- A la Fundación Casa del Burro de Rute, en la persona de D. Pascual Rovira García, por la cesión de los animales.
- Al personal del Hospital Clínico Veterinario de la Universidad de Córdoba que ha colaborado con las recogidas.
- A mi amigos Jordi Crusellas, Guillermo Vizuete, Álvaro Benítez, Rocío Gómez, Macarena Sánchez Martel y Paula Tarrero Martín.
- A Rafael Moral y a Javier Barberá, personas que lo dan todo sin esperar nada a cambio. Por tener un corazón que no les cabe en el pecho. Por todas esas risas que nos hemos echado, y las que nos quedan.
- A María Rodríguez Amores, mi María. Por su amistad, su positividad diaria y por sus consejos, claros y concisos, como a mí me gustan.
- A la Dra. Carmen Pineda, por su amistad y apoyo diario. Por ayudarme incondicionalmente.

- A mi hermano Ramón Acha y a mi cuñada María Martínez, que aunque nos veamos poco, se que siempre estarán ahí. Que Londres y Cádiz tampoco están tan lejos.
- A mis tíos Juan Valls, Pepa e Isabel Acha.
- A mis padres, Tomás Acha y Esperanza Valls, y a mi tía, María Valls, mis referentes. Por tantas cosas, que no hay papel ni tinta para escribirlas. Gracias.

Índice

Índice

Resumen

Resumen

La raza asnal Andaluza está catalogada actualmente como raza autóctona española en peligro de extinción (RD 2129/2008, Catálogo Oficial de Razas de Ganado de España). Hasta la fecha, sólo 94 garañones están considerados como individuos de alto valor genético (DAD-IS-FAO 2013). En este contexto, es necesaria la aplicación de técnicas de reproducción asistida como la inseminación artificial y la criopreservación de espermatozoides con el objetivo de mejorar la variabilidad genética de la raza y evitar así su extinción. Para ello, consideramos imprescindible realizar un estudio previo de las características reproductivas y los parámetros seminales propios de la raza. Hoy en día, en el análisis de las características seminales de numerosas razas de mamíferos ha sido objeto de gran interés el estudio de las subpoblaciones espermáticas presentes en los eyaculados, observándose una correlación directa entre la presencia de determinadas subpoblaciones espermáticas, la resistencia a la criopreservación y las tasas de fertilidad obtenidas. Por lo tanto, es importante estudiar la distribución de las subpoblaciones espermáticas que conforman el eyaculado del asno Andaluz para establecer la calidad espermática de las muestras seminales recolectadas, así como para predecir su congelabilidad y fertilidad. Actualmente, son escasos los estudios publicados sobre refrigeración y congelación del semen de burro, siendo inexistentes en el caso de la raza asnal Andaluza. Por ello, es necesario establecer un protocolo adecuado, así como seleccionar aquellos diluyentes y crioprotectores con los que obtener muestras seminales con buena calidad tras los procesos de refrigeración y crioconservación.

En la primera publicación se estudiaron las características seminales (volumen, pH, concentración, movimiento y morfología espermática) del eyaculado del asno andaluz con el objetivo de poder establecer los parámetros de normalidad propios de la raza. Asimismo, se comprobó la existencia de diferentes subpoblaciones de espermatozoides dentro del eyaculado del asno andaluz así como la influencia del individuo y del eyaculado dentro de un mismo individuo en su frecuencia de distribución. Además, se investigó la correlación existente entre la edad y el peso corporal del garañón, las características de calidad espermática y la distribución de las subpoblaciones espermáticas. Para ello, se recolectaron un total de 60 eyaculados de 12

asnos andaluces (5 eyaculados por burro), con edades comprendidas entre los 4 y 15 años (edad media: 9,9 ± 0,4 años) y pesos corporales de 313 a 435 kg (peso medio: 381,1 ± 5,8 kg). Mediante un análisis estadístico multivariante se evidenció la existencia de cuatro subpoblaciones espermáticas (sP) con patrones cinéticos específicos: sP1, constituida por espermatozoides lentos y no progresivos (15,4%); sP2, constituida por espermatozoides moderadamente lentos pero progresivos (35,9%); sP3, constituida por espermatozoides muy activos pero con movimiento no progresivo (18,5%); sP4, constituida por espermatozoides muy activos y con movimiento progresivo (30,2%). La proporción de las diferentes subpoblaciones espermáticas existentes dentro del eyaculado no se vio afectada por el peso o la edad del garañón ($P>0,05$). Sin embargo, factores como el individuo, el eyaculado, el movimiento total o la concentración media del eyaculado sí influyeron significativamente ($P<0,05$) en la distribución de las subpoblaciones espermáticas dentro del eyaculado. Por otra parte, se demostró una correlación significativa ($P<0,05$) entre el peso corporal de los garañones y el pH del eyaculado, el movimiento (porcentaje de movimiento total y progresivo) y la morfología espermática. Por el contrario, la correlación entre la edad de los garañones y las características del eyaculado fue baja y no significativa ($P>0,05$).

En la segunda publicación se compararon las características seminales evaluadas en el análisis espermático convencional así como los patrones de movimiento de las diferentes subpoblaciones espermáticas presentes en los eyaculados de asnos con diferente fertilidad, y se estableció su relación con la fertilidad *in vivo*. Durante el periodo experimental se recolectaron un total de 30 eyaculados de 6 asnos andaluces. La fertilidad de los garañones fue clasificada atendiendo a la tasa de gestación por ciclo, considerando a aquellos asnos con tasas de gestación por ciclo ≥ 60% como "fértiles" ($n = 3$) y a aquellos con tasas de preñez por ciclo < 40% como "subfértiles" ($n = 3$). Se observaron diferencias significativas ($P<0,001$) entre el grupo de asnos "fértiles" y el grupo de "subfértiles" para los valores de movimiento total y progresivo así como para la velocidad lineal. Por otro lado, se demostró que las características del eyaculado que producen un aumento significativo ($P<0,05$) de la tasa de gestación por ciclo son el movimiento total ($r = 0,37$) y progresivo ($r = 0,53$), la velocidad curvilínea ($r = 0,44$), la rectitud ($r = 0,39$), la frecuencia de cruzamiento ($r = 0,44$) y el volumen del eyaculado ($r = 0,53$). También evidenciamos que tres de las cuatro subpoblaciones espermáticas (sP2, sP3 y sP4) existentes en el eyaculado del asno andaluz, descritas con anterioridad

en la primera publicación, están significativamente (P<0,05) correlacionadas con la fertilidad del garañón (sP2, r = 0,54; sP3, r = 0,45; sP4, r = 0,56). Así, se observó una presencia significativamente (P<0,001) baja de la Subpoblación 4 en los eyaculados de los asnos pertenecientes al grupo de "subfértiles". En conclusión, este estudio demostró la relación existente entre los parámetros de movimiento espermático valorados *in vitro* mediante el sistema de análisis de esperma asistido por ordenador (CASA) y la fertilidad *in vivo* del eyaculado de asno.

En la tercera publicación buscamos seleccionar un diluyente apropiado para refrigerar semen de asno andaluz. Para ello, se evaluó la eficacia de dos diluyentes comerciales ampliamente utilizados para la refrigeración de semen equino (Gent® A e INRA 96®) para preservar semen de asno andaluz a 5ºC durante 72 horas. Además, se estudió el efecto de la incorporación en el diluyente de refrigeración de tres aminoácidos (glutamina, prolina y taurina) a diferentes concentraciones (0, 20, 40 y 60 mM). Por último, se investigó la influencia de la estacionalidad en las características seminales del asno andaluz. Durante un año, 50 eyaculados fueron recolectados de un total de 10 asnos andaluces. El movimiento (mediante el sistema de análisis CASA, Sperm Class Analyzer), la morfología espermática (en muestras teñidas con Diff-Quik®), y la integridad de la membrana plasmática (Vital-Test® kit) y acrosómica (tinción doble con los fluorocromos Isocianato de fluoresceína conjugado con la *lectina Arachis hipogea* e Ioduro de Propidio) fueron evaluados antes de la refrigeración (muestras frescas) y tras su almacenamiento durante 72 horas a 5ºC. El diluyente Gent® A preservó significativamente (P<0,05) mejor que el INRA 96® el movimiento espermático tras 72 horas de refrigeración. La incorporación de glutamina, prolina o taurina en el diluyente Gent® A mejoró significativamente (P<0,001) el movimiento del espermatozoide de asno andaluz. Se observó una influencia significativa (P<0,05) de la estación sobre todas las características seminales evaluadas, excepto sobre el volumen del eyaculado, la concentración espermática, el movimiento total y algunos parámetros cinéticos (oscilación, WOB; linealidad, LIN; rectitud, STR). En conclusión, nuestros resultados demostraron la eficacia del diluyente comercial Gent® A para refrigerar semen de asno andaluz. Además, se evidenció el efecto crioprotector de los aminoácidos testados sobre el espermatozoide de asno durante el proceso de refrigeración, obteniéndose mejores resultados de movimiento espermático en aquellas muestras en las que el diluyente Gent® A fue suplementado con diferentes concentraciones de

glutamina, prolina o taurina en comparación con aquellas en las que el Gent® A no fue suplementado. Por último, se demostró un efecto estacional en la calidad espermática del eyaculado del asno andaluz.

En la cuarta publicación, con el objetivo de seleccionar un diluyente adecuado para congelar semen de asno andaluz, comparamos el efecto de dos diluyentes de congelación, ampliamente usados para la congelación de semen de caballo (Gent® B e INRA 96®, este último suplementado con 2% de yema de huevo y 2,5% de glicerol), sobre la calidad espermática a la descongelación. Posteriormente, se estudió el efecto de sustituir el glicerol presente en el diluyente de congelación por otros crioprotectores de menor peso molecular: etilenglicol (EG; 1 o 1,5%), dimetilsulfóxido (DMSO; 1,5 o 2%), dimetilformamida (DMFA; 1 o 2,5%) o una combinación de EG 1% y DMFA 1,5%. Para tal fin, 74 eyaculados procedentes de 10 asnos adultos fueron recolectados. La metodología para valorar los parámetros de calidad seminal antes de la congelación (muestras frescas) y tras su almacenamiento en un tanque de nitrógeno líquido a -196 ºC fue similar a la empleada en la publicación precedente. El diluyente Gent® B proporcionó valores de movimiento espermático significativamente ($P<0,01$) superiores a los obtenidos con el INRA 96® modificado. La integridad de la membrana plasmática tras la descongelación fue significativamente ($P<0,001$) superior cuando se empleó EG 1% como crioprotector, mientras que el DMSO afectó negativamente ($P<0,001$) al movimiento espermático y a la integridad de la membrana plasmática. La DMFA 2,5% proporcionó los mejores resultados de calidad espermática tras el proceso de crioconservación, incrementando significativamente ($P<0,001$) el movimiento espermático y la integridad de la membrana plasmática. De los resultados obtenidos en este estudio podemos concluir que el Gent® B mejora la calidad espermática *in vitro* del espermatozoide de asno a la descongelación. Sin embargo, la sustitución del glicerol por otros crioprotectores de menor peso molecular, como el EG 1% y la DMFA 2,5%, mejora la protección del espermatozoide durante el proceso de congelación y descongelación. Además, este estudio demuestra que el uso del DMSO para criopreservar semen de asno es insatisfactorio, y se sospecha pueda tener un efecto tóxico para los espermatozoides de esta especie. Finalmente, los diluyentes de congelación evaluados en el presente estudio deberían ser incluidos en futuros estudios en los que se determine el diluyente de congelación más adecuado para cada asno.

Summary

Summary

The Andalusian donkey (*Equus asinus*) is currently considered in Spain as an endangered breed (Real Decreto 2129/2008, regulation of the National Catalogue of Endangered Species). Currently, only 94 individuals are considered as breeding males (DAD-IS-FAO 2013). In such situation, the application of assisted reproductive technologies such as artificial insemination and semen cryopreservation becomes necessary for improving gene distribution and for reducing the effects of excessive inbreeding. However, for this purpose, a previous study of the reproductive features and/or semen characteristics of the Andalusian donkey is required. Nowadays, the identification of sperm subpopulations within mammalian ejaculates has become an issue of the utmost interest for the sound evaluation of the ejaculates. In this sense, a direct relationship between the presence of discrete motile sperm subpopulations, the resistance to cryopreservation and the fertility rates obtained has been demonstrated. Therefore, it is interesting to characterize the distribution of subpopulations in the ejaculate of the Andalusian donkey in order to study the relationship between the quality and the reproductive performance of semen samples, and to predict its freezability and fertility. To date, published studies concerning on cooling or freezing donkey semen are scarce, and, to our knowledge, no previous studies reporting of Andalusian donkey semen cryopreservation have been published. Consequently, it is important to develop a suitable technique and select those extenders and cryoprotectants which provide the highest sperm quality after cooling or freezing Andalusian donkey spermatozoa.

In the first study the physiological sperm characteristics of Andalusian donkey (gel-free volume, pH, sperm concentration, motility and morphology) raw ejaculates were assessed to establish the average values normal for this donkey breed. Moreover, we studied the existence of sperm subpopulations with specific motion characteristics in raw Andalusian donkey ejaculates, evaluate the effects of individual donkey and ejaculates within the same male on the distribution of the subpopulations found, and explore the relationship between the age and the body weight of donkey donors, the sperm quality parameters, and the sperm subpopulations structure. To this end, 60 ejaculates (5 ejaculates per donkey) were collected from 12 Andalusian donkeys whose

age ranged from 4 to 15 years (mean: 9.9 ± 0.4 years) and their body weight varied from 313 to 435 kg (mean: 381.1 ± 5.8 kg). A multivariate clustering procedure evidenced the existence of four well-defined motile sperm subpopulations (sP) in Andalusian donkey ejaculates: sP1, consisting of slow and non-progressive spermatozoa (15.4%); sP2, consisting of moderately slow but progressive spermatozoa (35.9%); sP3 consisting of highly active but non-progressive spermatozoa (18.5%); sP4, consisting of highly active and progressive spermatozoa (30.2%). The proportions of sperm subpopulations in the ejaculates did not vary with age and body weight ($P>0.05$). However, the distribution of these subpopulations varied significantly ($P<0.05$) according to several parameters such as the individual donkey, the ejaculate of the same male, the total motility or the overall sperm concentration. On the other hand, significant ($P<0.05$) correlations were established between the body weight of donkeys and the pH, sperm motility (percentages of total and progressive motile spermatozoa) and total sperm abnormalities, respectively. Nevertheless, the correlations of the age with the measures of semen quality were low and not significant ($P>0.05$).

In the second study, we compared the conventional sperm parameters, plus the characteristics of the motility patterns of the different sperm subpopulations, of donkey donors with different fertility level, to determine their relationships to *in vivo* fertility. During the experimental period, 30 ejaculates from 6 Andalusian donkeys were collected. The fertility of donkeys was classified on the basis of pregnancy rates per cycle, where donkeys with a per cycle pregnancy rate ≥ 60% were considered to be "fertile" (n = 3) and those with a per cycle pregnancy rate < 40% were categorized to be "sub-fertile" (n = 3). Significant differences ($P<0.001$) between the "fertile" and the "sub-fertile" group were found for total and progressive motility, and for straight line velocity. Sperm variables associated ($P<0.05$) with an increase in percent pregnant per cycle included total motility ($r = 0.37$), progressive motility ($r = 0.53$), curvilinear velocity ($r = 0.44$), straightness ($r = 0.39$), beat cross frequency ($r = 0.44$) and gel-free volume ($r = 0.53$). Similarly, three of the sperm subpopulations (sP2, sP3 and sP4) present in Andalusian donkey ejaculates, previously described in the first study, were related ($P<0.05$) to fertility (sP2, $r = 0.54$; sP3, $r = 0.45$; sP4, $r = 0.56$). Thus, a lower percentage ($P<0.001$) of sP4 spermatozoa was observed in the "sub-fertile" group. In conclusion, we were able to relate the *in vitro* measures of sperm motility using

computer-assisted sperm analysis techniques (CASA) with the *in vivo* fertility of donkeys.

In the third study, we tried to select a suitable semen extender for cooling Andalusian donkey semen. For this purpose, we evaluated the efficacy of two commercially available liquid stallion semen extenders (Gent® A and INRA 96®) for the preservation of Andalusian donkey semen at 5ºC for up to 72 h. Moreover, we assessed the effect of amino acid addition (glutamine, proline, or taurine) on sperm quality of cooled donkey semen. In addition, this study investigated the effect of seasons on the semen quality of the Andalusian donkey. Throughout a year, 50 ejaculates were collected from 10 adult donkeys. Fresh semen and chilled samples were evaluated for sperm motility (assessed by computerized-assisted semen analysis - CASA), morphology (Diff-Quick® staining), acrosome integrity (PI/peanut agglutinin-fluorescein isothiocyanate double stain), and plasma membrane integrity (Vital-Test® kit). Sperm motility variables were greater ($P<0.05$) in Gent® A than in INRA 96®. The addition of glutamine, proline, or taurine to Gent® A improved ($P<0.001$) the motility of Andalusian donkey spermatozoa. A seasonal influence ($P<0.05$) was observed for all the sperm characteristics evaluated, with the exception of gel-free volume, sperm concentration, total motility and some sperm kinematic variables: wobble (WOB), linearity (LIN) and straightness (STR). In conclusion, this study showed the efficacy of Gent® A for cooling and preserving Andalusian donkey semen. Moreover, the protective effect of amino acids during cold storage was evidenced. Thus, the sperm motility was greater when Gent® A was supplemented at different concentrations of glutamine, proline, or taurine than Gent® A with no supplementation. Finally, the present study clearly showed a seasonal influence on the characteristics of fresh semen of the Andalusian donkey.

In the fourth study, in order to obtain an appropriate freezing semen extender for the cryopreservation of Andalusian donkey semen, we compared the effect of two equine semen extenders (Gent® B and INRA 96® supplemented with 2% clarified egg yolk and 2.5% glycerol) on sperm quality of frozen-thawed Andalusian donkey semen. Secondly, we investigated the replacement of glycerol by other lower molecular weight cryoprotectants on the freezing extender: ethylene glycol (EG; 1% or 1.5%), dimethyl sulfoxide (DMSO; 1.5% or 2%), dimethylformamide (DMFA; 1% or 2.5%) or a

combination of EG 1% and DMFA 1.5%. To this end, 74 ejaculates were collected from 10 healthy Andalusian donkeys. Methodology for sperm quality assessment prior (fresh samples) and after cryopreservation was similar to that used in the previous study. Gent® B yielded a higher ($P<0.01$) post-thaw sperm motility than modified INRA 96. EG 1% increased the sperm membrane integrity ($P<0.001$), whereas DMSO affected sperm motility and membrane integrity ($P<0.001$). DMFA 2.5% yielded higher ($P<0.001$) values for sperm motility and membrane integrity. We concluded that Gent B improves in vitro post-thaw sperm quality of donkey spermatozoa, but the replacement of glycerol with 1% EG or 2.5% DMFA increases sperm protection against cryodamage. The use of DMSO for freezing donkey semen was unsuccessful and a toxic effect is suspected. These extenders should be included in the prefreezing test for each donkey.

Introducción

Introducción

Durante siglos el asno (*Equus asinus*) andaluz al igual que otras razas españolas, ha sido demandado principalmente para la cría mular (Rodero y col., 1998). Sin embargo, esta raza asnal se encuentra actualmente sumida en una profunda crisis reflejo directo del hundimiento de la cultura rural y de los sistemas de explotación agraria y ganadera sostenible, lo que se traduce en un rápido descenso en el número de ejemplares y consecuentemente en su variabilidad genética. De acuerdo con el último censo elaborado por la FAO (Food and Agriculture Organization), el asno andaluz se localiza fundamentalmente en Andalucía y Extremadura y cuenta con 749 animales, de los cuales solo 94 individuos están considerados de alto valor genético (DAD-IS-FAO, 2013). Por ello, la raza asnal Andaluza está, hoy en día, catalogada como raza autóctona española en peligro de extinción *(Catálogo Oficial de Razas de Ganado de España, BOE 23/2009, RD 2129/2008, 26 de diciembre)*.

En esta situación, el desarrollo de técnicas de reproducción asistida tales como la criopreservación de semen y la inseminación artificial (IA) resulta indispensable para gestionar e integrar las poblaciones de esta raza asnal en peligro de extinción. No obstante, es imprescindible realizar un estudio previo de las características reproductivas y de los parámetros seminales de esta raza para posteriormente desarrollar y optimizar los protocolos de conservación de esperma. Recientemente, algunos estudios han investigado la estacionalidad reproductiva en otras razas extranjeras de asnos, con el objetivo de mejorar su eficacia reproductiva. Así, se ha demostrado una influencia estacional tanto en el comportamiento sexual (Gastal y col., 1996; Henry y col., 1998) como en las características seminales (Carluccio y col., 2013; Contri y col., 2010; Gastal y col., 1997) de los garañones. Sin embargo, estos estudios llevados a cabo en otras razas localizadas en áreas con condiciones climatológicas diferentes, son difícilmente extrapolables (Carluccio y col., 2013) a otras razas de asnos (p.ej. el asno andaluz). Según nuestro conocimiento, actualmente no existen estudios que hayan analizado la influencia de la estacionalidad sobre las características seminales del asno andaluz.

El análisis convencional en el laboratorio de la calidad espermática incluye una serie de parámetros (volumen eyaculado, concentración espermática, movimiento total y progresivo y morfología) que son fáciles de valorar y tienen un coste relativamente bajo. La relación entre estos parámetros espermáticos y la fertilidad ha sido investigada en muchas especies de mamíferos (Gadea, 2005; Kastelic y Thundathil, 2008; Love, 2011; O' Meara y col., 2008). Sin embargo, según la bibliografía consultada, no existen estudios que establezcan la relación existente entre estos parámetros de calidad espermática y la fertilidad de los eyaculados de asno.

En los mamíferos, el movimiento espermático juega un papel importante en el transporte del esperma dentro del tracto reproductor femenino, principalmente para la penetración de la zona pelúcida. Actualmente, la identificación de subpoblaciones de espermatozoides móviles dentro de un mismo eyaculado en mamíferos se ha convertido en un tema de máximo interés para la correcta evaluación de los eyaculados. Es ampliamente conocido que los eyaculados de diferentes especies de mamíferos están compuestos por distintas subpoblaciones de espermatozoides (Abaigar y col., 1999; Dorado y col., 2010a; Holt, 1996; Miró y col., 2005; Quintero-Moreno y col., 2003, 2007; Rigau y col., 2001), las cuales se caracterizan por parámetros cinéticos específicos, obtenidos por los sistemas de análisis de esperma asistido por ordenador (Computer-Assisted Sperm Analysis, CASA). De esta manera, la presencia de estas subpoblaciones cinéticas se ha relacionado con la resistencia a la criopreservación (Flores y col., 2009; Martinez-Pastor y col., 2005; Núñez-Martínez y col., 2006a, 2006b), la presencia de estimulantes (Abaigar y col., 1999), la respuesta a la refrigeración (Dorado y col., 2011) y la fertilidad (Quintero-Moreno y col., 2003, 2004). Aunque algunos estudios han demostrado la existencia de subpoblaciones espermáticas en los eyaculados de otras razas de asno (Flores y col., 2008; Miró y col., 2005, 2009), no se ha encontrado en la bibliografía actual ningún estudio que identifique la presencia de estas subpoblaciones en los eyaculados del asno andaluz. Tampoco existen estudios hasta la fecha que correlacionen los parámetros cinéticos obtenidos mediante sistemas CASA y la estructura subpoblacional con la edad y el peso corporal del garañón, así como con la calidad espermática del eyaculado. Por otro lado, si bien en el caballo sí se ha establecido la correlación existente entre los parámetros cinéticos del espermatozoide y las subpoblaciones cinéticas con la fertilidad *in vivo* (Jasko y col., 1992; Love, 2011),

según nuestro conocimiento, en el garañón aún no se han realizado estudios de estas características.

Las técnicas de reproducción asistida como la refrigeración de esperma y su posterior uso en programas de IA permiten transportar dosis seminales procedentes de individuos de alto valor genético entre poblaciones asnales aisladas, sin necesidad de trasladar a los animales. Además de permitirse así una óptima y eficiente distribución del material genético, se reduce también el riesgo de endogamia y se potencia el desarrollo de programas de conservación de razas de protección especial (Rota y col., 2008). En los últimos años, la refrigeración de semen de caballo ha sido ampliamente estudiada hasta el punto de convertirse en una técnica reproductiva de uso rutinario en la industria equina debido a los buenos índices de fertilidad obtenidos y los costes relativamente bajos de esta técnica (Contri y col., 2010a; Aurich, 2008). En el caso del asno, no obstante, son escasos los estudios publicados hasta la fecha sobre refrigeración de semen (Miro y col., 2009; Rota y col., 2008; Cottorello y col., 2002; Santos y col., 1995; Serres y col., 2002; Vidament y col., 2009). Algunos estudios han comparado el uso de diferentes diluyentes para preservar el semen de garañón a 5ºC, obteniéndose resultados muy variables. Sin embargo, el uso de diluyentes a base de yema de huevo ha demostrado tener un efecto beneficioso sobre el movimiento espermático tras la refrigeración (Cottorello y col., 2002; Mello y col., 2000), por lo que parecen más apropiados. Entre los diluyentes lácteos usados en el garañón, los diluyentes INRA 82 y Kenney preservaron mejor el movimiento espermático que la leche desnatada (Gomez-Cuetara y col., 2004), mientras que el INRA 96® mejoró los porcentajes de movimiento en comparación con el E-Z Mixin (Contri y col., 2010). Rota y col. (2008) publicaron que el uso de INRA 96® produce resultados de calidad espermática similares a los obtenidos con INRA 82, tras el almacenamiento de semen de asno Amiata a 5ºC durante 72 horas. Hasta la fecha, no existen estudios que evalúen la eficacia del diluyente comercial de refrigeración de semen equino Gent® A para preservar semen de garañón a 5ºC. En cualquier caso, debido a la inexistencia de trabajos de este tipo en el asno andaluz, se requieren estudios individualizados en esta raza que garanticen la obtención de los mejores resultados posibles.

Actualmente, los esfuerzos para mejorar la conservación del esperma equino mediante refrigeración se centran en la modificación de diluyentes específicos mediante

la adición de diferentes componentes como azúcares, antioxidantes o electrolitos, con el fin de proteger la membrana plasmática, prevenir el estrés oxidativo o preservar el movimiento de los espermatozoides (Samper, 2000). Hoy en día es ampliamente conocido el efecto protector que proporcionan los aminoácidos a las células animales frente a la hipotermia (Heinz y col., 1990; Kruuv y Glofcheski, 1990; Lalonde y col., 1991). En los espermatozoides, algunos estudios han demostrado que la adición de taurina al diluyente mejora la calidad espermática del semen refrigerado en el gato (Baran y col., 2009), el conejo (Alvarez y Storey, 1983a), el carnero (Bucak y col., 2007) y el caballo (Ijaz y Ducharme, 1995). El efecto protector durante el proceso de congelación y descongelación tanto de la glutamina (Khlifaoui y col., 2005; Trimeche y col., 1996, 1999) como de la prolina (Rudolph y Crowe, 1985; Trimeche y col., 1999) sobre los espermatozoides de caballo y asno también ha sido publicado. Sin embargo, Phetudomsinsuk y col. (2009) demostraron que la inclusión de glutamina a concentraciones de 50 mM en el diluyente de congelación produce una disminución significativa a la descongelación del movimiento espermático y de la integridad de la membrana acrosómica del espermatozoide de caballo. La disparidad de resultados obtenidos en estos estudios sugiere un efecto especie dependiente, no solo del tipo de aminoácido empleado, sino de la concentración utilizada, lo que fue sugerido previamente por Cabrita y col. (2011). Hoy en día, no existen estudios que valoren el efecto protector de los aminoácidos sobre los espermatozoides de asno andaluz durante el proceso de refrigeración.

Debido a la situación actual de la raza asnal Andaluza se hace necesario también el desarrollo de la metodología de criopreservación de esperma de asno que permita la creación de bancos de germoplasma basados en la congelación de muestras seminales de aquellos garañones de mayor valor genético, como requisito para la conservación de esta raza asnal (Andrabi y Maxwell 2007; Smits y col., 2012). Hasta la fecha, aunque algunos estudios han comparado el efecto de diferentes protocolos de criopreservación (Trimeche y col., 1998; Canisso y col., 2008; Cortés-Gutiérrez y col., 2008; Flores y col., 2008; Contri y col., 2012b), diluyentes de congelación (Serres y col., 2004a; Canisso y col., 2011) y crioprotectores (Alvarez y col., 2004; Serres y col., 2004a; Alvarez y col., 2006; Oliveira y col., 2006; Rota y col., 2012; Madison y col., 2013) sobre la calidad del espermatozoide de asno tras la descongelación, los resultados obtenidos en los programas de IA con semen congelado de asno no son satisfactorios

(Vidament y col., 2005; Vidament y col., 2009; Rota y col., 2012; Serres y col., 2014). Por ello, el desarrollo de un protocolo para la correcta criopreservación de espermatozoides de asno andaluz es necesario para obtener buenos resultados en los programas de IA con semen congelado. Sin embargo, según nuestro conocimiento, no existen estudios previos sobre la congelación de semen de esta raza asnal.

El proceso de congelación y descongelación provoca daños irreversibles sobre la ultraestructura y la funcionalidad de los espermatozoides, afectando así a los índices de fertilidad obtenidos tras la IA con dosis descongeladas (Watson, 1995). La adición de crioprotectores a los diluyentes de congelación de semen reduce el daño espermático producido durante el proceso de criopreservación (Oldenhof y col., 2010). Durante los últimos 50 años el glicerol ha sido considerado el crioprotector penetrante de referencia para la criopreservación de esperma equino (Hoffmann y col., 2011), siendo también empleado satisfactoriamente para congelar semen de asno (Trimeche y col., 1998; Serres y col., 2004a; Oliveira y col., 2006; Vidament y col., 2009; Rota y col., 2012; Ortiz y col., 2015). Sin embargo, el glicerol, debido a su baja permeabilidad (Ball y Vo, 2001), penetra más lentamente que el agua y otros crioprotectores a través de la membrana plasmática del espermatozoide de caballo (Glazar y col., 2009), induciendo así estrés osmótico durante el proceso de congelación y descongelación (Peña y col., 2011). Recientemente, ha sido demostrado (Macías García y col., 2012) que el efecto tóxico del glicerol sobre el espermatozoide de caballo se produce cuando se emplea a concentraciones superiores al 3,5%. Además, una mayor sensibilidad del espermatozoide de asno al glicerol en comparación con el espermatozoide de caballo ha sido sugerida (Vidament y col., 2009).

El empleo de crioprotectores que poseen un menor peso molecular que el glicerol (92,09 g/mol), como el etilenglicol (62,07 g/mol), el dimetilsulfóxido (78,13 g/mol) o la dimetilformamida (73,09 g/mol), reduce el daño osmótico sobre el espermatozoide de caballo (Glazar y col., 2009). En la última década, algunos autores han demostrado una mayor eficacia del etilenglicol (Alvarenga y col., 2000b; Ball y Vo, 2001; Henry y col., 2002; Mantovani y col., 2002; Squires y col., 2004), la dimetilformamida (Alvarenga y col., 2000a; Gomes y col., 2002; Medeiros y col., 2002; Alvarenga y col., 2005) y el dimetilsulfóxido (Chenier y col., 1998), en comparación con el glicerol, para preservar las características del espermatozoide de caballo durante

el proceso de crioconservación. Estas consideraciones nos permiten hipotetizar que estos crioprotectores penetrantes (etilenglicol, dimetilsulfóxido y dimetilformamida) podrían ser una buena opción para congelar semen de asno andaluz. Aunque algunos crioprotectores han sido comparados con anterioridad (Alvarez y col., 2004; Alvarez y col., 2005; Oliveira y col., 2006; Vidament y col., 2009; Rota y col., 2012), actualmente no existe ningún estudio en la bibliografía consultada que compare la eficacia del glicerol y de estos tres crioprotectores alternativos (etilenglicol, dimetilsulfóxido y dimetilformamida) para criopreservar espermatozoides de asno.

Recientemente, diluyentes ampliamente usados para la congelación de semen de caballo, como el Gent® B y el INRA 96® suplementado con 2% de yema de huevo y 2,5% de glicerol, han sido empleados con éxito (Flores y col., 2008; Rota y col., 2012; Ortiz y col., 2015) para congelar espermatozoides de asno. No obstante, según nuestro conocimiento, no se ha realizado ningún estudio que compare el efecto de estos diluyentes sobre la calidad del espermatozoide de asno tras la descongelación.

Objetivos

Objetivos

En la presente Tesis Doctoral se plantearon diversos objetivos específicos, desarrollados en cada una de las cuatro publicaciones siguientes:

- Primera Publicación: ***Sperm motility patterns in Andalusian donkey (Equus asinus) semen: Effects of body weight, age, and semen quality.*** *J. Dorado, D. Acha, M.J. Gálvez, I. Ortiz, J.J. Carrasco, B. Díaz, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Theriogenology 2013, 79: 1100-1109.

1. Estudiar la existencia de subpoblaciones espermáticas con patrones de movimiento específicos en el eyaculado del asno andaluz.

2. Investigar la influencia de los diferentes garañones empleados y de los sucesivos eyaculados de cada macho en la distribución de las subpoblaciones espermáticas identificadas en el eyaculado del asno andaluz.

3. Valorar la relación existente entre la edad y el peso corporal del garañón, las características de calidad espermática y la distribución de las subpoblaciones espermáticas.

- Segunda Publicación: ***Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (Equus asinus).*** *J. Dorado, D. Acha, I. Ortiz, M.J. Gálvez, J.J. Carrasco, B. Díaz, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Animal Reproduction Science 2013, 143: 64-71.

1. Comparar las características espermáticas convencionales y la distribución de las subpoblaciones espermáticas presentes en eyaculados de asnos con diferente tasa de fertilidad.

2. Determinar la relación existente entre las características espermáticas convencionales, así como de las diferentes subpoblaciones presentes en el eyaculado del asno andaluz, con la fertilidad *in vivo*, medida como tasa de preñez por ciclo.

- Tercera Publicación: ***Effect of extender and amino acid supplementation on sperm quality of cooled-preserved Andalusian donkey (Equus asinus) spermatozoa****. J. Dorado, D. Acha, I. Ortiz, M.J. Gálvez, J.J. Carrasco, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Animal Reproduction Science 2014, 146: 79-88.

1. Evaluar la eficacia de dos diluyentes comerciales de refrigeración de esperma equino (Gent® A e INRA 96®) para preservar semen de asno andaluz durante 72 horas a 5ºC.

2. Valorar el efecto de la adición al diluyente de refrigeración de diferentes concentraciones (0, 20, 40 y 60 mM) de glutamina, prolina y taurina, sobre los parámetros de calidad seminal.

3. Investigar la influencia de la estacionalidad en las características espermáticas del eyaculado del asno andaluz.

- Cuarta Publicación: ***Freezability of Andalusian donkey (Equus asinus) spermatozoa: effect of extenders and permeating*** **cryoprotectants.** *D. Acha, M. Hidalgo, M.J. Gálvez, I. Ortiz, J.J. Carrasco, V. Gómez-Arrones, J. Dorado.* Reproduction, Fertility and Development 2015, accepted 4 June 2015.

1. Comparar la eficacia de dos diluyentes de congelación de esperma equino que contienen glicerol como crioprotector (Gent® B e INRA 96® suplementado con un 2% de yema de huevo y un 2,5% de glicerol) para criopreservar semen de asno andaluz.

2. Evaluar el efecto de cuatro crioprotectores penetrantes (glicerol, etilenglicol, dimetilsulfóxido y dimetilformamida) sobre la calidad espermática a la descongelación de semen de asno andaluz.

Capítulo 1 / Chapter I

"Sperm motility patterns in Andalusian donkey (Equus asinus) semen: Effects of body weight, age, and semen quality".

J. Dorado, D. Acha, M.J. Gálvez, I. Ortiz, J.J. Carrasco, B. Díaz, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo. Theriogenology 2013, 79: 1100-1109.

Theriogenology 79 (2013) 1100–1109

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Sperm motility patterns in Andalusian donkey (*Equus asinus*) semen: Effects of body weight, age, and semen quality

J. Dorado [a,*], D. Acha [a], M.J. Gálvez [a], I. Ortiz [a], J.J. Carrasco [b], B. Díaz [b], V. Gómez-Arrones [b], R. Calero-Carretero [b], M. Hidalgo [a]

[a] Animal Reproduction Group, Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Cordoba, Córdoba, Spain
[b] Equine Reproduction Center, Centro de Selección y Reproducción Animal (CENSYRA-Extremadura Government), Badajoz, Spain

ARTICLE INFO

Article history:
Received 18 October 2012
Received in revised form 4 February 2013
Accepted 11 February 2013

Keywords:
Sperm subpopulations
Age
Body weight
Sperm quality
Donkey spermatozoa

ABSTRACT

The aims of this study were to (1) identify sperm subpopulations with specific motion characteristics in fresh Andalusian donkey ejaculates; (2) evaluate the effects of individual donkey and ejaculates within the same donkey on the distribution of the subpopulations found; and (3) explore the relationship between the age and the body weight of donkey donors, the sperm quality parameters, and the sperm subpopulations structure. Sixty ejaculates from 12 Andalusian donkeys (five ejaculates per donkey), ranging in age from 4 to 15 years, were collected. Immediately after collection, sperm characteristics (volume, sperm concentration, objective sperm motility, and sperm morphology) were assessed. Donkeys were evaluated for body weight. Significant ($P < 0.05$) correlations were established between the body weight of the donkeys and the pH ($r = -0.52$), sperm motility (percentage of motile spermatozoa: $r = -0.31$; percentage of progressive motile spermatozoa: $r = -0.34$), and total sperm abnormalities ($r = 0.38$). The correlations of the age with the measures of semen quality were low and not significant ($P > 0.05$). A multivariate clustering procedure separated 65,342 motile spermatozoa into four subpopulations: subpopulation 1, consisting of slow and nonprogressive spermatozoa (15.4%), subpopulation 2, consisting of moderately slow but progressive spermatozoa (35.9%), subpopulation 3, consisting of highly active but nonprogressive spermatozoa (18.5%), and subpopulation 4, consisting of highly active and progressive spermatozoa (30.2%). The distribution of these subpopulations varied significantly ($P < 0.05$) according to several parameters such as the individual donkey, the ejaculate of the same donkey, the total motility, and the overall sperm concentration. Our results show the existence of four well-defined motile sperm subpopulations in Andalusian donkey ejaculates, and suggest a high heterogeneity in the ejaculate structure in donkey. The relationship between the distribution of the sperm subpopulations and individual donkey, total motility, and sperm concentration shows that the spermatozoa of each have different motility patterns. However, the proportions of sperm subpopulations in the ejaculates did not vary with age and body weight. Finally, the study of discrete subpopulations of motile spermatozoa could lead to a substantial increase in information acquired during donkey semen analysis.

1. Introduction

The Andalusian donkey (*Equus asinus*) is found mainly in Andalusia and Extremadura (southwest Spain). The value of this breed in mule breeding has been known for centuries. However, the Andalusian donkey, among other Spanish

* Corresponding author. Tel.: +0034 957 212136; fax: +0034 957 211093.
E-mail address: jdorado@uco.es (J. Dorado).

0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2013.02.006

breeds of donkey, is currently considered in Spain as endangered (Real Decreto 2129/2008, regulation of the National Catalogue of Endangered Species). According to the Food and Agricultural Organization, the Andalusian donkey is at a critical risk of disappearing because of its small population size [1]. In such a situation, assisted reproductive technologies such artificial insemination and semen cryopreservation are powerful tools to manage and integrate populations of endangered species [2,3]. However, the scant information available regarding the assessment of sperm quality in Spanish breeds of donkeys is not consistent with the risk of the extinction of these populations. In this context, greater knowledge of the reproductive features and semen characteristics of the Andalusian donkey will be required (e.g., sperm motile subpopulations and other sperm quality parameters) to increase the population of this breed of donkey and help avoid its extinction.

The laboratory assessment of sperm quality is essential if assisted reproduction in domestic species is to be efficient. In mammals, sperm motility is important for sperm transport within the female reproductive tract and for penetration into the oocyte. Distinct sperm subpopulations showing forward progressive motility or, in contrast, nonprogressive patterns of movement will have different probability to cross the uterotubal junction and enter the oviduct [4]. Currently, the identification of subpopulations within the mammalian ejaculate has become an issue of utmost interest for the sound evaluation of the ejaculates. It is widely known that the ejaculates of many mammalian species contain different subpopulations of motile spermatozoa [5–11], which are characterized by precise values of the motion parameters obtained after a computer-assisted sperm analysis (CASA). Moreover, the presence of discrete motile sperm subpopulations has been related to resistance to cryopreservation [12–15], presence of stimulants [6], storage [16], and fertility [8,17]. Although several studies have identified sperm motile subpopulations in donkeys [9,18,19], no studies on the characterization of these subpopulations in Andalusian donkeys were found in the literature. Moreover, until now, no studies have correlated either CASA measurements or the proportions of sperm subpopulations in the ejaculates with the age, the body weight, or sperm quality of donkeys.

The aims of the present study were therefore to (1) identify sperm subpopulations with specific motion characteristics in fresh Andalusian donkey ejaculates; (2) study the variation of the subpopulation motility characteristics among different donkeys and between successive ejaculates within the same donkey; and (3) explore the relationship between the age and the body weight of donkey donors, the sperm quality parameters, and the sperm subpopulations found. The results obtained could lead to new ways for investigating the peculiarities of the Andalusian donkey semen, and to study the association between sperm characteristics and reproduction performance.

2. Materials and methods

2.1. Animals

Twelve mature Andalusian donkeys located at the Equine Center for Assisted Reproduction of the Centro de Selección y Reproducción Animal - CENSYRA (Badajoz, Spain) were used. All these animals were considered of high genetic value and were clinically healthy. Donkeys were weighed at the time of semen collection and the body weight and age were recorded. All donkeys were maintained under the same feeding and management conditions.

2.2. Semen collection and evaluation

Semen collections were performed on a regular basis (two collections per week) during two breeding seasons (2011 and 2012). Sixty ejaculates (five ejaculates per donkey) were collected from 12 donkeys of different age groups using a Missouri-model artificial vagina (Minitüb, Tiefenbach, Germany) lubricated with a sterile non-spermicidal gel (Minitüb) and prewarmed to 45 °C to 50 °C, fitted with an in-line gel filter (Minitüb) to separate the gel fraction. A jenny in natural or prostaglandin-induced estrus (Luprostiol 7.5 mg im, Prosolvin; Intervet International B.V., Boxmeer, The Netherlands) was used to induce copulatory activity.

After collection the gel-free volume was measured in a graduated tube. Sperm concentration was calculated with a photometer (SpermaCue; Minitüb), as described by Vidament et al. [20], and pH was determined using a pH meter (HI 2211-02; Hanna Instruments Inc., Woonsocket, RI, USA) [19]. The photometer was calibrated every day, using a control cuvette provided by the manufacturer. The percentage of spermatozoa with abnormal morphology was estimated on Diff-Quik stained smears (Baxter DADE Diagnostics AG, Düdingen, Switzerland) [21]. The sperm motility was evaluated using a CASA system. Before the assessment of movement, semen aliquots were diluted with dry skimmed milk extender with a defined glucose composition [22] to reach a sperm concentration of approximately 50×10^6 spermatozoa per mL [23] and incubated at 37 °C for 5 minutes.

2.3. Computer-assisted sperm motility analysis

Motion characteristics of the samples were assessed just after collection using a CASA system (Sperm Class Analyzer; Microptic SL, Barcelona, Spain), as described by Miró et al. [9] for donkey semen. The analysis was based on the analysis of 25 consecutive, digitized images obtained from a single field. Images were taken in a time lapse of 1 second, corresponding to a velocity of image-capturing of 1 photograph every 40 milliseconds. For each evaluation, three consecutive 5-μL drops of each semen sample were then evaluated using a phase contrast microscope (Eclipse 50i; Nikon, Tokyo, Japan) with a prewarmed stage at 37 °C at magnification ×100. Two microscopic fields per drop were filmed at random, including a minimum of 200 spermatozoa. Objects incorrectly identified as spermatozoa were minimized by using the playback function. With respect to the setting parameters for the program, spermatozoa with a mean average path velocity (VAP) <10 μm/s were considered immotile. Spermatozoa with VAP >90 μm/s were considered rapid, and spermatozoa deviating <25% from a straight line were designated as linear motile.

The measured parameters of sperm motion were curvilinear velocity (VCL; total distance travelled by the sperm head per unit time), straight line velocity (VSL; net distance gain of the sperm head per unit time), VAP (length of a derived "average" path of sperm head movement per unit time), wobble (WOB; ratio [VAP/VCL] × 100), linearity (LIN; ratio [VSL/VCL] × 100), straightness (STR; ratio [VSL/VAP] × 100), beat cross frequency (BCF; number of times the curvilinear path crosses the average path per unit time; approximation to the flagellar beat frequency for seminal spermatozoa), and amplitude of lateral head displacement (ALH; width of the head movement envelope) [24].

2.4. Classification, ordination, and identification of sperm subpopulations

Data from all the motile spermatozoa obtained in evaluations of 60 ejaculates (12 donkeys × five ejaculates) were imported into a single data set that represented 65,342 spermatozoa, each one defined by the eight motility descriptors specified in section 2.3. A clustering procedure was used to classify the spermatozoa of the data set into a reduced number of subpopulations according to their patterns of movement as previously described [12]. Briefly, the PRINCOMP procedure was first applied to perform principal component analysis of the motility data. A nonhierarchical cluster analysis using the FASTCLUS procedure and the selected principal components as variables was then applied. The CLUSTER procedure, which performed a hierarchical clustering on the cluster obtained by the previous steps, was then applied using the average linkage method (AVERAGE) for joining the clusters. To determine the final number of clusters (step four), 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.

2.5. Statistical analysis

The results were expressed as mean ± SEM. Normality of data was assessed with the Kolmogorov–Smirnov test. Because data reported in this study were not normally distributed, percentages were subjected to arc sine transformation and absolute measures to logarithmic transformation. Spearman correlation analysis (PROC CORR) was used to correlate the sperm characteristics of individual donkey ejaculates (N = 60) with the body weight and age of the 12 donkeys. A general linear model (PROC GLM) was used to evaluate significant differences ($P < 0.05$) in which donkey (12 donkeys) and ejaculates of the same donkey (five ejaculates per donkey) were entered as random effects, and the Duncan multiple range test was applied to list these differences. To study the distributions of observations (individual motile spermatozoa) within donkeys, ejaculates, and subpopulations, and to relate the distribution of motile spermatozoa belonging to the different subpopulations with sperm quality (sperm concentration, total motility and abnormal sperm morphology), we used the general linear model (PROC GLM) and chi-square test (FREQ procedure). For this purpose, all the ejaculates were categorized by the FASTCLUS procedure as follows:

According to sperm concentration:
Group 1 ejaculates: $\leq 221 \times 10^6$ spermatozoa per mL (N = 16)
Group 2 ejaculates: >221 and $\leq 441 \times 10^6$ spermatozoa per mL (N = 30)
Group 3 ejaculates: $>441 \times 10^6$ spermatozoa per mL (N = 14)

According to total motility:
Group 1 ejaculates: total motility ≤86% (N = 15)
Group 2 ejaculates: total motility >86% and ≤98% (N = 29)
Group 3 ejaculates: total motility >98% (N = 16)

According to sperm abnormalities:
Group 1 ejaculates: sperm abnormalities ≥21% (N = 13)
Group 2 ejaculates: sperm abnormalities <21% and ≥9% (N = 32)
Group 3 ejaculates: sperm abnormalities <9% (N = 15)

The main objective of these categories was to provide a reference that allowed changes in the distribution of subpopulations of motility characteristics to be detected. To complement the statistical analyses, the effects of age and body weight of donkey donors on the proportions of sperm subpopulations in the ejaculates were analyzed by Spearman correlation analysis (PROC CORR). All analyses were performed with the SAS statistical package v9.0 [25]. Significance was set at $P < 0.05$.

3. Results

Donkeys ranged in age from 4 to 15 years (mean: 9.9 ± 0.4 years) and their body weight varied from 313 to 435 kg (mean: 381.1 ± 5.8 kg). These donkeys were grouped into various ages which were 4 to 8 (N = 3), 9 and 10 (N = 5), 11 to 13 (N = 2), and ≥14 (N = 2) years.

3.1. Mean results for the semen quality analysis of donkey ejaculates: Correlation of sperm characteristics with body weight and age of the donkey

Table 1 shows the sperm characteristics for the 60 ejaculates collected from 12 Andalusian donkeys. Means values of most of the sperm parameters examined in this study varied between donkeys ($P < 0.01$), with the exception of the mean percentage of spermatozoa with head and midpiece defects ($P > 0.05$), and even between ejaculates of the same donkey ($P < 0.05$). However, when data were pooled across donkeys, there were no differences ($P > 0.05$) in semen quality parameters between different experimental days.

The correlations of the different evaluated sperm characteristics with the body weight and age of the donkey, respectively, are summarized in Table 2. The body weight of the donkeys was negatively correlated with the pH ($r = -0.52$; $P < 0.01$), sperm motility (percentage of motile spermatozoa: $r = -0.31$; $P < 0.05$; percentage of progressive motile spermatozoa: $r = -0.34$; $P < 0.05$), and total sperm abnormalities ($r = 0.38$; $P < 0.05$). The correlations of the age with other variables of semen quality were not statically significant ($P > 0.05$).

Table 1
Seminal parameters measured in fresh ejaculates (N = 60) from 12 different Andalusian donkeys.

Characteristic	Mean ± SEM[a]	Confidence interval (95%)
Gel-free volume (mL)	67.5 ± 3.8	59.9–75.1
Sperm concentration ($\times 10^6$ per mL)	337.8 ± 18.7	300.1–375.4
Total motility (%)[b]	91.7 ± 1.1	89.5–93.9
Progressive motility (%)[c]	75.0 ± 1.8	71.4–78.6
pH	7.3 ± 0.0	7.2–7.3
Total abnormalities (%)	16.4 ± 1.1	14.2–18.6
Head morphological abnormalities (%)	0.6 ± 0.0	0.4–0.8
Neck and midpiece abnormalities, including cytoplasmic droplets (%)	3.5 ± 0.5	2.4–4.6
Tail abnormalities (%)	12.3 ± 1.2	9.9–14.7
VCL (μm/s)	135.5 ± 5.5	124.4–146.5
VSL (μm/s)	90.8 ± 2.6	85.5–96.0
VAP (μm/s)	116.6 ± 4.3	108.1–125.2
LIN (%)	65.6 ± 1.2	63.2–68.1
STR (%)	75.2 ± 1.0	73.2–77.1
WOB (%)	84.3 ± 0.7	82.9–85.7
ALH (μm)	3.0 ± 0.2	2.7–3.4
BCF (Hz)	7.9 ± 0.1	7.6–8.1

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble.

[a] Five ejaculates were assessed from each donkey.

[b] Total motility is defined as the percentage of spermatozoa with a mean velocity >10 μm/s.

[c] Progressive motility is defined as the percentage of spermatozoa with a mean velocity >90 μm/s and straightness >75%.

3.2. Motile sperm subpopulation

Two principal components with eigenvalues >1 were identified by the principal component analysis, which accounted for 76.8% of the variance. Considering the scores of CASA parameters, the first principal component was related to fast and linear movement, and the second principal component was related to fast and irregular movement.

Four sperm subpopulations were defined by the application of nonhierarchical and subsequent hierarchical classification of 65,342 individual motile spermatozoa using the eight motility parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF). The spermatozoa were distributed among the donkeys in the following manner: 7371 motile spermatozoa were from Donkey 1, 5919 from Donkey 2, 5469 from Donkey 3, 4377 from Donkey 4, 3452 from Donkey 5, 3210 from Donkey 6, 4816 from Donkey 7, 6026 from Donkey 8, 6735 from Donkey 9, 5760 from Donkey 10, 5827 from Donkey 11, and 6380 from Donkey 12.

Summary statistics for the motility characteristics of the subpopulations are shown in Table 3. Subpopulation 1 (sP1) contained the lowest number of spermatozoa (15.4%) and included spermatozoa whose movements were less vigorous (low VCL, VAP, ALH, and BCF) and less progressive (low VSL, LIN, and STR) than in all other groups. Subpopulation 2 (sP2) contained the largest number of spermatozoa (35.9%) and included spermatozoa with relatively low velocity (medium VCL, VSL, and VAP) but with high progressiveness (high LIN, STR, WOB, BCF, and low ALH). Subpopulation 3 (sP3) included highly active but nonprogressive spermatozoa, as indicated by the high values of VCL and ALH together with low LIN and STR values and moderate BCF. The total motile spermatozoa included in this population was 18.5%. Subpopulation 4 (sP4) were the most vigorous spermatozoa (highest VCL and BCF and high ALH) and progressive (highest VSL and VAP), yielding 30.2% of the total motile population. The sperm trajectories were regular and linear (high LIN and STR), showing a moderate lateral head displacement (medium ALH).

Table 2
Correlations and P values between sperm quality parameters and the body weight and age of the donkeys (N = 60).

Characteristic	Age (y)		Weight (kg)	
	Correlation	P	Correlation	P
Gel-free volume (mL)	0.19	0.18	−0.29	0.12
Sperm concentration ($\times 10^6$ per mL)	−0.05	0.74	0.32	0.09
Total motility (%)	0.01	0.92	**−0.31**	**0.05**
Progressive motility (%)	−0.03	0.83	**−0.34**	**0.03**
pH	0.23	0.11	**−0.52**	**0.00**
Total abnormalities	0.03	0.86	**0.38**	**0.04**
VCL (μm/s)	0.02	0.91	−0.17	0.27
VSL (μm/s)	0.07	0.59	−0.20	0.23
VAP (μm/s)	0.04	0.75	−0.19	0.24
LIN (%)	0.11	0.42	−0.01	0.94
STR (%)	0.07	0.59	0.08	0.61
WOB (%)	0.13	0.31	−0.12	0.45
ALH (μm)	−0.08	0.57	−0.02	0.93
BCF (Hz)	0.10	0.44	−0.01	0.96

Values in bold are statistically significant ($P < 0.05$).
Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble.

3.2.1. Effect of individual variability on the distribution of spermatozoa within subpopulations

There were significant ($P < 0.001$) differences in the distribution of the four sperm subpopulations in diluted

Table 3
Motility parameters for the four sperm subpopulations (sP1, sP2, sP3, and sP4) defined after pattern analysis in Andalusian donkey ejaculates.

Sperm motility parameters	Sperm subpopulation			
	sP1	sP2	sP3	sP4
N	10,080	23,439	12,113	19,710
(%)	15.4	35.9	18.5	30.2
VCL (μm/s)	94.9 ± 0.4[a]	104.1 ± 0.1[b]	198.1 ± 0.3[c]	203.1 ± 0.2[d]
VSL (μm/s)	34.3 ± 0.2[a]	85.7 ± 0.1[b]	88.9 ± 0.2[c]	161.8 ± 0.2[d]
VAP (μm/s)	71.2 ± 0.4[a]	95.4 ± 0.1[b]	155.7 ± 0.2[c]	183.4 ± 0.2[d]
LIN (%)	38.6 ± 0.2[a]	82.6 ± 0.1[d]	46.0 ± 0.1[b]	80.6 ± 0.1[c]
STR (%)	53.7 ± 0.2[a]	89.9 ± 0.1[d]	58.9 ± 0.2[b]	88.6 ± 0.1[c]
WOB (%)	73.8 ± 0.1[a]	91.6 ± 0.1[d]	78.8 ± 0.1[b]	90.7 ± 0.0[c]
ALH (μm)	3.1 ± 0.0[b]	2.1 ± 0.0[a]	5.3 ± 0.0[d]	3.7 ± 0.0[c]
BCF (Hz)	6.6 ± 0.0[a]	8.4 ± 0.0[c]	7.6 ± 0.0[b]	9.3 ± 0.0[d]

Values are mean ± SEM.
Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble.

[a–d] Different letters indicate significant differences between subpopulations ($P < 0.05$). Raw data are shown but statistical comparisons were calculated after arc sine and logarithmic transformations.

ejaculates depending on the individual donkey donor (Fig. 1). Spermatozoa moving forward actively (sP4) ranged from 0.3% to 56.8%, and spermatozoa moving forward slowly (sP2) ranged from 4.8% to 86.5%. Subpopulation 1 (poorly motile nonprogressive spermatozoa) varied from 6.7% to 21.9% and sP3 (highly active but nonprogressive spermatozoa) from 0.2% to 35.5%. In addition, significant ($P < 0.001$) differences were seen between ejaculates of the same donkey with respect to the distributions of the four subpopulations (data not shown).

Exploration of the factors determining the relative proportions of sperm subpopulations in the ejaculates revealed that none of the sperm subpopulation frequencies were correlated with age or body weight. Moreover, body weights were negatively correlated ($r = -0.65$; $P < 0.001$) with the age of donkeys.

3.2.2. *Effect of sperm quality on the distribution of spermatozoa within subpopulations*

Relationship of sperm quality (sperm concentration, total motility, and abnormal sperm morphology) to sperm subpopulation proportions is presented in Table 4. Significant ($P < 0.001$) differences were seen when the distributions of the four sperm subpopulations were compared with the classification of the ejaculates according to their percentages of total motility, with the exception of the sP1. The greater percentages of spermatozoa included in sP3 and sP4 were observed in ejaculates of groups 3 and 4 total motility (motility >86%), and the greater percentage of spermatozoa included in sP2 (72.7%) was observed in group 1 ejaculates (total motility ≤86%). Similarly, the distribution of these subpopulations also varied significantly ($P < 0.05$) depending the sperm concentration of the ejaculates (Table 4). The greater percentages of spermatozoa included in sP1 and sP2 (18.3% and 57.9%, respectively) were observed in group 1 ejaculates (sperm concentration $\leq 221 \times 10^6$ spermatozoa per mL). Conversely, the greater percentages of spermatozoa included in sP3 and sP4 were observed in groups 2 and 3 ejaculates (sperm concentration $>221 \times 10^6$ spermatozoa per mL). As in the case of total motility, this indicates that the precise subpopulation structure could also be related to the overall sperm concentration. However, no significant ($P > 0.05$) differences were found when the distributions of the four sperm subpopulations were compared with the classification of the ejaculates according to their percentages of morphology (Table 4).

The proportion of sP2, sP3, and sP4 spermatozoa in diluted ejaculates was correlated with motility (sP2: $r = -0.66$, $P < 0.001$; sP3: $r = 0.68$, $P < 0.001$; sP4: $r = 0.49$, $P < 0.001$) and sperm concentration (sP2: $r = -0.40$, $P < 0.01$; sP3: $r = 0.40$, $P < 0.01$; sP4: $r = 0.37$, $P < 0.01$). None of the sperm subpopulation frequencies were correlated with morphology.

3.3. *Effects of body weight and age of the donkey on sperm characteristics and on the subpopulation distribution of motile spermatozoa*

Relationship of age with sperm parameters and the proportions of spermatozoa in the different subpopulations

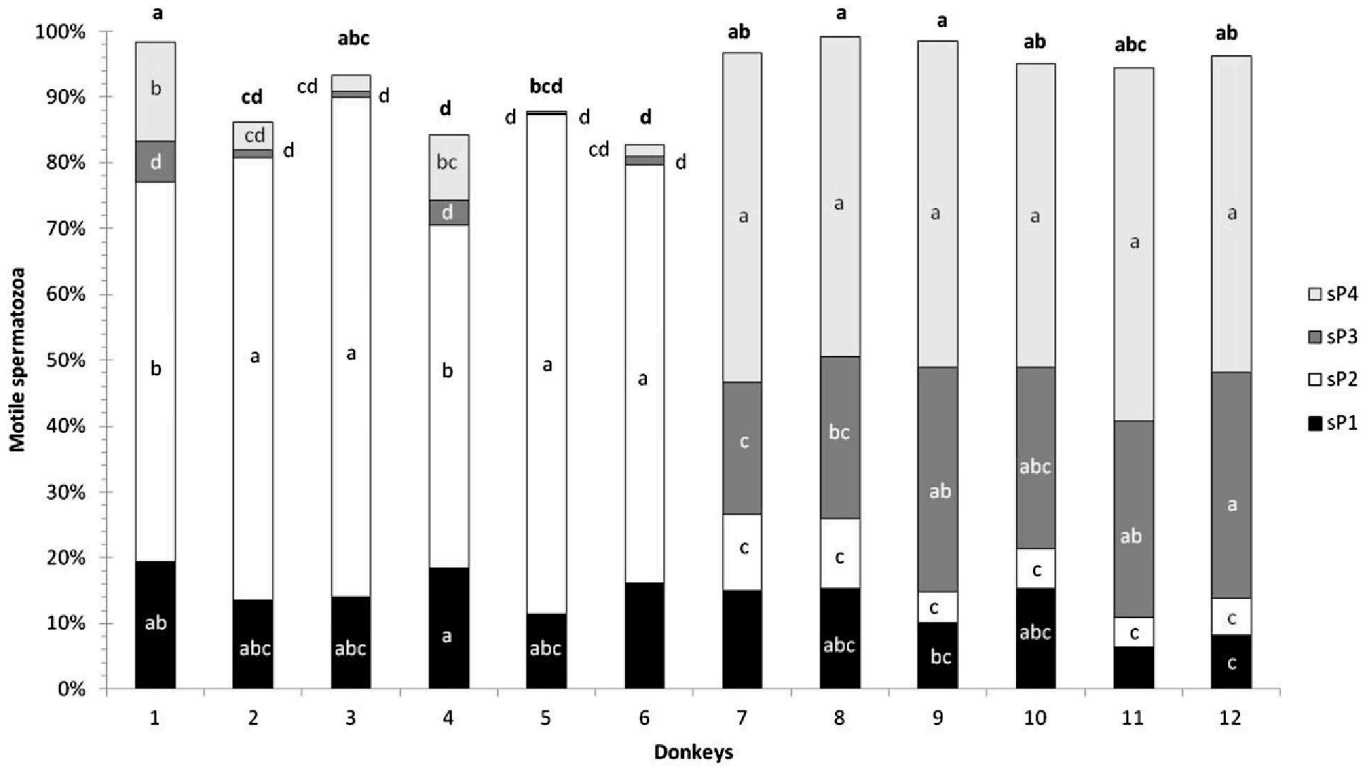


Fig. 1. Relative frequency distribution of motile spermatozoa (mean percentages; N = 60) within subpopulations (sP1, sP2, sP3, and sP4) between donkeys in fresh ejaculates. Different letters (a–d) inside or beside columns indicate significant differences within subpopulations between donkeys ($P < 0.05$). Different letters (a–d) above the columns indicate significant differences in total sperm motility between donkeys ($P < 0.05$).

is presented in Table 5. Mean gel-free volume was similar at all ages apart from 11 to 13 years of age where it was lower ($P < 0.05$). Mean pH was higher ($P < 0.05$) in group 3 donkeys (11–13 years of age), and mean sperm concentration and morphological abnormalities did not change ($P > 0.05$) with age. No significant ($P > 0.05$) differences between age groups were found for all of the evaluated sperm kinematic parameters (except for BCF). Moreover, the distributions of the four sperm populations did not change ($P > 0.05$) with age (Table 5).

Relationship of body weight with sperm parameters and sperm subpopulation proportions is presented in Table 6. Mean values of sperm motility (total and progressive motility) and VAP were lower ($P > 0.05$) in group 3 donkeys (weight >400 kg), and the opposite was found for the mean morphological abnormalities ($P < 0.05$). Mean pH was also higher ($P < 0.001$) in groups 1 and 2 donkeys (weight ≤400 kg). Although the distribution of the subpopulations did not change ($P > 0.05$) with weight of the donkeys (Table 6), the proportion of spermatozoa present in sP4 (7.0%) was minimally lower in group 3 donkeys (weight >400 kg).

4. Discussion

To our knowledge, this study is the first attempt to describe sperm characteristics, including the characteristics of motility patterns, of the Andalusian donkey. All physiological characteristics of freshly ejaculated semen that we observed were comparable with those previously recorded in other donkey breeds [9,19,26–29].

Although it is assumed by the scientific community that the presence of distinct subpopulations of motile spermatozoa is either an important reproductive strategy or an unavoidable consequence of sperm production and/or the microenvironment of the sperm [30], their mechanism of formation and physiological role are not known. Until now, the presence of different sperm subpopulations in mammalian ejaculates has been related to the ability to undergo capacitation and fertilize [31,32], and that freezability might vary significantly among sperm subpopulations [12,33]. Therefore, it is interesting to characterize the distribution of subpopulations in the ejaculate of the Andalusian donkey to study the relationship between the quality and the reproductive performance of semen samples, and to predict the ejaculate freezability and fertility.

The results obtained showed that ejaculates from these donkeys could be divided into four subpopulations of motile spermatozoa. A simple interpretation of this sperm subpopulation structure is that these four groups represent spermatozoa in different physiological states [6], either because cell metabolism is damaged or because they are hyperactive spermatozoa [9] or because a progressive sequence in population structure is produced during epididymal storage [34], reflected by very different swimming behaviors. The smallest subpopulation (sP1) was characterized by low speed, nonlinear motion. This subpopulation might represent a subgroup of metabolically compromised spermatozoa, that were soon to lose their motility altogether. The largest sperm subpopulation (sP2) included highly progressive but not very vigorous spermatozoa. It might be understood that this group of spermatozoa, which have complex but overall straight trajectories, is a stage of evolution of the rapid progressive group (sP4). The sP3 spermatozoa were moderately vigorous but poorly progressive. The low LIN values indicate that these cells possibly show a form of circular or uncoordinated and/or erratic movements. Likewise, high VCL values coupled with remarkably high ALH values indicated that this subpopulation is typified by wide lateral excursions of the sperm head. This result might be indicative that sperm were affected by sample management or by other intrinsic factors, and had begun a degenerative process. Subpopulation 4 represents the highest quality spermatozoa (spermatozoa with rapid and progressive movement). Recent studies indicated that the spermatozoa belonging to this subpopulation can be considered the ones with the highest fertilizing potential [8,13,35], and the ejaculates with the largest populations of rapid progressive spermatozoa are also the most resistant to cryopreservation injury [36]. Therefore, it is reasonable to presume that sP4 spermatozoa exhibited the pattern movement most suitable for being part of the fertilizing subpopulation.

The presence of four well-defined motile sperm subpopulations has been demonstrated in different species [8,11,34,36], and, as indicated by our current and previous results [9,18,19], the Spanish breeds of donkeys do not seem

Table 4
Sperm subpopulation proportions in donkey semen and their change with sperm quality.

Sperm subpopulation	Sperm concentration ($\times 10^6$ spermatozoa per mL)			Total motility (%)			Abnormal sperm morphology (%)		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
	≤221	>221 and ≤441	>441	≤86	>86 and ≤98	>98	<9	≥9 and <21	≥21
1	18.3[a]	11.7[b]	15.3[a,b]	16.3[a]	13.1[a]	16.4[a]	15.8[a]	13.1[a]	14.9[a]
2	57.9[a]	33.0[b]	12.0[b]	72.7[a]	36.3[b]	18.3[b]	47.9[a]	31.3[a]	29.0[a]
3	7.7[b]	20.0[a]	26.6[a]	2.9[b]	17.6[a]	25.2[a]	11.4[a]	19.7[a]	21.6[a]
4	16.1[b]	35.3[a]	46.1[a]	8.1[b]	33.0[a]	40.1[a]	24.9[a]	35.9[a]	34.5[a]

Sperm subpopulations are described in Section 3.2. Results express the percentages of the sperm subpopulations distributed among the three groups depending on the sperm concentration, total motility, and abnormal sperm morphology values of the ejaculates. The results were obtained from 60 ejaculates; five ejaculates from each animal. The spermatozoa were distributed among the ejaculates in the following manner: 13,466 motile spermatozoa were from ejaculate 1; 13,736 from ejaculate 2; 13,018 from ejaculate 3; 12,870 from ejaculate 4; and 12,252 from ejaculate 5. The total number of motile spermatozoa analyzed was 65,342.

[a,b] Different superscript letters between rows in the same group indicate significant differences ($P < 0.05$).

Table 5
Relationship of age to sperm quality characteristics (mean ± SEM) and relative proportions of sperm subpopulations 1 through 4 (%) in 12 Andalusian donkeys (4–15 years of age).

Variables	Age (y)			
	4–8 (N = 3)	9–10 (N = 5)	11–13 (N = 2)	>14 (N = 2)
Sperm characteristics				
Gel-free volume (mL)	57.4 ± 0.1[a,b]	75.1 ± 0.2[a]	47.9 ± 0.1[b]	79.3 ± 0.2[a]
Sperm concentration (×10^6 per mL)	382.3 ± 1.1[a]	299.0 ± 0.8[a]	361.1 ± 0.9[a]	364.5 ± 1.5[a]
Total motility (%)[c]	90.8 ± 0.1[a]	93.5 ± 0.0[a]	90.1 ± 0.1[a]	90.0 ± 0.1[a]
Progressive motility (%)[d]	72.0 ± 0.1[a]	78.7 ± 0.1[a]	76.0 ± 0.1[a]	69.0 ± 0.1[a]
pH	7.2 ± 0.0[b]	7.3 ± 0.0[b]	7.4 ± 0.0[a]	7.2 ± 0.0[b]
Total abnormalities	14.7 ± 0.1[a]	16.4 ± 0.1[a]	19.8 ± 0.1[a]	15.2 ± 0.1[a]
VCL (μm/s)	133.2 ± 0.4[a]	153.3 ± 0.3[a]	147.5 ± 0.6[a]	134.9 ± 0.5[a]
VSL (μm/s)	94.6 ± 0.4[a]	103.09 ± 0.3[a]	102.6 ± 0.6[a]	99.3 ± 0.5[a]
VAP (μm/s)	115.9 ± 0.4[a]	132.6 ± 0.3[a]	129.7 ± 0.6[a]	120.7 ± 0.5[a]
LIN (%)	72.1 ± 0.2[a]	67.7 ± 0.1[a]	71.4 ± 0.2[a]	74.2 ± 0.2[a]
STR (%)	81.3 ± 0.2[a]	77.6 ± 0.1[a]	79.7 ± 0.2[a]	82.0 ± 0.2[a]
WOB (%)	87.3 ± 0.1[a]	86.0 ± 0.1[a]	88.5 ± 0.1[a]	89.2 ± 0.1[a]
ALH (μm)	3.0 ± 0.0[a]	3.3 ± 0.0[a]	3.1 ± 0.0[a]	2.7 ± 0.0[a]
BCF (Hz)	8.0 ± 0.0[b]	8.2 ± 0.0[b]	9.3 ± 0.0[a]	8.1 ± 0.0[b]
Sperm population				
1	15.8[a]	15.9[a]	9.8[a]	15.6[a]
2	49.3[a]	31.6[a]	45.7[a]	44.9[a]
3	13.3[a]	19.4[a]	15.8[a]	11.0[a]
4	21.6[a]	33.1[a]	28.7[a]	28.5[a]

Sperm populations are described in Section 3.2. Results express the percentages of the sperm populations distributed among the four groups depending on the age of the donkeys. The results were obtained from 60 ejaculates; five ejaculates from each animal. The total number of motile spermatozoa analyzed was 65,342. The spermatozoa were distributed among the ejaculates in the following manner: 13,466 motile spermatozoa were from ejaculate 1; 13,736 from ejaculate 2; 13,018 from ejaculate 3; 12,870 from ejaculate 4; and 12,252 from ejaculate 5.
Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble.
[a,b] Different superscript letters in a row indicate significant differences ($P < 0.05$).
[c] Total motility is defined as the percentage of spermatozoa with a mean velocity >10 μm/s.
[d] Progressive motility is defined as the percentage of spermatozoa with a mean velocity >90 μm/s and straightness >75%.

to be an exception. However, the specific parameters and the specific percentages of each of the motile sperm subpopulations present in Andalusian donkey ejaculates seem to differ to those observed in the ejaculates of Catalonian donkeys [9]. These results could suggest differences between breeds in the sperm subpopulation structure within donkey species. Similar findings have been recently reported in bulls [36]. In fact, it should be emphasized that Andalusian donkey spermatozoa are more rapid and more progressive than Catalonian donkey spermatozoa. It should also be noted that the sperm number and total motility was higher in the ejaculates of Andalusian donkey compared with those in Catalonian donkey ejaculates. In addition, the individuals who defined the sperm subpopulations in this study were different from the one in the present study. The influence of several variables such as the individual donkey, total motility, and total sperm per mL on the specific subpopulation of an ejaculate has been demonstrated in a large number of studies [6,8,9,11,37].

Considering the characteristics of the sperm analysis using CASA, it is difficult to compare the movement patterns of the sperm subpopulations identified because of the fact that either the CASA system (hardware or software) or the kinematic parameters used to define the sperm subpopulations in previous studies were different from the one used in the present study. Moreover, the identification of motile sperm subpopulations in ejaculates might depend on the account of the methods used for the study of the clusters, as it has been previously suggested by Bravo et al. [37]. To improve the results and facilitate comparison among studies, standardization and improvement of the statistical methods used to disclose the subpopulation pattern have also been recommended in a recent study [38].

The present methodology, a multistep procedure, is one of the preferred methods currently used in sperm subpopulations analyses [38]. From a practical point of view, it might be desirable, when the subpopulation patterns are characterized, to use validated datasets as a guide to classify new datasets, instead of repeating the clustering process. Holt [5] used discriminat analysis (a supervised classification system) to assign cluster memberships to unclustered datasets, using an initial dataset that had been classified using cluster analysis. Other studies [39,40] examined sperm motility patterns in capacitating conditions for ram and for mouse spermatozoa, using the support vector machines methodology. Ramón et al. [41], more recently, used the support vector machines method to characterize the sperm motile population structure in Iberian red deer related to freezability. The application of these statistical tests on validated clustered data sets would allow us to develop automated supervised classification of sperm samples.

It is worth noting that the specific motile subpopulation structure of an ejaculate depends on the donkey from which the sample came. Most studies demonstrate the influence of the animal on the distribution of motile sperm subpopulations in the ejaculate [6,8,9,14,42,43], and there appear to be genetic bases for variations in the distribution of sperm subpopulations in the ejaculate between individuals [34]. The sperm subpopulation structure was also

Table 6
Relationship of body weight to sperm quality characteristics (mean ± SEM) and relative proportions of sperm subpopulations 1 through 4 (%) in 12 Andalusian donkeys (weight 313–435 kg).

Variables	Body weight (kg)		
	≤370 (N = 3)	>370 and ≤400 (N = 5)	>400 (N = 4)
Sperm characteristics			
Gel-free volume (mL)	68.4 ± 0.2[a]	84.1 ± 0.2[a]	58.4 ± 0.2[a]
Sperm concentration ($\times 10^6$ per mL)	226.5 ± 0.3[a]	306.2 ± 0.8[a]	316.2 ± 1.3[a]
Total motility (%)[b]	91.8 ± 0.1[a]	93.7 ± 0.0[a]	80.1 ± 0.1[c]
Progressive motility (%)[d]	72.6 ± 0.1[a]	74.6 ± 0.1[a]	56.8 ± 0.1[b]
pH	7.2 ± 0.0[a]	7.3 ± 0.0[a]	7.0 ± 0.0[b]
Total abnormalities	11.2 ± 0.0[b]	13.0 ± 0.1[a,b]	18.5 ± 0.1[a]
VCL (μm/s)	127.7 ± 0.4[a]	125.5 ± 0.3[a]	101.2 ± 0.3[a]
VSL (μm/s)	93.5 ± 0.3[a]	91.5 ± 0.3[a]	78.0 ± 0.4[a]
VAP (μm/s)	114.6 ± 0.3[a]	111.4 ± 0.3[a,b]	90.8 ± 0.3[b]
LIN (%)	74.3 ± 0.2[a]	73.1 ± 0.1[a]	75.9 ± 0.3[a]
STR (%)	81.8 ± 0.2[a]	81.8 ± 0.1[a]	84.5 ± 0.2[a]
WOB (%)	89.6 ± 0.1[a]	88.3 ± 0.1[a]	88.7 ± 0.1[a]
ALH (μm)	2.6 ± 0.0[a]	2.5 ± 0.0[a]	2.3 ± 0.0[a]
BCF (Hz)	7.8 ± 0.0[b]	8.9 ± 0.0[a]	8.2 ± 0.0[a,b]
Sperm populations			
1	15.4[a]	16.1[a]	20.7[a]
2	56.6[a]	52.3[a]	69.2[a]
3	9.1[a]	9.1[a]	3.1[a]
4	18.9[a]	22.5[a]	7.0[a]

Sperm populations are described in Section 3.2. Results express the percentages of the sperm populations distributed among the three groups depending upon the body weight of the donkeys. The results were obtained from 60 ejaculates; five ejaculates from each animal. The total number of motile spermatozoa analyzed was 65,342. The spermatozoa were distributed among the ejaculates in the following manner: 13,466 motile spermatozoa were from ejaculate 1; 13,736 from ejaculate 2; 13,018 from ejaculate 3; 12,870 from ejaculate 4; and 12,252 from ejaculate 5.

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble.

[a,b] Different superscript letters in a row indicate significant differences ($P < 0.05$).

[c] Total motility is defined as the percentage of spermatozoa with a mean velocity >10 μm/s.

[d] Progressive motility is defined as the percentage of spermatozoa with a mean velocity >90 μm/s and straightness >75%.

different between ejaculates for the same donkey, suggesting the presence of functionally different spermatozoa in a single ejaculate of Andalusian donkey.

The present study is the first, to the best of our knowledge, to investigate the effect of some factors responsible for this interejaculate variation (e.g., age and body weight) on donkey semen quality. Likewise, this is the first attempt to elucidate the influence of these factors on the specific subpopulation structure present in the ejaculates of Andalusian donkey. It has been clearly demonstrated that aging has detrimental effects on testicular function, affecting sperm quality in humans [44] and animals [6,34,45]. In contrast, no reports have been published on this topic in donkeys. In our study conditions, none of the evaluated sperm characteristics were affected by the age of the donkeys. Indeed, no correlation was found between age and semen characteristics. In dogs, it has been demonstrated using a CASA system that there was no effect of age on the motility [45]. In addition, in bulls, it has been demonstrated using conventional sperm evaluation techniques that the percentages of motile and morphologically normal spermatozoa did not change with age [46].

The body weight of the donkeys was significantly correlated with pH, sperm motility (total and progressive motility), and morphology of spermatozoa. In the present study, donkeys with higher body weights tend to produce ejaculates which have lower percentages of progressively motile and morphologically normal spermatozoa. These results are in accordance with previous findings by Rijsselaere et al. [45]. In their study, dogs with higher body weights tend to produce a greater proportion of spermatozoa with lower intrinsic velocity. It has previously been suggested that differences in food intake might be the reason for the body weight changes [47]. For this reason, in the present study, all animals were fed sufficiently to maintain their body weights and nutritional status at a stable level throughout the experiment. So, our results indicate that body weight has a direct effect on sperm quality characteristics of these donkeys, and, as has already been suggested in bovine, it might be possible that the increased body weight might affect scrotal or testicular thermoregulation by reducing the amount of heat that can be radiated and evaporated from the scrotal neck. Consequently, the temperature of the testes and the scrotum might be increased, thereby negatively influencing the sperm quality parameters [48]. However, no effects of age and body weight on the relative frequency distribution of motile spermatozoa within subpopulation were demonstrated. This conclusion is not consistent with earlier findings in the Mohor gazelle [34], which showed that heavier animals tend to produce a greater proportion of highly motile spermatozoa. These differences might be partially because of different species (i.e., genetics, selection, etc.) and different experimental conditions applied (i.e., location, feeding management, etc.). Another explanation could be because the donkeys were in good nutritional condition, allowing all the male donkeys to have an adequate seminal quality, including sperm motion parameters, and in consequence similar motility patterns. In any case, a comparison of motility parameters among species is difficult because of the intrinsic and specific properties of spermatozoal movement and because of different settings of CASA instruments [28].

The comparison between motile subpopulation structure and sperm quality indicates that sperm trajectories vary according to the sperm number and total motility. This is logical if it is assumed that the motility of a single spermatozoon depends on the different interactions established with other sperm cells [8]. Hence, these factors strongly affect the percentages and the motility characteristics of the motile subpopulations of Andalusian donkey ejaculates. Although the hemocytometer is regarded as the gold standard for the evaluation of sperm concentration, this method is often not applied in routine laboratory practice, especially where semen of several donkeys is collected daily [23]. Because of this, the photometer SpermaCue (Minitüb), an accurate and precise analytic technique for routine use in artificial insemination centers, was used for assessing sperm concentration of raw semen of adult Andalusian donkeys. Regardless of the method used, our results are in accord

with previous studies [8,11,17], in which sperm concentration was measured using a hemocytometer chamber.

Motility is considered as a manifestation of structural and functional competence of spermatozoa, and progressive motility is usually positively correlated with normal morphology [49]. However, in the present study, the distributions of the four sperm populations did not vary according to the sperm morphology. Moreover, none of the sperm subpopulation frequencies were correlated with morphology. Although sperm morphology might play a role in sperm subpopulation structure, a number of factors have also been proposed to explain the existence of discrete motile sperm subpopulations. In fowl, a distinction between sperm with high and low motility was attributed to phenotypic differences in rates of ATP synthesis [50,51]. Functionality of mitochondria has also been reported as a basis for sperm heterogeneity in humans [52]. Conceivably, heterogeneity in mitochondrial function might influence sperm motility characteristics and underlie the presence of discrete sperm subpopulations [30]. Furthermore, the existence of discrete subpopulations of motile sperm might, at least in part, be associated with the chemical nature of the sperm's environment [53]. Therefore, further studies are required to clarify this question and to explain the relevance on motile sperm subpopulation structure.

5. Conclusions

Our results confirm that four sperm subpopulations with specific motility patterns are present in ejaculates from this group of Andalusian donkeys, and suggest a high heterogeneity in the ejaculate structure in donkey. Therefore, the relationship between the distribution of the sperm subpopulations and individual donkey, total motility, and sperm concentration shows that the spermatozoa of each have different motility patterns. However, the proportions of sperm subpopulations in the ejaculates did not vary with age and body weight. Moreover, for the first time, this study highlights that body weight in the donkey are significantly correlated with sperm motility, morphology, and pH, whereas none of the evaluated sperm characteristics were correlated with the age of the donkey. Finally, the study of discrete subpopulations of motile spermatozoa could lead to a substantial increase in information acquired during donkey semen analysis. Further studies should be performed, however, to assess the effect of cold storage and freezing on subpopulations, and determine the relative importance of the different sperm motile subpopulations coexisting in a donkey ejaculate when predicting its *in vivo* fertility.

Acknowledgments

The authors thank the Fundación Casa del Burro (Rute, Cordoba, Spain) for supplying the animals. This work was supported by Grant RZ2009-00006-00-00 (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Ministerio de Ciencia e Innovación, Spain). Part of this study was presented at the 15th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR), September 15-17, 2011, Antalaya, Turkey.

References

[1] Domestic Animal Diversity Information Service (DAD-IS). http://dad.fao.org/ [accessed 08.03.12].
[2] Holt WV, Pickard AR. Role of reproductive technologies and genetic resource banks in animal conservation. Rev Reprod 1999;4:143–50.
[3] Wildt DE, Ellis S, Howard JG. Linkage of reproductive sciences: from 'quick fix' to 'integrated' conservation. J Reprod Fertil Suppl 2001; 57:295–307.
[4] Scott MA. A glimpse at sperm function in vivo: sperm transport and epithelial interaction in the female reproductive tract. Anim Reprod Sci 2000;60–61:337–48.
[5] Holt WV. Can we predict fertility rates? Making sense of sperm motility. Reprod Domest Anim 1996;31:17–24.
[6] Abaigar T, Holt WV, Harrison RAP, Del Barrio G. Sperm subpopulations in Boar (*Sus scrofa*) and Gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol Reprod 1999;60:32–41.
[7] Rigau T, Farré M, Ballester J, Mogas T, Pea A, Rodríguez-Gil JE. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology 2001;56:801–15.
[8] Quintero-Moreno A, Miró J, Teresa Rigau A, Rodríguez-Gil JE. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 2003;59:1973–90.
[9] Miró J, Lobo V, Quintero-Moreno A, Medrano A, Peña A, Rigau T. Sperm motility patterns and metabolism in Catalonian donkey semen. Theriogenology 2005;63:1706–16.
[10] Quintero-Moreno A, Rigau T, Rodríguez-Gil JE. Multivariate cluster analysis regression procedures as tools to identify motile sperm subpopulations in rabbit semen and to predict semen fertility and litter size. Reprod Domest Anim 2007;42:312–9.
[11] Dorado J, Molina I, Muñoz-Serrano A, Hidalgo M. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Florida goats. Theriogenology 2010;74:795–804.
[12] Martinez-Pastor F, Garcia-Macias V, Alvarez M, Herraez P, Anel L, De Paz P. Sperm subpopulations in Iberian red deer epididymal sperm and their changes through the cryopreservation process. Biol Reprod 2005;72:316–27.
[13] Núñez Martínez I, Morán JM, Peña FJ. Two-step cluster procedure after principal component analysis identifies sperm subpopulations in canine ejaculates and its relation to cryoresistance. J Androl 2006; 27:596–603.
[14] Núñez-Martínez I, Moran JM, Peña FJ. A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: changes after cryopreservation. Reprod Domest Anim 2006;41:408–15.
[15] Flores E, Fernández-Novell JM, Peña A, Rodríguez-Gil JE. The degree of resistance to freezing-thawing is related to specific changes in the structures of motile sperm subpopulations and mitochondrial activity in boar spermatozoa. Theriogenology 2009;72:784–97.
[16] Dorado J, Gálvez MJ, Murabito MR, Muñoz-Serrano A, Hidalgo M. Identification of sperm subpopulations in canine ejaculates: effects of cold storage and egg yolk concentration. Anim Reprod Sci 2011; 127:106–13.
[17] Quintero-Moreno A, Rigau T, Rodríguez-Gil JE. Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. Theriogenology 2004;61:673–90.
[18] Flores E, Taberner E, Rivera MM, Peña A, Rigau T, Miró J, et al. Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates. Theriogenology 2008;70:936–45.
[19] Miró J, Taberner E, Rivera M, Peña A, Medrano A, Rigau T, et al. Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen. Theriogenology 2009;72: 1017–22.
[20] Vidament M, Vincent P, Martin FX, Magistrini M, Blesbois E. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. Anim Reprod Sci 2009; 112:22–35.
[21] Brito LFC. Evaluation of stallion sperm morphology. Clin Tech Equine Pract 2007;6:249–64.
[22] Kenney RM, Bergman RV, Cooper WL, Morse GW. Minimal contamination techniques for breeding mares: techniques and preliminary findings. Proc Am Assoc Equine Pract 1975;21:327–36.
[23] Harald S. Semen evaluation. In: Samper JC, editor. Equine breeding management and artificial insemination. St. Louis: Saunders Elsevier; 2009. p. 57–74.
[24] Dorado J, Rodríguez I, Hidalgo M. Cryopreservation of goat spermatozoa: comparison of two freezing extenders based on post-thaw

sperm quality and fertility rates after artificial insemination. Theriogenology 2007;68:168–77.
[25] SAS Institute Inc. SAS, 2002. SAS/STAT software. Cary, NC: SAS Institute Inc.; 2002.
[26] Cortés-Gutiérrez EI, Crespo F, Gosálvez A, Dávila-Rodríguez MI, López-Fernández C, Gósalvez J. DNA fragmentation in frozen sperm of *Equus asinus*: Zamorano-Leonés, a breed at risk of extinction. Theriogenology 2008;69:1022–32.
[27] Contri A, De Amicis I, Veronesi MC, Faustini M, Robbe D, Carluccio A. Efficiency of different extenders on cooled semen collected during long and short day length seasons in Martina Franca donkey. Anim Reprod Sci 2010;120:136–41.
[28] Gloria A, Contri A, De Amicis I, Robbe D, Carluccio A. Differences between epididymal and ejaculated sperm characteristics in donkey. Anim Reprod Sci 2011;128:117–22.
[29] Contri A, Gloria A, Robbe D, De Amicis I, Carluccio A. Characteristics of donkey spermatozoa along the length of the epididymis. Theriogenology 2012;77:166–73.
[30] Kanuga MK, Drew RE, Wilson-Leedy JG, Ingermann RL. Subpopulation distribution of motile sperm relative to activation medium in steelhead (*Oncorhynchus mykiss*). Theriogenology 2012;77:916–25.
[31] Harrison RAP. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. Reprod Fertil Dev 1996;8:581–94.
[32] Petrunkina AM, Waberski D, Günzel-Apel AR, Töpfer-Petersen E. Determinants of sperm quality and fertility in domestic species. Reproduction 2007;134:3–17.
[33] Muiño R, Peña AI, Rodríguez A, Tamargo C, Hidalgo CO. Effects of cryopreservation on the motile sperm subpopulations in semen from Asturiana de los Valles bulls. Theriogenology 2009;72:860–8.
[34] Abaigar T, Cano M, Pickard AR, Holt WV. Use of computer-assisted sperm motility assessment and multivariate pattern analysis to characterize ejaculate quality in Mohor gazelles (*Gazella dama mhorr*): effects of body weight, electroejaculation technique and short-term semen storage. Reproduction 2001;122:265–73.
[35] Cremades T, Roca J, Rodriguez-Martinez H, Abaigar T, Vazquez JM, Martinez EA. Kinematic changes during the cryopreservation of boar spermatozoa. J Androl 2005;26:610–8.
[36] Muiño R, Tamargo C, Hidalgo CO, Peña AI. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: effects of cryopreservation and between-bull variation. Anim Reprod Sci 2008;109:27–39.
[37] Bravo JA, Montanero J, Calero R, Roy TJ. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Ile de France rams. Anim Reprod Sci 2011;129:22–9.
[38] Martínez-Pastor F, Tizado EJ, Garde JJ, Anel L, de Paz P. Statistical series: opportunities and challenges of sperm motility subpopulation analysis. Theriogenology 2011;75:783–95.
[39] Vulcano GJ, Moses DF, Valcárcel A, De Las Heras MA. A lineal equation for the classification of progressive and hyperactive spermatozoa. Math Biosci 1998;149:77–93.
[40] Goodson SG, Zhang Z, Tsuruta JK, Wang W, O'Brien DA. Classification of mouse sperm motility patterns using an automated multiclass support vector machines model. Biol Reprod 2011;84:1207–15.
[41] Ramón M, Martínez-Pastor F, García-Álvarez O, Maroto-Morales A, Soler AJ, Jiménez-Rabadán P, et al. Taking advantage of the use of supervised learning methods for characterization of sperm population structure related with freezability in the Iberian red deer. Theriogenology 2012;77:1661–72.
[42] Rivera MM, Quintero-Moreno A, Barrera X, Rigau T, Rodríguez-Gil JE. Effects of constant, 9 and 16-h light cycles on sperm quality, semen storage ability and motile sperm subpopulations structure of boar semen. Reprod Domest Anim 2006;41:386–93.
[43] Dorado J, Muñoz-Serrano A, Hidalgo M. The effect of cryopreservation on goat semen characteristics related to sperm freezability. Anim Reprod Sci 2010;121:115–23.
[44] Schwartz D, Mayaux MJ, Spira A. Semen characteristics as a function of age in 833 fertile men. Fertil Steril 1983;39:530–5.
[45] Rijsselaere T, Maes D, Hoflack G, De Kruif A, Van Soom A. Effect of body weight, age and breeding history on canine sperm quality parameters measured by the Hamilton-Thorne analyser. Reprod Domest Anim 2007;42:143–8.
[46] Ahmad E, Ahmad N, Naseer Z, Aleem M, Khan MS, Ashiq M, et al. Relationship of age to body weight, scrotal circumference, testicular ultrasonograms, and semen quality in Sahiwal bulls. Trop Anim Health Prod 2011;43:159–64.
[47] Argo CM, Smith JS, Kay RNB. Seasonal changes of metabolism and appetite in Soay rams. Anim Sci 1999;69:191–202.
[48] Hoflack G, Van Soom A, Maes D, de Kruif A, Opsomer G, Duchateau L. Breeding soundness and libido examination of Belgian Blue and Holstein Friesian artificial insemination bulls in Belgium and The Netherlands. Theriogenology 2006;66:207–16.
[49] Peña Martínez AI. Canine fresh and cryopreserved semen evaluation. Anim Reprod Sci 2004;82–83:209–24.
[50] Bowling ER, Froman DP, Davis AJ, Wilson JL. Attributes of broiler breeder males characterized by low and high sperm mobility. Poultry Sci 2003;82:1796–801.
[51] Froman DP, Kirby JD. Sperm mobility: phenotype in roosters (*Gallus domesticus*) determined by mitochondrial function. Biol Reprod 2005;72:562–7.
[52] Gallon F, Marchetti C, Jouy N, Marchetti P. The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. Fertil Steril 2006;86:1526–30.
[53] Lahnsteiner F, Radner M. Lysozyme activities and immunoglobulin concentrations in seminal plasma and spermatozoa of different teleost species and indications on its significance for sperm function. Theriogenology 2010;74:246–54.

Capítulo II / Chapter II

"Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (Equus asinus)".

J. Dorado, D. Acha, I. Ortiz, M.J. Gálvez, J.J. Carrasco, B. Díaz, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo. Animal Reproduction Science 2013, 143: 64-71.

Animal Reproduction Science 143 (2013) 64–71

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (*Equus asinus*)

J. Dorado[a,*], D. Acha[a], I. Ortiz[a], M.J. Gálvez[a], J.J. Carrasco[b], B. Díaz[b], V. Gómez-Arrones[b], R. Calero-Carretero[b], M. Hidalgo[a]

[a] Animal Reproduction Group, Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Cordoba, 14071 Córdoba, Spain

[b] Equine Reproduction Center, Centro de Selección y Reproducción Animal (CENSYRA-Extremadura Government), 06007 Badajoz, Spain

ARTICLE INFO

Article history:
Received 2 July 2013
Received in revised form 7 October 2013
Accepted 13 October 2013
Available online 21 October 2013

Keywords:
Sperm subpopulations
Semen characteristics
Fertility
Andalusian donkey

ABSTRACT

Sperm quality has an important role in determining fertility. The aims of this study were to compare the conventional sperm parameters, plus the characteristics of the motility patterns of the different sperm subpopulations, of donkey donors with different fertility level, and to determine their relationships to fertility. Thirty ejaculates from 6 Andalusian donkeys were assessed for gel-free volume, pH, sperm concentration, motility and morphology. The fertility of donkeys was classified on the basis of pregnancy rates per cycle, where donkeys with a per cycle pregnancy rate ≥60% were considered to be "fertile" ($n=3$) and those with a per cycle pregnancy rate <40% were categorized to be "sub-fertile" ($n=3$). Significant differences ($P<0.001$) between the "fertile" and the "sub-fertile" group were found for total and progressive motility, and for straight line velocity. Sperm variables associated ($P<0.05$) with an increase in percent pregnant per cycle included total motility ($r=0.37$), progressive motility ($r=0.53$), curvilinear velocity ($r=0.44$), straightness ($r=0.39$), beat cross frequency ($r=0.44$), and gel-free volume ($r=0.53$). Four sperm subpopulations (sP) were identified in fresh semen: sP1 (slow and non-progressive spermatozoa, 20%), sP2 (moderately slow but progressive spermatozoa, 71.2%), sP3 (highly active but non-progressive spermatozoa, 2.9%), and sP4 (highly active and progressive spermatozoa, 5.9%). The lowest percentage (3.1%; $P<0.001$) of sP4 spermatozoa was observed in the "sub-fertile" group. Three of the sperm subpopulations were related ($P<0.05$) to fertility (sP2, $r=0.54$; sP3, $r=0.45$; sP4, $r=0.56$). In conclusion, we were able to relate the fertility of donkeys with *in vitro* measures of sperm motility using computer-assisted sperm analysis techniques.

1. Introduction

As other Spanish breeds of donkey, the Andalusian donkey (*Equus asinus*) is currently considered in Spain as endangered (Real Decreto 2129/2008, regulation of the National Catalog of Endangered Species) because of its small population size (101 jackasses and 447 jennies in 2011) (DAD-IS-FAO, 2011). In such situation, greater knowledge of the reproductive features and/or semen characteristics of the Andalusian donkey will be required to increase the population of this breed of donkey and help to avoid its extinction. This strategy is particularly important when the information available regarding the assessment of sperm quality in endangered Spanish donkey breeds is scarce.

* Corresponding author at: Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Cordoba, Campus de Rabanales (Edif. Hospital Clínico Veterinario), Ctra. Madrid-Cádiz, km 396, 14071 Córdoba, Spain. Tel.: +34 957 212136; fax: +34 957 211093.
E-mail address: jdorado@uco.es (J. Dorado).

0378-4320/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.anireprosci.2013.10.003

The laboratory assessment of sperm quality includes a number of conventional sperm parameters (volume of the ejaculate, sperm concentration, total and progressive motility, and morphology) that are simple to perform and at relatively low cost. The relationships between these sperm features and fertility have been investigated in males of many domestic species (Gadea, 2005; Kastelic and Thundathil, 2008; Love, 2011; O' Meara et al., 2008). In the case of sperm morphology, it has been demonstrated that a decrease in the number of morphologically normal spermatozoa in stallion ejaculates leads to reduced fertility (Jasko et al., 1990, 1992; Love, 2011). In mammals, sperm motility is important for sperm transport within the female reproductive tract and for penetration into the oocyte. Distinct sperm subpopulations showing forward progressive motility or, in contrast, non-progressive patterns of movement will have different probability to cross the utero-tubal junction and enter the oviduct (Scott, 2000). In practice, computer-assisted semen motility analysis (CASA) of spermatozoa has also been correlated with fertility of fresh stallion semen (Jasko et al., 1992; Love, 2011).

The identification of subpopulations within the mammalian ejaculate has become an issue of utmost interest for the sound evaluation of the ejaculates. It is widely known that the ejaculates of many mammalian species contain different subpopulations of motile spermatozoa (Abaigar et al., 1999; Dorado et al., 2010a; Holt, 1996; Miró et al., 2005; Quintero-Moreno et al., 2003, 2007; Rigau et al., 2001), which are characterized by precise values of the sperm motility descriptors obtained by CASA analysis. Moreover, the presence of discrete motile sperm subpopulations has been related to resistance to cryopreservation (Flores et al., 2009; Martinez-Pastor et al., 2005; Núñez Martínez et al., 2006a, 2006b), presence of stimulants (Abaigar et al., 1999), storage (Dorado et al., 2011) and fertility (Quintero-Moreno et al., 2003, 2004). Although several studies have identified sperm motile subpopulations in donkeys (Flores et al., 2008; Miró et al., 2005, 2009), no studies have explored the relationship between either sperm quality parameters or proportions of sperm subpopulations in the ejaculates and the fertility of donkeys.

Using semen from 6 donkeys grouped according to their *in vivo* fertility, the aims of the present study were to: (1) compare the conventional sperm characteristics and the sperm subpopulations found in ejaculates of donkey donors with different fertility, and (2) determine their relationships to fertility, as measured by per cycle pregnancy rate. The results obtained could lead to identify which parameters in the ejaculate of the Andalusian donkey were useful in determining *in vivo* fertility differences.

2. Materials and methods

2.1. Animals and fertility data

Semen was collected from six healthy mature Andalusian donkeys (4–15 years of age, 313–435 kg in weight). The jackasses were housed in individual paddocks at the Equine Center for Assisted Reproduction of the Centro de Selección y Reproducción Animal – CENSYRA (Badajoz, Spain).

In a retrospective study, the per cycle pregnancy rates following insemination of 27 Andalusian jennies in 76 estrous cycles during two breeding seasons (2011 and 2012) were examined. Fresh semen inseminations were performed with semen collected from six fertile Andalusian donkeys used in the CENSYRA' routine artificial insemination (AI) program. On each estrous cycle, the jennies were inseminated within 1 h of semen collection with 800×10^6 total spermatozoa diluted in skim milk-glucose extender (Kenney et al., 1975) (20 mL total volume) every other day until ovulation. Finally, fertility was measured according to the per cycle pregnancy rate, as defined previously by Vidament et al. (2009). The number of jennies inseminated per donkey and fertility rates are presented in Table 1.

Based on their *in vivo* fertility, the jackasses were grouped arbitrarily into two groups: donkeys with a pregnancy rate per cycle of 60% or higher were considered to be "fertile", whereas they were categorized to be "sub-fertile" if they yielded low pregnancy rates per cycle (<40%) (Casey et al., 1997; Novak et al., 2010).

2.2. Semen collection and evaluation

Semen collections were performed on a regular basis (two collections per week) during two breeding seasons (2011 and 2012). Semen ($n = 30$; five ejaculates per donkey) was collected from donkeys using a Missouri-model artificial vagina (Minitüb, Tiefenbach, Germany) in the presence of a jenny in natural or prostaglandin-induced estrus (Luprostiol, 7.5 mg intramuscularly; Prosolvin, Intervet International B.V., Boxmeer, The Netherlands) was used to induce copulatory activity.

Table 1
Estimates of fertility (based on pregnancy rate per cycle in jennies following artificial insemination with fresh semen) for individual donkeys used in the present study (breeding seasons 2011 and 2012).

Donkey ID	No. of jennies assigned to each donkey	No. of estrous cycles inseminated	No. of pregnancies	Donkey fertility[a]	Donkey category[b]
1	3	4	3	75.0	Fertile
2	4	13	5	38.5	Sub-fertile
3	11	28	9	32.1	Sub-fertile
4	8	16	10	62.5	Fertile
5	2	4	3	75.0	Fertile
6	6	11	4	36.4	Sub-fertile

[a] Fertility was based on pregnancy diagnosis by rectal ultrasound scanning after AI (Vidament et al., 2009).
[b] Categorized on the basis of estimated fertility.

After collection the gel-free volume was measured in a graduated tube. Sperm concentration was calculated with a photometer (SpermaCue, Minitüb, Tiefenbach, Germany), as described by Vidament et al. (2009), and pH was determined using a pH meter (HI 2211-02, Hanna Instruments Inc., Woonsocket, USA) (Miró et al., 2009). The percentage of spermatozoa with abnormal morphology was estimated on Diff-Quik stained smears (Baxter DADE Diagnostics AG, Düdingen, Switzerland) (Brito, 2007). The sperm motility was evaluated by means of a CASA system. Prior to the assessment of movement, semen aliquots were diluted with skim milk-glucose extender to reach a sperm concentration of approximately 50×10^6 spermatozoa/mL (Harald, 2009) and incubated at 37 °C for 5 min.

2.3. Computer-assisted sperm motility analysis

Motion characteristics of the samples were assessed just after collection using a CASA system (Sperm Class Analyzer, Microptic SL, Barcelona, Spain), as described by Miró et al. (2005) for donkey semen. The analysis was based on the analysis of 25 consecutive, digitized images obtained from a single field. Images were taken in a time lapse of 1 s, corresponding to a velocity of image-capturing of 1 photograph every 40 ms. For each evaluation, three consecutive 5 μL drops of each semen sample were then evaluated using a phase contrast microscope (Eclipse 50i, Nikon, Tokyo, Japan) with a pre-warmed stage at 37 °C at 100× magnification. Two microscopic fields per drop were filmed at random, including a minimum of 200 spermatozoa. Objects incorrectly identified as spermatozoa were minimized by using the playback function. With respect to the setting parameters for the program, spermatozoa with a mean average path velocity (VAP) <10 μm/s were considered immotile. Spermatozoa with VAP >90 μm/s were considered as rapid, while spermatozoa deviating <25% from a straight line were designated as linear motile.

The measured parameters of sperm motion were: curvilinear velocity, VCL (total distance traveled by the sperm head per unit time); straight line velocity, VSL (net distance gain of the sperm head per unit time); average path velocity, VAP (length of a derived "average" path of sperm head movement per unit time); linearity, LIN (ratio [straight line velocity/curvilinear velocity] × 100); straightness, STR (ratio [straight line velocity/average path velocity] × 100); wobble, WOB (ratio [average path velocity/curvilinear velocity] × 100); amplitude of lateral head displacement, ALH (width of the head movement envelope) and beat cross frequency, BCF (number of times the curvilinear path crosses the average path per unit time; approximation to the flagellar beat frequency for seminal spermatozoa) (Dorado et al., 2007).

2.4. Classification, ordination and identification of sperm subpopulations

Data from all the motile spermatozoa obtained in evaluations of 30 ejaculates (6 donkeys × 5 ejaculates) were imported into a single data set that represented 29,798 spermatozoa, each one defined by the 8 motility descriptors specified above. A clustering procedure was used to classify the spermatozoa of the data set into a reduced number of subpopulations according to their patterns of movement as previously described (Martinez-Pastor et al., 2005). Briefly, the PRINCOMP procedure was first applied to perform principal component analysis (PCA) of the motility data. A nonhierarchical cluster analysis using the FASTCLUS procedure and the selected principal components as variables was then applied. The CLUSTER procedure, which performed a hierarchical clustering on the cluster obtained by the previous steps, was then applied using the average linkage method (AVERAGE) for joining the clusters. To determine the finally number of clusters (step four), 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.

2.5. Statistical analysis

The study was performed under blind conditions and the fertility rate data was obtained from CENSYRA after the assessment of semen samples from the various donkeys was completed. The results were expressed as mean ± SEM. Normality of data was assessed with the Kolmogorov–Smirnov test. Since data reported in this study were not normally distributed, percentages were subjected to arc sine transformation and absolute measures to logarithmic transformation. The sperm quality characteristics between fertility groups were compare using a general linear model (PROC GLM), in which donkey (six donkeys) and ejaculates of the same donkey (5 ejaculates per donkey) were entered as random effects, and the Duncan's Multiple Range test was applied to list these differences. The proportions of sperm subpopulations between fertility groups were also compared using the general linear model (PROC GLM) and Chi-square test (FREQ procedure). The relationships between semen traits and fertility were determined by linear regression analysis (PROC REG). All analyses were performed with SAS statistic package v9.0 (SAS Institute Inc., Cary, NC, USA). Significance was set at $P < 0.05$.

3. Results

Ejaculates used in this study ($n = 30$) had the following characteristics: gel-free volume 76.3 ± 6.6 mL (range: 36–160 mL), sperm concentration $240.3 \pm 21.5 \times 10^6$ spermatozoa/mL (range: $121–531 \times 10^6$ spermatozoa/mL), pH 7.2 ± 0.0 (range 6.8–7.6), total motility 89.1 ± 1.8% (range 71.5–98.9%), progressive motility 67.2 ± 2.9% (range 46.6–87.4%), total abnormalities 12.9 ± 1.4% (range 3.6–22.6%). Other sperm quality characteristics for the collected ejaculates are described in Table 4.

As shown in Table 2, a significant ($P < 0.05$) effect of the donkey on the quality of the fresh ejaculate was recorded in gel-free volume, pH, sperm concentration, and morphology as well as in some CASA-derived parameters (MT, MP, and VCL). No significant ($P > 0.05$) differences were recorded between ejaculates of the same animal for any sperm quality parameter. In addition, when data were pooled across

Table 2
Mean measurements of semen quality from six donkeys categorized as "fertile" or "sub-fertile" based on *in vivo* fertility data of breeding season.

Sperm characteristics	Fertile group			Sub-fertile group			*P*-values
	Donkey 1	Donkey 4	Donkey 5	Donkey 2	Donkey 3	Donkey 6	
Gel-free volume (mL)	112.5^{a}	88.1^{ab}	62.7^{b}	54.3^{b}	54.4^{b}	62.5^{b}	0.011
Sperm concentration ($\times 10^6$/mL)	152.7^{c}	193.0^{c}	229.7^{bc}	404.3^{a}	293.0^{b}	228.0^{bc}	0.001
Total motility (%)[a]	98.4^{a}	90.6^{ab}	90.7^{ab}	82.3^{b}	84.5^{b}	81.6^{b}	0.008
Progressive motility (%)[b]	83.9^{a}	65.4^{bc}	70.3^{b}	55.0^{c}	62.3^{bc}	55.0^{c}	0.001
pH	7.3^{a}	7.2^{a}	7.1^{ab}	6.9^{c}	7.2^{a}	7.0^{bc}	0.004
Total abnormalities (%)	6.0	12.2	8.3	16.5	18.6	20.6	0.000
VCL (μm/s)	116.0^{a}	104.5^{ab}	106.0^{ab}	101.3^{bc}	93.3^{c}	97.2^{bc}	0.002
VSL (μm/s)	82.6^{a}	76.6^{a}	81.3^{a}	76.1^{a}	72.9^{a}	74.5^{a}	0.309
VAP (μm/s)	101.4^{a}	92.8^{a}	95.7^{a}	90.5^{a}	84.5^{a}	86.3^{a}	0.060
LIN (%)	70.7^{a}	73.4^{a}	76.7^{a}	74.0^{a}	77.8^{a}	75.6^{a}	0.513
STR (%)	80.5^{a}	81.4^{a}	84.1^{a}	82.5^{a}	85.4^{a}	84.9^{a}	0.509
WOB (%)	86.7^{a}	88.7^{a}	90.0^{a}	88.3^{a}	90.3^{a}	87.9^{a}	0.593
ALH (μm)	2.2^{a}	2.3^{a}	2.3^{a}	2.3^{a}	2.1^{a}	2.3^{a}	0.717
BCF (Hz)	9.9^{a}	8.2^{a}	7.8^{a}	8.0^{a}	9.0^{a}	8.2^{a}	0.150

Different superscript letters (a–d) indicate significant differences between donkeys ($P<0.05$). Raw data are shown but statistical comparisons were calculated after arc sine and logarithmic transformations.

Table 3
Fresh semen sperm subpopulations (sP1, sP2, sP3, and sP4) and motility descriptors.

Sperm motility parameters	Sperm subpopulations (sP)			
	1	2	3	4
N	5954	21,208	861	1775
(%)	20.0	71.2	2.9	5.9
VCL (μm/s)	92.1 ± 0.5^{d}	103.3 ± 0.1^{c}	155.1 ± 0.6^{a}	145.7 ± 0.3^{b}
VSL (μm/s)	33.1 ± 0.2^{d}	86.0 ± 0.1^{c}	91.1 ± 0.7^{b}	130.9 ± 0.3^{a}
VAP (μm/s)	70.0 ± 0.4^{d}	95.1 ± 0.1^{c}	124.3 ± 0.6^{b}	137.9 ± 0.3^{a}
LIN (%)	38.5 ± 0.2^{d}	83.5 ± 0.1^{b}	59.3 ± 0.4^{c}	90.1 ± 0.2^{a}
STR (%)	52.4 ± 0.3^{d}	90.5 ± 0.1^{b}	74.3 ± 0.6^{c}	95.0 ± 0.1^{a}
WOB (%)	75.4 ± 0.1^{d}	91.9 ± 0.0^{b}	80.4 ± 0.3^{c}	94.9 ± 0.1^{a}
ALH (μm)	2.9 ± 0.0^{b}	2.0 ± 0.0^{d}	4.0 ± 0.0^{a}	2.2 ± 0.0^{c}
BCF (Hz)	7.6 ± 0.0^{c}	8.7 ± 0.0^{b}	10.2 ± 0.1^{a}	10.4 ± 0.1^{a}

Values are mean ± SEM for 30 semen samples from 6 Andalusian donkeys. The total number of spermatozoa analyzed was 29,798.
VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.
Different superscript letters (a–d) indicate significant differences between subpopulations ($P<0.05$). Raw data are shown but statistical comparisons were calculated after arc sine and logarithmic transformations.

Table 4
Sperm quality characteristics of two groups of donkeys ("fertile" and "sub-fertile"; $n=6$).

Sperm characteristics	Total group ($n=6$)	Fertile group ($n=3$)	Sub-fertile group ($n=3$)
Gel-free volume (mL)	76.3 ± 6.6	80.6 ± 11.1^{a}	71.2 ± 5.9^{a}
Sperm concentration ($\times 10^6$/mL)	240.3 ± 21.5	259.4 ± 37.6^{a}	216.9 ± 12.4^{a}
Total motility (%)[a]	89.1 ± 1.8	90.2 ± 2.7^{a}	87.6 ± 2.3^{a}
Progressive motility (%)[b]	67.2 ± 2.9	70.1 ± 4.1^{a}	63.6 ± 3.8^{a}
pH	7.2 ± 0.0	7.2 ± 0.1^{a}	7.1 ± 0.0^{a}
Total abnormalities (%)	12.9 ± 1.4	12.2 ± 2.1^{a}	13.7 ± 2.0^{a}
VCL (μm/s)	105.1 ± 0.2	106.6 ± 0.2^{a}	103.5 ± 0.2^{b}
VSL (μm/s)	78.2 ± 0.2	78.5 ± 0.3^{a}	77.9 ± 0.2^{a}
VAP (μm/s)	93.4 ± 0.2	94.4 ± 0.2^{a}	92.4 ± 0.2^{b}
LIN (%)	74.2 ± 0.1	73.3 ± 0.2^{b}	75.1 ± 0.2^{a}
STR (%)	82.7 ± 0.1	82.2 ± 0.2^{b}	83.2 ± 0.2^{a}
WOB (%)	88.5 ± 0.1	88.0 ± 0.1^{b}	89.0 ± 0.1^{a}
ALH (μm)	2.3 ± 0.0	2.2 ± 0.0^{b}	2.3 ± 0.0^{a}
BCF (Hz)	8.6 ± 0.0	9.2 ± 0.0^{a}	8.0 ± 0.0^{b}

Results are expresses as mean ± SEM for 30 ejaculates from 6 Andalusian donkeys. The jackasses were divided arbitrary into two different groups according to their fertility ($n=76$ estrous cycles from 27 jennies).
VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.
Different superscript letters (a, b) in a row indicate significant differences ($P<0.05$).
[a] Total motility is defined as the percentage of spermatozoa with a mean velocity >10 μm/s.
[b] Progressive motility is defined as the percentage of spermatozoa with a mean velocity >90 μm/s and straightness >75%.

donkeys, there were no differences ($P > 0.05$) in semen quality parameters between different experimental days.

3.1. Motile sperm subpopulation

Four sperm subpopulations were defined by the application of nonhierarchical and subsequent hierarchical classification of 29,798 individual motile spermatozoa. Table 3 shows the mean values for each motility variable in each subpopulation.

Subpopulation 1 (sP1) included spermatozoa whose movements were less vigorous (low VCL, VAP, and BCF) and less progressive (low VSL, LIN, and STR) than in all other groups, yielding 20% of the total motile population. Subpopulation 2 (sP2) included spermatozoa with relatively low velocity (medium VCL, VSL, and VAP) but with high progressiveness (high LIN, STR, WOB, BCF, and low ALH), yielding 71.2% of the total motile population. Subpopulation 3 (sP3) included highly active but non-progressive spermatozoa (high values of VCL, ALH, and BCF together with low LIN and STR values), yielding 2.9% of the total motile population. Subpopulation 4 (sP4) were the most vigorous spermatozoa (high VCL and BCF) and progressive (highest VSL and VAP), yielding 5.9% of the total motile population. The sperm trajectories were regular and linear (high LIN and STR), showing a moderate lateral head displacement (medium ALH).

There were significant ($P < 0.001$) differences in the distribution of the four sperm subpopulations in diluted ejaculates depending on the individual donkey donor. Spermatozoa moving forward actively (sP4) ranged from 0.3% to 13.4%, whereas spermatozoa moving forward slowly (sP2) ranged from 60.0% to 85.8%. sP1 (poorly motile non-progressive spermatozoa) varied from 13.7% to 23.3% and sP3 (highly active but non-progressive spermatozoa) from 0.2% to 6.5%. In addition, significant ($P < 0.001$) differences were seen between ejaculates of the same male with respect to the distributions of the four subpopulations.

3.2. Variations in conventional sperm characteristics and in subpopulation distribution of motile spermatozoa for pregnancy rate per cycle in jennies

Sperm quality parameters and sperm subpopulation proportions for the total group of donkeys ($n = 6$) and for the two different subgroups ("fertile" and "sub-fertile") are summarized in Tables 4 and 5. Significant differences ($P < 0.001$) between the "fertile" and the "sub-fertile" group were found for all of the evaluated sperm motility parameters (except for MT, MP, and VSL). Mean VCL, VAP, and BCF values were higher in "fertile" donkeys, whereas the opposite was found for LIN, STR, WOB, and ALH. No significant ($P > 0.05$) differences were found between the "fertile" and the "sub-fertile" group for the conventional sperm parameters of fresh semen (Table 4). Finally, the proportions of spermatozoa assigned to the Subpopulations 3 (highly active but non-progressive spermatozoa) and 4 (rapid and progressive spermatozoa) were significantly ($P < 0.001$) lower in the "sub-fertile" group (1.7% and 3.1%, respectively), whereas the opposite was found for sP2 (67.7% *versus* 74.8%; Table 5). The percentage of spermatozoa assigned to sP1 did not differ between groups ($P > 0.05$).

Table 5
Relative proportions of sperm subpopulations 1–4 (%) of two groups of donkeys ("fertile" and "sub-fertile"; $n = 6$).

Sperm populations (sP)	Total group ($n = 6$)	Fertile group ($n = 3$)	Sub-fertile group ($n = 3$)
1	20.0	19.5[a]	20.4[a]
2	71.2	67.7[b]	74.8[a]
3	2.9	4.1[a]	1.7[b]
4	5.9	8.7[a]	3.1[b]

Sperm populations are described in Section 3.2. Results express the percentages of the sperm populations distributed among the two groups depending upon the fertility of the donkeys. The results were obtained from 30 ejaculates, five ejaculates from each animal. The spermatozoa were distributed among the ejaculates in the following manner: 8146 motile spermatozoa were from ejaculate 1, 7349 from ejaculate 2, 4052 from ejaculate 3, 4810 from ejaculate 4 and 5441 from ejaculate 5. The total number of motile spermatozoa analyzed was 29,798.
VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.
Different superscript letters (a, b) in a row indicate significant differences ($P < 0.05$).

3.3. Relationships between sperm characteristics and percent pregnant per cycle

Correlations between per cycle pregnancy rate and MT ($r = 0.37$) and STR ($r = 0.39$) were low, but significant ($P < 0.05$). Correlations were higher when per cycle pregnancy rate were associated with MP ($r = 0.53$, $P = 0.003$), VCL ($r = 0.44$, $P = 0.014$), and BCF ($r = 0.44$, $P = 0.016$). Gel-free volume was positively correlated with per cycle pregnancy rate ($r = 0.53$, $P = 0.015$). Three of the sperm subpopulations (sP2, sP3, and sP4) were positively correlated ($P < 0.05$) with per cycle pregnancy rate ($r = 0.54$, $r = 0.45$, and $r = 0.56$, respectively).

4. Discussion

To the best of our knowledge, the present study is the first to report the relationship between the ejaculated sperm characteristics and field fertility in a group of Andalusian donkeys. To date, other studies have reported relationships between sperm quality (motility and morphologic features) and fertility outcome in the stallion (Jasko et al., 1990, 1992; Love, 2011; Love et al., 2000; Morrell et al., 2008), but these studies varied depending on how fertility was defined and the design may have been limited by small numbers of stallions (Love, 2011). In addition, methodologies used to determine sperm quality criteria vary, thereby introducing bias that may alter interpretation of findings (Rodríguez-Martínez, 2003).

Sperm production capacity as reflected in sperm concentration, semen volume, and number of spermatozoa in the ejaculate gives valuable information with regard to the number of mares a stallion can cover, or insemination doses he can produce, per day. However, the relationship between fertility and these sperm parameters is very poor (Colenbrander et al., 2003). In our study, no differences were observed between "fertile" and "sub-fertile" groups

regarding gel-free volume or sperm concentration, which is in consonance with previous findings (Parlevliet and Colenbrander, 1999). However, a relationship between gel-free volume and fertility was found. In this connection, it has been reported that a large number of factors (breed, age, housing, *etc.*) can affect sperm production capacity (Gadea, 2005). On the other hand, the pH of gel-free volume of the "fertile" donkeys tended to be greater ($P = 0.392$) when compared with the measurement of "sub-fertile" donkeys; however, a significant difference was not detected. Differences in pH could be accounted for by the contribution of secretions from accessory glands fluids (Brun et al., 2002).

It has been demonstrated that a decrease in the number of motile, morphologically normal spermatozoa in stallion ejaculates leads to reduced fertility (Jasko et al., 1990, 1992; Love, 2011). In contrast, in donkeys, no reports have been published on this topic. Under our experimental conditions, there were no differences between "fertile" and "sub-fertile" donkeys for both sperm parameters. This limited usefulness to relate either sperm motility or morphology to fertility results has already become evident in stallions (Malmgren, 1992; Voss et al., 1981). With regard to sperm morphology, intra- and inter-stallion variations in sperm morphology have been reported (Voss et al., 1981), suggesting that it has a more limited value in determining the potential fertility in the stallion (Kuisma et al., 2006). In fact, differences are noticeable among donkeys for conventional sperm parameters (gel-free volume, pH, sperm concentration, and morphology), and for total and progressive motility (CASA). Therefore, these variations add to the already complicated process of attempting to predict potential fertility *in vivo*. However, it is possible that tests based on more animals and/or ejaculates per donkey would improve the predictive value of the *in vitro* process.

The majority of the CASA motion parameters used in the present study have been previously identified as the most reliable markers of sperm quality in humans (De Geyter et al., 1998; Larsen et al., 2000) and in domestic animals (Dorado et al., 2009; Holt et al., 1997). Moreover, some of those CASA-derived parameters (VSL, VAP, LIN, STR, ALH, and BCF) have been previously associated with fertility in humans (Macleod and Irvine, 1995) and in domestic animals (Brun et al., 2002; Farrell et al., 1998; Holt et al., 1997; Vidament et al., 2000). Our results show that only three specific CASA parameters (VCL, VAP, and BCF) were poorer in donkeys with lower *in vivo* fertility results. Sperm motility parameters that were different between groups also tended to be correlated. Therefore, VCL, STR, and BCF were positively related to per cycle pregnancy rate. Similar to our study, Jasko et al. (1992) and Love (2011) reported that VAP were higher in the group of higher fertility based on percent pregnant per cycle. Studies in dogs (Silva et al., 2006) and boars (Holt et al., 1997) have demonstrated that high sperm velocities are landmarks of fertility both *in vivo* and *in vitro*. It has also been reported previously that VCL is of key importance for formation of the sperm reservoir at the utero-tubal junction in mice (Olds-Clarke, 1996). Moreover, BCF has been positively correlated with pregnancy rate or *in vitro* fertilization rate (Suarez et al., 1991).

The importance of VSL to the fertilizing of the spermatozoa has been observed in many species (Gillan et al., 2008; Silva et al., 2006; Van den Bergh et al., 1998) and it may play a role in sperm transport through the reproductive tract and penetration of the oocyte (Gillan et al., 2008). However, under our experimental conditions, no differences were observed between "fertile" and "sub-fertile" donkeys. Likewise, VSL was not related to per cycle pregnancy rate. Other authors have failed to determine an association between motion parameters of semen samples and fertility after AI in bull (Bailey et al., 1994), sheep (Sánchez-Partida et al., 1999), goat (Dorado et al., 2010b), and stallion (Nie et al., 2002), due in part to the many factors involved in the fertilization process and in the fertility of animals (Gadea, 2005). In any case, it is well known that the positive relationship between single semen quality characteristics such as motility and fertility is either of low significance or measurable in some sires but not in others, as reported by Gil et al. (2005).

The ALH was shown to affect the outcome of *in vivo* fertility (IVF) in humans (Barlow et al., 1991) and rats (Moore and Akhondi, 1996). However, this parameter has to be considered with caution, as it depends on the capacitation status of the spermatozoon (Iguer-ouada and Verstegen, 2001). In fact, mean ALH values were significantly greater in the "sub-fertile" group than in the "fertile" one. Because LIN is the ratio of VSL to VCL and is negatively correlated to IVF rate, the infertile spermatozoa may tend to have a high proportion of VSL to VCL (Verstegen et al., 2002). This study produced results which corroborate these findings.

This study has also been aimed to characterize those sperm subpopulations based on motile characteristics that could be related with fertility in Andalusian donkeys. In our conditions, the percentage of the subpopulation with the most rapid and progressive spermatozoa (sP4), and with high values of VCL, VSL, and VAP was lower in donkeys with poor *in vivo* fertility results. In addition, three of the subpopulations identified (sP2, sP3, and sP4) were related to per cycle pregnancy rate. Our findings suggest the relationship between the predominance of a particular motile subpopulation and fertility, as has been concluded in other studies (Harrison, 1996; Martinez-Pastor et al., 2005; Petrunkina et al., 2007). Therefore, knowing the percentage of the subpopulation with the most rapid and progressive spermatozoa and with high velocities, together with BCF, might be used as a powerful tool to predict the fertilizing ability of an animal.

Problems confronted in previous fertility studies are also obvious here. The number of breeding per donkey was small in comparison with other species such as bovine, ovine, caprine, or porcine, and fertility of jackass can vary between years. Many other factors in addition to semen characteristics influence fertility. Percent pregnant per cycle is a more accurate and reliable measure of fertility than other ones (*i.e.* seasonal pregnancy rate, foaling rate), due to this estimate separates the highly fertile jackass that required few estrous period to impregnate a jenny from the less fertile individual that required more opportunities (Love, 2011). However, this measure of fertility does not eliminate other factors affecting fertility results, such as management and reproduction performance of jennies, their previous reproductive status, and possible genetic factors (Jasko et al., 1992). Standardizing all these variables is clearly not possible.

5. Conclusions

Relationships between the evaluated sperm characteristics and fertility of this group of donkeys have been evidenced. Among CASA-derived sperm motion measures, mean VCL, VAP, and BCF values were higher in donkeys achieving a percent pregnant per cycle ≥60%. Per cycle pregnancy rate seemed to be related to computer-assisted measurements of sperm motility. Based on our findings, we could consequently elucidate specific sperm characteristics which are indicative for the IVF in donkeys. In addition, the subpopulation characterized by rapid and progressive spermatozoa was significantly lower in donkeys with poor *in vivo* fertility results. Moreover, fertility was related to the subpopulations identified. Our results stress the importance of using the CASA techniques to detect subtle differences in sperm motility between "fertile" and "sub-fertile" donkeys. In addition, the study of discrete subpopulations of motile spermatozoa could add additional insight into the detection of sub-fertility.

Acknowledgments

We are extremely grateful to Fundación Casa del Burro (Rute, Cordoba, Spain) for supplying the animals. This work has been supported by Grant RZ2009-00006-00-00 (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Ministerio de Ciencia e Innovación, Spain).

References

Abaigar, T., Holt, W.V., Harrison, R.A.P., Del Barrio, G., 1999. Sperm subpopulations in Boar (*Sus scrofa*) and Gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol. Reprod. 60, 32–41.

Bailey, J.L., Buhr, M.M., Robertson, L., 1994. Relationships among in vivo fertility, computer-analysed motility and in vitro Ca^{2+} flux in bovine spermatozoa. Can. J. Anim. Sci. 74, 53–58.

Barlow, P., Delvigne, A., Van Dromme, J., Van Hoeck, J., Vandenbosch, K., Leroy, F., 1991. Predictive value of classical and automated sperm analysis for in-vitro fertilization. Hum. Reprod. 6, 1119–1124.

Brito, L.F.C., 2007. Evaluation of stallion sperm morphology. Clin. Tech. Equine Pract. 6, 249–264.

Brun, J.M., Theau-Clément, M., Bolet, G., 2002. The relationship between rabbit semen characteristics and reproductive performance after artificial insemination. Anim. Reprod. Sci. 70, 139–149.

Casey, P.J., Gravance, C.G., Davis, R.O., Chabot, D.D., Liu, I.K.M., 1997. Morphometric differences in sperm head dimensions of fertile and subfertile stallions. Theriogenology 47, 575–582.

Colenbrander, B., Gadella, B.M., Stout, T.A.E., 2003. The predictive value of semen analysis in the evaluation of stallion fertility. Reprod. Domest. Anim. 38, 305–311.

DAD-IS-FAO, 2011. Information System Domestic Animal Diversity. FAO, Rome, Italy.

De Geyter, C., De Geyter, M., Koppers, B., Nieschlag, E., 1998. Diagnostic accuracy of computer-assisted sperm motion analysis. Hum. Reprod. 13, 2512–2520.

Dorado, J., Rodríguez, I., Hidalgo, M., 2007. Cryopreservation of goat spermatozoa: comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. Theriogenology 68, 168–177.

Dorado, J., Hidalgo, M., Muñoz, A., Rodríguez, I., 2009. Assessment of goat semen freezability according to the spermatozoa characteristics from fresh and frozen samples. Anim. Reprod. Sci. 112, 150–157.

Dorado, J., Molina, I., Muñoz-Serrano, A., Hidalgo, M., 2010a. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Florida goats. Theriogenology 74, 795–804.

Dorado, J., Muñoz-Serrano, A., Hidalgo, M., 2010b. The effect of cryopreservation on goat semen characteristics related to sperm freezability. Anim. Reprod. Sci. 121, 115–123.

Dorado, J., Gálvez, M.J., Murabito, M.R., Muñoz-Serrano, A., Hidalgo, M., 2011. Identification of sperm subpopulations in canine ejaculates: effects of cold storage and egg yolk concentration. Anim. Reprod. Sci. 127, 106–113.

Farrell, P.B., Presicce, G.A., Brockett, C.C., Foote, R.H., 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology 49, 871–879.

Flores, E., Taberner, E., Rivera, M.M., Peña, A., Rigau, T., Miró, J., Rodríguez-Gil, J.E., 2008. Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates. Theriogenology 70, 936–945.

Flores, E., Fernández-Novell, J.M., Peña, A., Rodríguez-Gil, J.E., 2009. The degree of resistance to freezing-thawing is related to specific changes in the structures of motile sperm subpopulations and mitochondrial activity in boar spermatozoa. Theriogenology 72, 784–797.

Gadea, J., 2005. Sperm factors related to in vitro and in vivo porcine fertility. Theriogenology 63, 431–444.

Gil, M.A., Roca, J., Cremades, T., Hernández, M., Vázquez, J.M., Rodríguez-Martínez, H., Martínez, E.A., 2005. Does multivariate analysis of post-thaw sperm characteristics accurately estimate in vitro fertility of boar individual ejaculates? Theriogenology 64, 305–316.

Gillan, L., Kroetsch, T., Chis Maxwell, W.M., Evans, G., 2008. Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. Anim. Reprod. Sci. 103, 201–214.

Harald, S., 2009. Semen evaluation. In: Samper, J.C. (Ed.), Equine Breeding Management and Artificial Insemination. Saunders Elsevier, St. Louis, USA, pp. 57–74.

Harrison, R.A.P., 1996. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. Reprod. Fertil. Dev. 8, 581–594.

Holt, W.V., 1996. Can we predict fertility rates? Making sense of sperm motility. Reprod. Domest. Anim. 31, 17–24.

Holt, C., Holt, W.V., Moore, H.D.M., Reed, H.C.B., Curnock, R.M., 1997. Objectivily measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. J. Androl. 18, 312–323.

Iguer-ouada, M., Verstegen, J.P., 2001. Evaluation of the "Hamilton thorn computer-based automated system" for dog semen analysis. Theriogenology 55, 733–749.

Jasko, D.J., Lein, D.H., Foote, R.H., 1990. Determination of the relationship between sperm morphologic classifications and fertility in stallions: 66 cases (1987–1988). J. Am. Vet. Med. Assoc. 197, 389–394.

Jasko, D.J., Little, T.V., Lein, D.H., Foote, R.H., 1992. Comparison of spermatozoal movement and semen characteristics with fertility in stallions: 64 cases (1987–1988). J. Am. Vet. Med. Assoc. 200, 979–985.

Kastelic, J.P., Thundathil, J.C., 2008. Breeding soundness evaluation and semen analysis for predicting bull fertility. Reprod. Domest. Anim. 43, 368–373.

Kenney, R.M., Bergman, R.V., Cooper, W.L., Morse, G.W., 1975. Minimal contamination techniques for breeding mares: techniques and preliminary findings. In: Proc. 21st Annu. Conv. Am. Assoc. Equine Pract, p. 327.

Kuisma, P., Andersson, M., Koskinen, E., Katila, T., 2006. Fertility of frozen-thawed stallion semen cannot be predicted by the currently used laboratory methods. Acta Vet. Scand., 48.

Larsen, L., Scheike, T., Jensen, T.K., Bonde, J.P., Ernst, E., Hjollund, N.H., Zhou, Y., Skakkebæk, N.E., Giwercman, A., Bonde, J.P.E., Hjollund, N.H.I., Henriksen, T.B., Kolstad, H.A., Anderson, A.M., Olsen, J., 2000. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. Hum. Reprod. 15, 1562–1567.

Love, C.C., 2011. Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology 76, 547–557.

Love, C.C., Varner, D.D., Thompson, J.A., 2000. Intra- and inter-stallion variation in sperm morphology and their relationship with fertility. J. Reprod. Fertil. Suppl., 93–100.

Macleod, I.C., Irvine, D.S., 1995. Andrology: the predictive value of computer-assisted semen analysis in the context of a donor insemination programme. Hum. Reprod. 10, 580–586.

Malmgren, L., 1992. Sperm morphology in stallions in relation to fertility. Acta Vet. Scand. Suppl. 88, 39–47.

Martinez-Pastor, F., Garcia-Macias, V., Alvarez, M., Herraez, P., Anel, L., De Paz, P., 2005. Sperm subpopulations in Iberian red deer epididymal sperm and their changes through the cryopreservation process. Biol. Reprod. 72, 316–327.

Miró, J., Lobo, V., Quintero-Moreno, A., Medrano, A., Peña, A., Rigau, T., 2005. Sperm motility patterns and metabolism in Catalonian donkey semen. Theriogenology 63, 1706–1716.

Miró, J., Taberner, E., Rivera, M., Peña, A., Medrano, A., Rigau, T., Peñalba, A., 2009. Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen. Theriogenology 72, 1017–1022.

Moore, H.D.M., Akhondi, M.A., 1996. Fertilizing capacity of rat spermatozoa is correlated with decline in straight-line velocity measured by continuous computer-aided sperm analysis: epididymal rat spermatozoa from the proximal cauda have a greater fertilizing capacity in vitro than those from the distal cauda or vas deferens. J. Androl. 17, 50–60.

Morrell, J.M., Johannisson, A., Dalin, A.M., Hammar, L., Sandebert, T., Rodriguez-Martinez, H., 2008. Sperm morphology and chromatin integrity in Swedish warmblood stallions and their relationship to pregnancy rates. Acta Vet. Scand., 50.

Nie, G.J., Wenzel, J.G.W., Johnson, K.E., 2002. Comparison of pregnancy outcome in mares among methods used to evaluate and select spermatozoa for insemination. Anim. Reprod. Sci. 69, 211–222.

Novak, S., Smith, T.A., Paradis, F., Burwash, L., Dyck, M.K., Foxcroft, G.R., Dixon, W.T., 2010. Biomarkers of in vivo fertility in sperm and seminal plasma of fertile stallions. Theriogenology 74, 956–967.

Núñez-Martínez, I., Moran, J.M., Peña, F.J., 2006a. A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: changes after cryopreservation. Reprod. Domest. Anim. 41, 408–415.

Núñez Martínez, I., Morán, J.M., Peña, F.J., 2006b. Two-step cluster procedure after principal component analysis identifies sperm subpopulations in canine ejaculates and its relation to cryoresistance. J. Androl. 27, 596–603.

O' Meara, C.M., Hanrahan, J.P., Prathalingam, N.S., Owen, J.S., Donovan, A., Fair, S., Ward, F., Wade, M., Evans, A.C.O., Lonergan, P., 2008. Relationship between in vitro sperm functional tests and in vivo fertility of rams following cervical artificial insemination of ewes with frozen-thawed semen. Theriogenology 69, 513–522.

Olds-Clarke, P., 1996. How does poor motility alter sperm fertilizing ability? J. Androl. 17, 183–186.

Parlevliet, J.M., Colenbrander, B., 1999. Prediction of first season stallion fertility of 3-year-old Dutch Warmbloods with prebreeding assessment of percentage of morphologically normal live sperm. Equine Vet. J. 31, 248–251.

Petrunkina, A.M., Waberski, D., Günzel-Apel, A.R., Töpfer-Petersen, E., 2007. Determinants of sperm quality and fertility in domestic species. Reproduction 134, 3–17.

Quintero-Moreno, A., Miró, J., Teresa Rigau, A., Rodríguez-Gil, J.E., 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 59, 1973–1990.

Quintero-Moreno, A., Rigau, T., Rodríguez-Gil, J.E., 2004. Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. Theriogenology 61, 673–690.

Quintero-Moreno, A., Rigau, T., Rodríguez-Gil, J.E., 2007. Multivariate cluster analysis regression procedures as tools to identify motile sperm subpopulations in rabbit semen and to predict semen fertility and litter size. Reprod. Domest. Anim. 42, 312–319.

Rigau, T., Farré, M., Ballester, J., Mogas, T., Pea, A., Rodríguez-Gil, J.E., 2001. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology 56, 801–815.

Rodríguez-Martínez, H., 2003. Laboratory semen assessment and prediction of fertility: still utopia? Reprod. Domest. Anim. 38, 312–318.

Sánchez-Partida, L.G., Windsor, D.P., Eppleston, J., Setchell, B.P., Maxwell, W.M.C., 1999. Fertility and its relationship to motility characteristics of spermatozoa in ewes after cervical, transcervical, and intrauterine insemination with frozen-thawed ram semen. J. Androl. 20, 280–288.

Scott, M.A., 2000. A glimpse at sperm function in vivo: sperm transport and epithelial interaction in the female reproductive tract. Anim. Reprod. Sci. 60-61, 337–348.

Silva, A.R., Cardoso, R.d.C.S., Silva, L.D.M., Chirinéa, V.H., Lopes, M.D., Souza, F.F., 2006. Prognostic value of canine frozen-thawed semen parameters on in vitro sperm-oocyte interactions. Theriogenology 66, 456–462.

Suarez, S.S., Katz, D.F., Owen, D.H., Andrew, J.B., Powell, R.L., 1991. Evidence for the function of hyperactivated motility in sperm. Biol. Reprod. 44, 375–381.

Van den Bergh, M., Emiliani, S., Biramane, J., Vannin, A.S., Englert, Y., 1998. A first prospective study of the individual straight line velocity of the spermatozoon and its influences on the fertilization rate after intracytoplasmic sperm injection. Hum. Reprod. 13, 3103–3107.

Verstegen, J., Iguer-Ouada, M., Onclin, K., 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57, 149–179.

Vidament, M., Magistrini, M., Palmer, E., Clement, F., 2000. Equine artificial insemination in French National Studs. Reprod. Domest. Anim. 35, 61–66.

Vidament, M., Vincent, P., Martin, F.X., Magistrini, M., Blesbois, E., 2009. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. Anim. Reprod. Sci. 112, 22–35.

Voss, J.L., Pickett, B.W., Squires, E.L., 1981. Stallion spermatozoal morphology and motility and their relationships to fertility. J. Am. Vet. Med. Assoc. 178, 287–289.

Capítulo III / Chapter III

"Effect of extender and amino acid supplementation on sperm quality of cooled-preserved Andalusian donkey (Equus asinus) spermatozoa".

J. Dorado, D. Acha, I. Ortiz, M.J. Gálvez, J.J. Carrasco, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo. Animal Reproduction Science 2014, 146: 79-88.

Animal Reproduction Science 146 (2014) 79–88

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Effect of extender and amino acid supplementation on sperm quality of cooled-preserved Andalusian donkey (*Equus asinus*) spermatozoa

J. Dorado[a,*], D. Acha[a], I. Ortiz[a], M.J. Gálvez[a], J.J. Carrasco[b], V. Gómez-Arrones[b], R. Calero-Carretero[b], M. Hidalgo[a]

[a] Animal Reproduction Group, Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Cordoba, 14071 Córdoba, Spain

[b] Equine Reproduction Center, Centro de Selección y Reproducción Animal, (CENSYRA-Extremadura Government), 06007 Badajoz, Spain

ARTICLE INFO

Article history:
Received 25 October 2013
Received in revised form 5 February 2014
Accepted 7 February 2014
Available online 19 February 2014

Keywords:
Amino acids
Andalusian donkey
Cooled semen
Sperm quality

ABSTRACT

The main aim of this study was to evaluate the efficacy of two commercially available liquid stallion semen extenders for the preservation of Andalusian donkey semen at 5 °C for up to 72 h, and to evaluate the effect of amino acid addition on sperm quality of cooled donkey semen. In addition, this study investigated the effect of seasons on semen characteristics of Andalusian jackasses. Throughout a year, 50 ejaculates were collected from ten adult donkeys and a complete semen evaluation was performed immediately after collection. In Experiment 1, semen samples ($n = 32$) were pooled, divided into two aliquots, and cooled in either Gent® A or INRA 96®. In Experiment 2, pooled semen samples ($n = 9$) were cooled in Gent A® supplemented with 0 (as control), 20, 40, or 60 mM for each glutamine, proline, or taurine. Fresh semen and chilled samples were assessed for sperm motility, morphology, acrosome integrity, and plasma membrane integrity. Sperm motility variables were greater ($P < 0.05$) in Gent® A than in INRA 96®. The presence of glutamine, proline, or taurine in Gent® A improved ($P < 0.001$) the motility of Andalusian donkey spermatozoa. Differences ($P < 0.05$) in some sperm variables were observed among seasons. In conclusion, Gent® A maintained sperm motility characteristics after 72 h of cold storage to a greater extent than INRA 96®. Moreover, motility was greater when Gent® A supplemented at different concentrations of amino acids than Gent® A with no supplementation. An effect of seasons on the semen quality of the Andalusian donkey was demonstrated.

1. Introduction

One of the strategies to increase jackass breeding efficiency, especially in endangered breeds such as the Andalusian donkey (*Equus asinus*), is the use of cooled semen in artificial insemination (AI) programs as a tool for improving gene distribution and for reducing the effects of excessive inbreeding (Rota et al., 2008). *Artificial insemination* of mares with cooled-shipped semen has become a routine procedure in the equine breeding industry because of its good fertility rates and relatively low costs (Contri et al., 2010). Although the use of cooled semen in horse reproduction has been investigated (Aurich, 2008), far less studies are available for donkeys (Miro et al., 2009; Rota

* Corresponding author at: Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Cordoba, Campus de Rabanales (Edif. Hospital Clínico Veterinario), Ctra. Madrid-Cádiz, km 396, 14071 Córdoba, Spain. Tel.: +34 957 212136; fax: +34 957 211093.
E-mail address: jdorado@uco.es (J. Dorado).

http://dx.doi.org/10.1016/j.anireprosci.2014.02.009
0378-4320/

et al., 2008; Santos et al., 1995; Serres et al., 2002; Vidament et al., 2009). Few previous studies have compared different semen extenders for the preservation of donkey semen at 5 °C and the results vary between studies. However, the use of extenders containing egg yolk seems advantageous (Cottorello et al., 2002; Mello et al., 2000; Rota et al., 2008). Among the milk-based extenders used in donkeys, use of INRA 82 and Kenney resulted in maintenance of a greater sperm motility than use of skimmed milk alone (Gomez-Cuetara et al., 2004), whereas use of INRA 96® resulted in greater maintenance of motility than use of E-Z Mixin (Contri et al., 2010). Rota et al. (2008) reported that the use of INRA 96® produces similar results to INRA 82 during semen preservation at 5 °C for 72 h in Amiata donkeys. To date, there has not been an evaluation or comparison of the efficacy of Gent® A extender, a commercially available stallion semen extender, for cooling and preserving donkey semen. There are no studies concerning the preservation of semen quality during storage in the Andalusian donkey.

A wide variety of organisms are known to accumulate amino acids in response to cold temperatures (Anchordoguy et al., 1988). To date, it has also been reported that some amino acids protect several types of animal cells against hypothermia (Heinz et al., 1990; Kruuv and Glofcheski, 1990; Lalonde et al., 1991). In spermatozoa, several studies have demonstrated that the addition of taurine to semen extenders improves the quality of chilled semen in cats (Baran et al., 2009), rabbits (Alvarez and Storey, 1983a), rams (Bucak et al., 2007), and stallions (Ijaz and Ducharme, 1995). The protective effect of both glutamine (Khlifaoui et al., 2005; Trimeche et al., 1996, 1999) and proline (Rudolph and Crowe, 1985; Trimeche et al., 1999) against freezing–thawing damage in stallion and donkey spermatozoa have also been reported. However, Phetudomsinsuk et al. (2009) reported that the inclusion of glutamine (50 mM) in freezing extenders resulted in a significant decrease in sperm motility and plasma membrane integrity of frozen–thawed stallion spermatozoa. The disparity of results obtained by these previous studies suggests a species-specific effect depending not only on the type of antioxidants used but also on the concentration used, which has been suggested by Cabrita et al. (2011). No previous comparative studies concerning the use of amino acids for cooling and preserving Andalusian donkey semen have been conducted.

To improve the reproductive performance of donkeys, several studies on reproductive seasonality have recently been performed. Therefore, previous studies have demonstrated the seasonal influences on both sexual behavior (Gastal et al., 1996; Henry et al., 1998) and seminal variables (Carluccio et al., 2013; Contri et al., 2010; Gastal et al., 1997) of jackasses. However, studies performed on different breeds or in climatically different study areas are poorly transferable to other breeds of donkeys (e.g., Andalusian donkeys) as has been suggested previously (Carluccio et al., 2013). To our knowledge, no studies have been conducted to evaluate semen quality of Andalusian donkeys throughout an annual cycle.

With this background, the aims of the present study were to: (1) evaluate the efficacy of two commercially available liquid stallion semen extenders (Gent® A compared with INRA 96®) for the preservation of Andalusian donkey semen at 5 °C for 72 h, (2) assess the effect of different concentrations (0, 20, 40, and 60 mM) of glutamine, proline, and taurine amino acids added to the extender on quality of cooled donkey semen, and (3) investigate the effect of seasons on semen characteristics of Andalusian jackasses.

2. Materials and methods

2.1. Experimental animals

This study was conducted in Badajoz (38°54′6″ N–6°58′28″ W; altitude of 182 m above sea level), Spain, between June 2011 and November 2012. Ten healthy mature Andalusian donkeys (4–15 years of age and weighting 313–435 kg) were used. The jackasses were housed in individual paddocks at the Equine Center for Assisted Reproduction of the Centro de Selección y Reproducción Animal (CENSYRA) and maintained outdoors under natural photoperiod. The animals were fed daily hay and grain, and water was freely available.

Semen was collected from the jackasses using a Missouri-model artificial vagina (Minitüb GmbH, Tiefenbach, Germany) with an in-line gel filter (Minitüb GmbH, Tiefenbach, Germany). A jenny in natural or prostaglandin-induced estrus (Luprostiol, 7.5 mg intramuscularly; Prosolvin, Intervet International B.V., Boxmeer, The Netherlands) was used to induce copulatory activity.

2.2. Analysis of semen quality variables

Immediately after collection the gel-free volume of each ejaculate was measured in a graduated tube. Sperm concentration was calculated with a photometer (SpermaCue, Minitüb GmbH, Tiefenbach, Germany) as described by Vidament et al. (2009), and seminal pH was determined within 5 min using a pH meter (HI 2211-02, Hanna Instruments Inc., Woonsocket, MA, USA) (Miro et al., 2009). At the same time, an aliquot of the ejaculate was diluted in pre-warmed (37 °C) skim milk-glucose extender (Kenney et al., 1975) to a final concentration of 25×10^6 spermatozoa/mL. Aliquots of the diluted semen were subsequently taken as needed to conduct the appropriate analyses.

Sperm motility was assessed using the computer-assisted sperm analyzer (CASA) system Sperm Class Analyzer (Microptic SL, Barcelona, Spain), as described by Miro et al. (2005) for donkey semen. Prior to the assessment of movement, aliquots of diluted semen were incubated at 37 °C for 5 (fresh semen) or 10 min (cooled samples). For each evaluation, three consecutive 5 μL drops of each diluted semen sample were evaluated using a phase contrast microscope (Eclipse 50i, Nikon, Tokyo, Japan) with a pre-warmed stage at 37 °C at 100× magnification. Two microscopic fields per drop were filmed randomly, including a minimum of 200 spermatozoa. Objects incorrectly identified as spermatozoa were minimized by using the playback function. Regarding the setting variables of the program, spermatozoa with a mean average path velocity (VAP) < 10 μm/s were considered immotile.

Spermatozoa with VAP > 90 μm/s were considered as rapid, while spermatozoa deviating < 25% from a straight line were designated as linear motile. The measured variables of sperm motion were total motility (MT; %), progressive motility (MP; %), curvilinear velocity (VCL; μm/s), straight line velocity (VSL; μm/s); average path velocity (VAP; μm/s), linearity (LIN, as VSL/VCL; %), straightness (STR, as VSL/VAP; %), wobble (WOB, as VAP/VCL; %), beat cross frequency (BCF; Hz), and amplitude of lateral head displacement (ALH; μm). Definitions of these descriptors of sperm movement can be found in Dorado et al. (2007).

Sperm morphology was examined by light microscopy evaluation (Olympus BH-2, Olympus Optical Co., Ltd., Tokyo, Japan) on smears stained with Diff-Quick® (Medion Diagnostics AG, Düdingen, Switzerland) staining (Brito, 2007). At least 200 spermatozoa per slide were counted to determine the percentage of spermatozoa with abnormal morphology (ASM, %).

Sperm membrane integrity was assessed using the double stain propidium iodide (PI) with acridine orange (AO) from the Vital-Test® kit (Halotech SL, Madrid, Spain), following the manufacturer' instructions. Briefly, an aliquot (10 μL) of diluted semen (15×10^6 spermatozoa/mL) was placed on a microscope slide at 37 °C and mixed with 1 μL PI stock solution and 1 μL AO stock solution. At least 200 spermatozoa per slide were counted with fluorescence microscopy (Olympus BX40, Tokyo, Japan), using a U-ND25-2 filter (a 460–490 nm excitation filter). The nuclei of spermatozoa with intact plasma membrane stained green with AO whereas those with a damaged membrane stained red with PI. The results are expressed as percentages of membrane-intact spermatozoa – MIS (AO+; %).

To evaluate the sperm acrosomes, the PI/peanut agglutinin–fluorescein isothiocyanate (FITC-PNA) double stain (Sigma–Aldrich Chemie GmbH, Steinheim, Germany), was used as described by Cheng et al. (1996) with minor modifications. In brief, a 10 μL aliquot of diluted semen (25×10^6 spermatozoa/mL) was spread on a microscope slide, air dried, fixed, and permeabilized with 70% (v:v) ethanol for 30 s. A mixture of 10 μL PI (0.1 mg/mL in ultra-pure water) and 20 μL FITC-PNA (0.1 mg/mL in PBS), previously incubated at 38 °C for 5 min in the dark, was then spread over each smear and the slides were incubated in a dark, moist chamber at 4 °C for 30 min. The slides were subsequently rinsed with deionized water at 4 °C, mounted with 10 μL of Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA), covered with a coverslip, and sealed with nail polish. The acrosome morphology of spermatozoa was observed using an epifluorescence microscope (Olympus BX40, Tokyo, Japan). At least 200 spermatozoa were evaluated on each slide under 1000× magnification. Ethanol-permeabilized donkey spermatozoa could be classified into two groups: (1) acrosome-intact spermatozoa – AIS (the acrosomal region of the sperm head displayed bright green fluorescence; PI+/FICT-PNA+), and (2) acrosome-reacted spermatozoa – ARS (green fluorescent at the equatorial segment or no anterior acrosomal staining; PI+/FICT-PNA−). All spermatozoa showed red fluorescence due to counterstaining with PI. Values were expressed as percentages.

2.3. Storage of semen at 5 °C

Semen quality was assessed before cooling as detailed below. The cooling protocol was based on those used in previous studies (Miro et al., 2009; Serres et al., 2002) including the following modifications. Briefly, aliquots (1 mL) of each ejaculate were pooled (two ejaculates per pool) and diluted 1:4 (v:v, semen:extender) with skim milk–glucose extender (EquiPro® A, Minitüb GmbH, Tiefenbach, Germany) without antibiotics, in sterile 15-mL Falcon centrifuge tubes (BDFalcon™ Tubes, BD Biosciences, Erembodegem, Belgium). Pooled semen samples were then centrifuged at 660 × *g* for 15 min at 20 °C. After supernatant aspiration, the pellet was re-suspended in the tested extenders (see experimental design) at a final concentration of 50×10^6 spermatozoa/mL, and then cooled at 22 °C for 10 min. Vials were placed in Equitainer™ I (Hamilton Research, Inc., Danvers, MA, USA) for the first 24 h of chilled preservation, and then transferred to a 5 °C refrigerator (Refrigerated Thermostat – FTC 90E, VELP Scientifica, Usmate, Italia) for preservation until hour 72.

Semen assessments were performed after recovery (in skim milk–glucose extender) and after 72 h of storage in the tested extenders. For assessment, aliquots of diluted semen were re-warmed to 37 °C and evaluated as described previously.

2.4. Experimental design

2.4.1. Experiment 1: effect of extender on sperm quality

The effects of two different commercial equine semen extenders, Gent® A (Minitüb GmbH, Tiefenbach, Germany) and INRA 96® (IMV Technologies, L'Aigle, France) on sperm quality of cooled donkey semen were evaluated. Gent extender was developed at the University of Ghent (Belgium), and it is marketed by Minitüb GmbH (REF. 13571/0045). Although the precise formulation of Gent® A has not been released by the manufacturer, it consists of buffers, milk, and egg yolk (Janett et al., 2012). INRA 96® is patented and available as a ready-to-use solution (REF. 01644). The INRA 96® extender contains a defined milk protein (native phosphocaseinate), which is then supplemented with Hank' balanced salt solution, glucose, and lactose (Batellier et al., 1998). Ejaculates ($n = 32$; four ejaculates per donkey) were collected from eight donkeys from June 2011 to November 2012. Ejaculates from two donkeys were pooled on each collection day to avoid uncontrolled male-to-male variation. Eight pooled semen samples (two ejaculates per pool; four replicates) were divided into two aliquots and cooled in either Gent® A or INRA 96®. Osmolality and pH of the extenders were determined (Gent® A: 312 mOSm/kg, 6.73; INRA 96®: 314 mOSm/kg, 7.01). All osmolalities were assessed using a freezing-point digital micro-osmometer Type 6 (Löser Messtechnik, Berlin, Germany).

2.4.2. Experiment 2: effect of amino acid supplementation to Gent A® extender

To evaluate the effect of amino acid addition on sperm quality of donkey semen preserved at 5 °C for 72 h, three ejaculates were collected from six donkeys (total of 18)

Table 1
Mean values (±SEM) of sperm morphology, acrosome integrity, membrane integrity, and sperm motility evaluated by the Sperm Class Analyzer system, in four ejaculates collected from eight Andalusian donkeys ($n = 32$) and 16 pooled semen samples of donkeys (two ejaculates per pool) after 72 h of cooled preservation in Gent® A and INRA 96®.semen extender.

Sperm characteristics	Fresh semen	Extenders	
		Gent® A	INRA 96®
MT (%)[a]	95.9 ± 0.4[a]	66.8 ± 5.5[b]	49.4 ± 3.9[c]
MP (%)[b]	73.2 ± 3.6[a]	51.3 ± 5.5[b]	36.5 ± 3.8[c]
VCL (μm/s)	143.3 ± 7.8[a]	128.1 ± 10.0[b]	107.2 ± 4.7[c]
VSL (μm/s)	89.8 ± 4.6[a]	95.5 ± 9.8[a]	75.3 ± 5.9[b]
VAP (μm/s)	121.0 ± 6.5[a]	111.9 ± 10.5[a]	84.4 ± 5.9[b]
LIN (%)	60.2 ± 0.8[b]	66.9 ± 3.2[a]	62.4 ± 0.3[ab]
STR (%)	70.6 ± 0.6[b]	78.9 ± 2.2[a]	80.7 ± 1.8[a]
WOB (%)	82.3 ± 0.6[a]	81.5 ± 2.4[a]	73.8 ± 2.6[b]
ALH (μm)	3.5 ± 0.2[a]	2.9 ± 0.2[b]	3.1 ± 0.2[ab]
BCF (Hz)	7.4 ± 0.2[c]	8.1 ± 0.4[b]	9.0 ± 0.5[a]
ASM (%)	10.7 ± 1.6[b]	17.4 ± 1.4[a]	20.1 ± 1.4[a]
AIS (%)	89.7 ± 2.3[a]	83.4 ± 2.9[b]	77.9 ± 4.8[b]
MIS (%)	79.5 ± 2.6[a]	65.3 ± 4.4[b]	60.8 ± 4.9[b]

MT, total motility; MP, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; ASM, abnormal sperm morphology; AIS, acrosome-intact spermatozoa; MIS, membrane-intact spermatozoa. Different superscript letters (a–c) in the same row indicate differences ($P < 0.05$).

[a] Total motility is defined as the percentage of spermatozoa with a mean velocity > 10 μm/s.

[b] Progressive motility is defined as the percentage of spermatozoa with a mean velocity > 90 μm/s and straightness > 75%.

from October 2011 to November 2012. The methodology was similar to that used in Experiment 1. The Gent A® extender was chosen for this experiment based on the results obtained in Experiment 1. Nine pooled semen samples (two ejaculates per pool; three replicates) were divided into ten aliquots and cooled in Gent A® supplemented with 0 (as control), 20, 40, or 60 mM for each glutamine, proline, or taurine. Osmolality and pH of the extenders were determined (Control: 312 mOSm/kg, 6.73; 20 mM glutamine: 333 mOSm/kg, 6.71; 40 mM glutamine 345 mOSm/kg, 6.69; 60 mM glutamine: 365 mOSm/kg, 6.67; 20 mM proline: 322 mOSm/kg, 6.82; 40 mM proline: 340 mOSm/kg, 6.80; 60 mM proline: 360 mOSm/kg, 6.78; 20 mM taurine: 323 mOSm/kg, 6.71; 40 mM taurine: 348 mOSm/kg, 6.70; 60 mM taurine: 370 mOSm/kg, 6.69). Semen quality was evaluated before cooling and 72 h after initiating the cooling process.

2.4.3. Experiment 3: effect of season on semen characteristics

To evaluate the seasonal influence on semen quality of donkeys, the same ejaculates as those used in Experiments 1 and 2 ($n = 50$) were analyzed during each season (winter, spring, summer, and autumn). The ejaculates were collected from ten donkeys (three or four ejaculates per donkey) from June 2011 to November 2012, with an even distribution of collection days across the seasons for each donkey. Calendar-based seasons were considered: winter (December 22–March 20), spring (March 21–June 20), summer (June 21–September 22), and autumn (September 23–December 21).

2.5. Statistical analysis

Results are expressed as mean ± SEM. All data were analyzed using the SAS statistical package (v9.0; SAS Institute Inc., Cary, NC, USA). Normality of data was assessed with the Kolmogorov–Smirnov test. Since data reported in this study were not normally distributed, percentages were subjected to arc sine transformation and absolute measures to logarithmic transformation.

In Experiments 1 and 2, semen characteristics of cooled semen were analyzed with a univariate approach using a repeated measure GLM. Extender treatment was considered to be fixed factor, whereas different collections were considered to be random. Differences between mean values were analyzed by the Duncan method.

In Experiment 3, the effect of season (winter, spring, summer, and autumn) on the quality of fresh donkey semen was tested using a multivariate general linear model (GLM), in which donkey (ten donkeys) and season (four seasons) were considered as fixed factors and different collections (three or four ejaculates per donkey) were considered as repeated measures. A Bonferroni correction was used for multiple post hoc comparisons. For all statistical procedures the level of significance was set at $P < 0.05$.

3. Results

3.1. Experiment 1: effect of extender on sperm quality

Ejaculates used in this study had the following characteristics: gel-free volume of 59.6 ± 4.9 mL (range: 41.4–107.5 mL), sperm concentration of 309.6 ± 19.7 × 10^6 spermatozoa/mL (range: 190.5–454.5 × 10^6 spermatozoa/mL), and pH at 7.2 ± 0.1 (range: 6.9–7.7).

Data for comparisons of semen quality analysis are provided in Table 1. Stored samples had a significant ($P < 0.01$) reduction in CASA-derived variables regardless of the extender used, with the exception of LIN, STR, and BCF. Sperm motility measurements differed between extenders (Table 1). Use of Gent® A resulted in greater ($P < 0.05$) values of MT, MP, VCL, VSL, VAP, and WOB compared to INRA 96®, whereas use of INRA 96® was found to provide for greater mean BCF values than use of Gent® A. Cold storage had an effect ($P < 0.001$) on mean ASM, AIS, and MIS values, but there were no differences ($P > 0.05$) between extenders used for these sperm quality variables (Table 1). An effect ($P < 0.001$) of different collections on the quality of cooled semen was recorded for all variables except LIN, STR, WOB, ALH, and ASM.

3.2. Experiment 2: effect of amino acid supplementation to Gent A® extender

Ejaculates used in this study had the following characteristics: a gel-free volume of 64.3 ± 8.3 mL (range: 30–104.5 mL), sperm concentration of 443 ± 30.3 × 10^6 spermatozoa/mL (range:

334.1–630.6 × 10^6 spermatozoa/mL), and pH of 7.3 ± 0.1 (range: 7.1–7.7).

Data in Table 2 show the effect of the supplementation of the Gent® A extender at different concentrations (0, 20, 40, or 60 mM) of glutamine, proline, and taurine on cooled-preserved spermatozoa. The results of this experiment showed that treatment with Gent® A without amino acid supplementation provided the least desirable results ($P < 0.001$) for the majority of CASA-derived motility variables. There were no significant ($P > 0.05$) differences for MT when 20 mM glutamine, 20 mM taurine, and Gent® A were used without amino acid supplementation (63.8 ± 15.9%, 67.1 ± 2.8%, and 69.8 ± 4.6% respectively). In addition, no significant ($P > 0.05$) differences were found in mean ALH values between treatments with 20 mM glutamine, 20 mM proline, 20 mM taurine, 40 mM taurine, and Gent® A with no supplementation (Table 2). No significant ($P = 0.073$) differences were found in ASM between treatments with Gent® A with no supplementation and Gent® A supplemented at different concentrations with amino acids. The additives glutamine, proline, and taurine did not improve the mean AIS and MIS values, compared to use of Gent® A without amino acid supplementation (Table 2).

When comparing the three amino acids at the different concentrations, the results were homogeneous for all evaluated variables (Table 2). The least ($P < 0.001$) mean values of MT were obtained at 20 mM for glutamine and taurine. The greatest MP ($P < 0.001$) was obtained at concentrations above 60 mM for all amino acids. There were no significant ($P > 0.05$) differences for VSL, VAP, LIN, STR, WOB, and BCF with treatment at the different amino acid concentrations. Improvement ($P < 0.001$) was observed at 60 mM for AIS when using glutamine and taurine; however, there was no improvement ($P > 0.05$) when glutamine, proline, or taurine were used at 20 or 40 mM (Table 2). The greatest ($P < 0.001$) sperm membrane integrity was obtained at 60 mM for glutamine and taurine. An effect ($P < 0.01$) of different collections on the quality of the cooled semen was recorded for all variables.

3.3. Experiment 3: effect of season on semen characteristics

Data in Table 3 show the mean characteristics of fresh ejaculates in the different seasons. Gel-free volume and sperm concentration are different ($P > 0.05$) between seasons. Seminal pH was less ($P < 0.001$) in autumn (7.1 ± 0.0) compared to winter (7.4 ± 0.1) and spring (7.5 ± 0.0).

The majority of the CASA-derived variables for jackass semen were different ($P < 0.001$) in the different seasons with VCL (184.3 ± 7.6 μm/s), VSL (113.1 ± 5.1 μm/s), VAP (155.6 ± 5.2 μm/s), ALH (4.2 ± 0.3 μm) and BCF (8.3 ± 0.4 Hz) all being greater in winter. There, however were not differences in MT ($P > 0.05$) during the different seasons of the year, whereas MP was less ($P < 0.001$) in the summer (76.7 ± 5.6%) and autumn (71.3 ± 2.9%). No differences ($P > 0.05$) in mean values of LIN, STR and WOB were observed among seasons.

Sperm abnormalities were less ($P < 0.001$) in the summer (11.4 ± 1.5%) and autumn (10.1 ± 1.6%) compared with

Table 2
Mean values (±SEM) of sperm morphology, acrosome integrity, membrane integrity, and sperm motility evaluated by the Sperm Class Analyzer system, in three ejaculates collected from six Andalusian donkeys ($n = 18$) and nine pooled semen samples of donkeys (two ejaculates per pool) after 72 h of cooled preservation in Gent® A supplemented with different concentrations (0, 20, 40, or 60 mM) of glutamine (Glu), proline (Pro) and taurine (Tau).

Parameters	Fresh semen	Cooled samples									
		Control	Glu 20	Glu 40	Glu 60	Pro 20	Pro 40	Pro 60	Tau 20	Tau 40	Tau 60
MT (%)[a]	91.9 ± 1.4^{a}	63.8 ± 15.9^{d}	67.1 ± 2.8^{cd}	73.7 ± 3.4^{bc}	79.8 ± 2.7^{b}	74.6 ± 2.8^{bc}	75.2 ± 2.4^{bc}	81.0 ± 1.8^{b}	69.8 ± 4.6^{cd}	74.4 ± 3.8^{bc}	82.4 ± 2.4^{b}
MP (%)[b]	86.2 ± 1.1^{a}	39.8 ± 12.1^{g}	59.2 ± 3.1^{f}	66.2 ± 12.0^{def}	74.4 ± 2.6^{bcd}	67.2 ± 2.1^{cdef}	69.2 ± 2.6^{bcde}	75.3 ± 1.8^{b}	62.3 ± 4.3^{ef}	68.1 ± 4.0^{bcde}	75.7 ± 2.9^{b}
VCL (μm/s)	192.9 ± 5.3^{a}	104.2 ± 9.7^{d}	153.1 ± 8.3^{c}	154.2 ± 6.4^{bc}	166.9 ± 5.0^{bc}	155.7 ± 6.0^{bc}	162.8 ± 7.8^{bc}	172.7 ± 5.4^{b}	152.7 ± 10.2^{c}	160.1 ± 6.9^{bc}	164.2 ± 7.2^{bc}
VSL (μm/s)	121.3 ± 6.3^{a}	71.4 ± 10.2^{b}	120.5 ± 7.8^{a}	121.0 ± 5.5^{a}	130.9 ± 4.5^{a}	117.3 ± 6.2^{a}	124.1 ± 6.4^{a}	130.4 ± 5.2^{a}	116.8 ± 9.2^{a}	126.7 ± 5.8^{a}	125.2 ± 5.4^{a}
VAP (μm/s)	164.4 ± 6.5^{a}	84.8 ± 12.4^{c}	139.3 ± 7.7^{b}	137.9 ± 6.0^{b}	150.0 ± 4.6^{ab}	140.2 ± 5.8^{b}	146.4 ± 7.2^{b}	153.9 ± 5.4^{ab}	136.8 ± 9.9^{b}	145.7 ± 6.3^{b}	145.8 ± 6.6^{b}
LIN (%)	60.9 ± 1.9^{b}	63.9 ± 5.0^{b}	74.3 ± 1.9^{a}	74.6 ± 1.7^{a}	76.0 ± 2.2^{a}	71.9 ± 1.9^{a}	73.7 ± 1.7^{a}	73.3 ± 2.1^{a}	73.0 ± 2.5^{a}	76.1 ± 2.0^{a}	73.4 ± 2.0^{a}
STR (%)	70.9 ± 1.2^{a}	79.4 ± 2.8^{b}	82.8 ± 1.7^{a}	84.4 ± 1.5^{a}	84.8 ± 2.0^{a}	80.7 ± 1.7^{a}	82.5 ± 1.6^{a}	82.4 ± 1.7^{a}	82.4 ± 2.0^{a}	84.3 ± 1.7^{a}	83.1 ± 1.9^{a}
WOB (%)	83.4 ± 1.6^{b}	78.5 ± 5.0^{c}	87.4 ± 0.7^{a}	86.5 ± 0.7^{ab}	87.8 ± 0.8^{a}	86.9 ± 0.8^{a}	87.6 ± 0.6^{a}	87.1 ± 1.0^{a}	86.4 ± 1.2^{ab}	88.4 ± 0.9^{a}	86.3 ± 0.9^{ab}
ALH (μm)	4.2 ± 0.2^{a}	2.9 ± 0.4^{d}	3.1 ± 0.2^{cd}	3.5 ± 0.1^{bc}	3.5 ± 0.1^{bc}	3.3 ± 0.1^{bcd}	3.5 ± 0.1^{bc}	3.7 ± 0.1^{b}	3.3 ± 0.2^{bcd}	3.3 ± 0.2^{bcd}	3.6 ± 0.1^{b}
BCF (Hz)	7.8 ± 0.4^{c}	7.8 ± 0.5^{c}	9.1 ± 0.3^{ab}	9.0 ± 0.2^{ab}	9.6 ± 0.2^{a}	8.7 ± 0.3^{b}	9.3 ± 0.2^{ab}	9.3 ± 0.3^{ab}	9.3 ± 0.3^{ab}	9.2 ± 0.2^{ab}	9.5 ± 0.3^{ab}
ASM (%)	19.0 ± 2.5	19.9 ± 2.7	22.5 ± 3.5	20.1 ± 2.5	15.8 ± 2.5	23.6 ± 3.1	21.6 ± 2.9	19.0 ± 2.8	23.8 ± 3.7	20.6 ± 2.8	16.9 ± 2.8
AIS (%)	92.7 ± 1.7^{a}	89.7 ± 3.0^{ab}	80.0 ± 2.6^{ef}	80.2 ± 3.7^{ef}	86.8 ± 2.5^{abcd}	76.8 ± 4.3^{f}	82.9 ± 3.4^{cdef}	84.8 ± 2.9^{bcde}	79.6 ± 4.5^{ef}	82.3 ± 3.1^{def}	89.2 ± 2.2^{ab}
MIS (%)	78.7 ± 4.3^{a}	73.2 ± 7.2^{ab}	59.3 ± 4.4^{c}	67.7 ± 4.4^{bc}	73.3 ± 3.0^{ab}	63.5 ± 2.3^{bc}	60.1 ± 2.1^{c}	66.0 ± 3.8^{bc}	62.6 ± 3.7^{bc}	66.3 ± 3.0^{bc}	69.2 ± 3.5^{abc}

MT, total motility; MP, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; ASM, abnormal sperm morphology; AIS, acrosome-intact spermatozoa; MIS, membrane-intact spermatozoa. Different superscript letters (a–g) in the same row indicate differences ($P < 0.05$).

[a] Total motility is defined as the percentage of spermatozoa with a mean velocity > 10 μm/s.

[b] Progressive motility is defined as the percentage of spermatozoa with a mean velocity > 90 μm/s and straightness > 75%.

Table 3
Mean values (±SEM) of sperm morphology, acrosome integrity, membrane integrity, and sperm motility evaluated by the Sperm Class Analyzer system, in fresh ejaculates ($n = 50$) from ten different Andalusian donkeys in the different seasons.

Sperm characteristics	Overall mean	Seasons			
		Winter	Spring	Summer	Autumn
Gel-free volume (mL)	62.6 ± 3.7	53.6 ± 12.9	59.6 ± 7.6	66.3 ± 12.5	65.9 ± 4.8
Sperm concentration ($\times 10^6$/mL)	351.9 ± 18.4	406.0 ± 30.7	360.4 ± 30.7	366.5 ± 51.6	331.1 ± 29.5
pH	7.3 ± 0.0	7.4 ± 0.1^{ab}	7.5 ± 0.0^{a}	7.2 ± 0.0^{bc}	7.1 ± 0.0^{c}
MT (%)[a]	94.3 ± 0.7	92.7 ± 0.9	96.5 ± 0.7	96.4 ± 1.5	93.3 ± 1.3
MP (%)[b]	77.5 ± 2.1	88.3 ± 1.3^{a}	84.9 ± 3.6^{a}	76.7 ± 5.6^{b}	71.3 ± 2.9^{b}
VCL (μm/s)	159.1 ± 5.3	184.3 ± 7.6^{a}	165.4 ± 10.9^{ab}	144.4 ± 13.7^{b}	153.1 ± 7.9^{b}
VSL (μm/s)	102.3 ± 3.6	113.1 ± 5.1^{a}	102.2 ± 6.3^{b}	102.4 ± 10.0^{b}	99.4 ± 5.7^{b}
VAP (μm/s)	136.4 ± 4.7	155.6 ± 5.2^{a}	139.7 ± 8.9^{ab}	128.9 ± 11.9^{b}	130.9 ± 7.3^{b}
LIN (%)	61.7 ± 1.0	60.2 ± 1.4	58.6 ± 1.4	63.5 ± 3.9	63.3 ± 1.5
STR (%)	71.8 ± 0.8	70.3 ± 0.8	69.0 ± 1.0	72.3 ± 3.2	73.4 ± 1.2
WOB (%)	83.3 ± 0.6	83.7 ± 1.9	81.8 ± 1.1	83.7 ± 2.2	83.7 ± 0.9
ALH (μm)	3.7 ± 0.1	4.2 ± 0.3^{a}	3.8 ± 0.2^{ab}	2.9 ± 0.2^{c}	3.6 ± 0.7^{b}
BCF (Hz)	7.6 ± 0.1	8.3 ± 0.4^{a}	7.7 ± 0.3^{ab}	6.9 ± 0.5^{c}	7.4 ± 0.1^{bc}
ASM (%)	13.4 ± 1.2	19.1 ± 3.6^{a}	17.8 ± 1.8^{a}	11.4 ± 1.5^{b}	10.1 ± 1.6^{b}
AIS (%)	90.6 ± 1.7	93.6 ± 2.6^{a}	88.2 ± 4.9^{a}	76.7 ± 11.0^{b}	93.1 ± 1.2^{a}
MIS (%)	79.9 ± 1.8	71.7 ± 5.0^{b}	73.3 ± 3.6^{b}	80.2 ± 6.4^{ab}	85.0 ± 1.9^{a}

MT, total motility; MP, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; ASM, abnormal sperm morphology; AIS, acrosome-intact spermatozoa; MIS, membrane-intact spermatozoa. Different superscripts (a–c) in the same row indicate differences ($P < 0.05$).

[a] Total motility is defined as the percentage of spermatozoa with a mean velocity > 10 μm/s.

[b] Progressive motility is defined as the percentage of spermatozoa with a mean velocity > 90 μm/s and straightness > 75%.

the winter (19.1 ± 3.6%) and spring (17.8 ± 1.8%). Acrosome integrity was less ($P < 0.05$) in the summer (76.7 ± 11.0%) compared to all the other seasons. Sperm membrane integrity was less ($P < 0.001$) in the winter (71.7 ± 5.0%) and spring (73.3 ± 3.6%) than in the autumn (85 ± 1.9%).

In the present study, an effect ($P < 0.05$) of the jackass on the quality of the fresh ejaculate was recorded in sperm concentration, morphology, acrosome integrity, plasma membrane integrity, and sperm kinematic variables with the exception of LIN, STR, and WOB. No significant ($P > 0.05$) differences were recorded between ejaculates of the same animal for any sperm quality variable with the exception of gel-free volume and MP.

4. Discussion

There are no previous reports on the effectiveness of the use of defined milk protein nor egg yolk-based semen extenders for maintenance of semen characteristics at 5 °C in Andalusian donkeys. In Experiment 1, two semen extenders (Gent® A and INRA 96®) already in use for stallion semen preservation, were tested in the Andalusian donkey. As the quality of cooled semen deteriorates with an increasing period of storage (England and Ponzio, 1996), the differences in semen characteristics between tested extenders appears also to increase with preservation time (Contri et al., 2010; Rota et al., 2008). Due to this fact, in the present study the quality of cooled semen samples was assessed after 72 h of cold storage.

The use of these two extenders greatly affected sperm motility characteristics of Andalusian donkey spermatozoa. After 72 h, some CASA-derived motility variables (MT, MP, VCL, VSL, VAP, and WOB) were greater for spermatozoa stored in Gent® A than in INRA 96®. In addition, semen extended with Gent® A had a 29.1% decrease in MT and 21.9% decrease in MP. When using INRA 96®, a reduction of more than 46% in MT and more than 36% in MP occurred. These results are consistent with previous studies (Cottorello et al., 2002; Mello et al., 2000) in which the use of extenders containing large amounts of egg yolk (e.g., Baken extender, modified Baken extender, and modified INRA 82) had a beneficial effect on sperm motility of cold-stored donkey semen. Similar findings have also been reported in stallions (Bedford et al., 1995; Jasko et al., 1991). In the present study, treatment with Gent® A resulted in greater sperm velocity (VCL, VSL, and VAP) than INRA 96® which is particularly interesting because the sperm velocities ascertained by CASA assessments have been previously correlated with in vivo fertility in mammals (Gomendio and Roldan, 2008). Conversely, sperm motility characteristics of semen cooled with INRA 96® was consistent with data reported in previous studies on donkey semen preservation (Contri et al., 2010; Santos et al., 1995). Further studies are needed to better understand the changes in motility patterns of donkey spermatozoa caused by semen extenders and the relationship with fertility.

No differences as a result of type of extender use were found, however, for sperm morphology, sperm membrane integrity, and acrosome integrity. These results may be explained by the special formulation of INRA 96® which is thought to create a physical protection for sperm membranes during preservation (Pillet et al., 2008). Moreover, the anti-oxidative properties of native phosphocaseinates have also been reported (Batellier et al., 2001).

Some amino acids (e.g., N-acetyl-L-cysteine, taurine, and hypotaurine) can reduce the impact of low temperatures during the sperm storage process, and thus improve the quality characteristics of cold-stored semen in stallions (Ijaz and Ducharme, 1995), boars (Funahashi and Sano, 2005), rams (Bucak et al., 2007), cats (Baran et al., 2009), and dogs (Michael et al., 2009). However, in donkeys there are no relevant data concerning the supplementation of

cooled semen extenders with amino acids to which findings in the present study could be compared. The results of the present study demonstrated, for the first time, that the addition of glutamine, proline, or taurine to the Gent A® extender significantly enhanced the motility variables of donkey spermatozoa after 72 h of cold storage when concentrations of 20–60 mM were used without affecting sperm morphology, sperm membrane integrity, and acrosome integrity. These results suggest that the cooling process was harmful to sperm motility and that the amino acid supplementation protected donkey spermatozoa against the temperature changes during the cooling process. However, the exact mechanisms are not completely understood as to how donkey spermatozoa stored in extenders supplemented at different concentrations with amino acids for 72 h are capable of maintaining motility in cold storage.

The beneficial effects associated with the addition of taurine may be explained by its ability to inhibit the loss of forward motility and to prevent lipid peroxidation of sperm membranes during cooling (Alvarez and Storey, 1983b). Membrane lipid peroxidation causes irreversible arrest of sperm motility (Bucak et al., 2010); however, whether this occurred under conditions used in the present study remains to be assessed. In addition, Meizel et al. (1980) have reported that taurine could be of further advantage to spermatozoa integrity due to its osmoregulatory and capacitating properties. No references have been found concerning the effects of both glutamine and proline on motility variables of cooled spermatozoa. In equine species, Trimeche et al. (1996) reported that glutamine has an active role in recovery of sperm cell metabolism by acting at the extracellular level. Previous studies indicate that proline helps to stabilize the structure of the sperm plasma membrane by interacting with the membrane phospholipids (Storey and Storey, 1990). Investigating the amino acid mechanism of cryo-protection in other cells, Marsh et al. (1990) concluded that the mechanism of action of amino acids may be related to preservation of mitochondrial function. In this regard, the functional status of the mitochondria is related to the energetic status and motility of spermatozoa (Martinez-Pastor et al., 2004).

In the current research, the effectiveness of glutamine, proline, or taurine on donkey semen tended to increase, though not significantly so, when added at a concentration of 60 mM. Previous studies have reported the toxicity of greater amino acid concentrations is related to an increase in osmotic pressure (Khlifaoui et al., 2005; Trimeche et al., 1996). The use of these three amino acids at 60 mM in the Gent® A medium increased the osmolality of the extender to 365, 360, and 370 mOSm/kg, respectively. However, at this concentration, the protective effect of the amino acids glutamine, proline, and taurine was not negated by the sensitizing effect of the hyper-tonicity.

The present study failed to elucidate the effect of the amino acids glutamine, proline, and taurine on acrosome integrity, sperm membrane integrity, and morphology of cooled donkey spermatozoa. Therefore, in comparison to Gent® A without amino acid supplementation, the addition of glutamine, proline, and taurine did not result in an improvement in sperm membrane and acrosome integrity at concentrations of 20–60 mM. In rams, Bucak et al. (2007) concluded that the addition of taurine at concentrations of 50 and 100 mM was ineffective in improving sperm membrane integrity and morphology during storage at 5 °C. In a study with cooled dog semen (Michael et al., 2009), taurine supplementation only had a positive influence on sperm motility characteristics. Aside from species differences, these contrasting findings can be explained by differences in the extenders and cooling procedures used. Several amino acids (e.g., glutamine, proline, and taurine) have been detected in spermatozoa, seminal plasma, and epididymal fluid of several mammals (Buff et al., 2001; Khlifaoui et al., 2005; Meizel et al., 1980; Sanchez Partida et al., 1992). Therefore, if these three amino acids exist in donkey spermatozoa the endogenous concentration might be enough for the protection of spermatozoa and the thus may explain the limited effects of exogenous amino acid supplementation. Another hypothesis is that perhaps these amino acids do not have the same protective effects as amino acids do on sperm integrity in other species or the protective action in donkey spermatozoa is not evident at the concentrations used in the present study. Further experiments must be conducted to elucidate the exact mechanism of sperm protection by these amino acids in cooled donkey semen.

With regard to seasonality, the present study clearly showed for the first time a seasonal influence on the characteristics of fresh semen of the Andalusian donkey. In general, the evaluated sperm characteristics were comparable with those previously recorded in Andalusian jackasses (Dorado et al., 2013) and confirmed the results obtained in other studies in which a large amount of good-quality semen was collected from donkeys (Carluccio et al., 2013; Cortes-Gutierrez et al., 2008; Miro et al., 2009; Quartuccio et al., 2011; Rota et al., 2012). However, the average gel-free semen volume (62.6 mL) was less than that with earlier studies (Contri et al., 2010; Quartuccio et al., 2011), whereas the mean annual sperm concentration (351.9×10^6 spermatozoa/mL) was greater than those reported for the Ragusano (Quartuccio et al., 2011) and Amiata donkeys (Rota et al., 2012). In this regard, individual differences in the amount of gel fraction produced by the donkeys may play an important role (Quartuccio et al., 2011). No seasonal variation was detected for gel-free semen volume as had been previously reported in donkeys (Gastal et al., 1997). Results from the present study are in contrast to earlier findings in which the greatest values were found in winter (Carluccio et al., 2013; Contri et al., 2010). The limited climatic and photoperiod variations in the present study probably contributed to this difference in results. Sperm concentration was not modified throughout the experimental period with reasonably constant mean values throughout the year which is in agreement with other published data (Contri et al., 2010; Gastal et al., 1997). However, Carluccio et al. (2013) observed seasonal differences in donkeys with greater sperm concentrations occurring during the spring and summer. Because gel-free volume is negatively related to semen concentration (Magistrini et al., 1987), overall sperm concentration was numerically greater in the winter, although there were no statistical differences. The differences observed between

studies might either be due to differences in latitude where animals resided, donkey management or frequency of semen collection (Carluccio et al., 2013; Janett et al., 2003). In addition, the number of experimental animals (sample size) as well as breed differences must be considered as possible contributors to the different results among studies (Carluccio et al., 2013; Dowsett and Knott, 1996).

The average value of seminal pH during the entire year was 7.3, which was somewhat less than that reported for the Catalonian donkey (Miro et al., 2009). However, this value is in agreement with that previously reported for the Andalusian (Dorado et al., 2013) and Martina Franca donkeys (Carluccio et al., 2013). The reason for this difference could be attributed to a greater contribution of fluid from the sexual accessory glands and/or a lesser contribution from the epididymis due to the increased frequency of ejaculations. In addition, a clear seasonal influence was also observed in seminal pH, which is in agreement with previous findings (Gastal et al., 1997).

A seasonal influence on total sperm motility was not detected in the present study similar to the findings observed in the Martina Franca jackasses in previous studies (Carluccio et al., 2013; Contri et al., 2010). However, a reduced progressive sperm motility was observed in summer and autumn, which is in agreement with data reported for Ragusano donkeys (Quartuccio et al., 2011). The mean values obtained (94.3% and 77.5%, respectively) were similar to those previously reported for Andalusian donkeys (Dorado et al., 2013) and for other breeds (Carluccio et al., 2013; Quartuccio et al., 2011; Rota et al., 2012), but greater compared to other Spanish breeds (Cortes-Gutierrez et al., 2008; Miro et al., 2009). In addition, seasonal variation was detected for several sperm motility variables including VCL, VSL, VAP, ALH, and BCF. The extent to which seasonal changes in sperm motility are influenced by breed and ambient conditions (temperature, humidity, etc.) is not understood. In this context, seasonal changes in seminal plasma constituents may also play an important role (Janett et al., 2003). Small and large plasma volumes will negatively affect sperm motility (Pickett et al., 1975).

The mean annual sperm morphology value in the present study is similar to that reported in some previous studies (Dorado et al., 2013; Miro et al., 2005; Quartuccio et al., 2011). These values are, however, less compared to those previously reported by Carluccio et al. (2013) in Martina Franca donkeys (approximately 3.7%). The differences between these results could be the consequence of individual differences and/or different interpretations of the abnormalities observed in the different studies. In addition, seasonal differences in sperm morphology were observed in the present study, and in particular a greater ASM has been found in the winter and spring. Results of the present study are not consistent with earlier findings in which sperm abnormalities did not appear to be influenced by season (Carluccio et al., 2013; Gastal et al., 1997).

In agreement with previous findings (Carluccio et al., 2013; Contri et al., 2010; Quartuccio et al., 2011), approximately 80% of spermatozoa in the ejaculates of donkeys used in Experiment 3 of the present study were cells with an intact plasma membrane. Regarding acrosome integrity, there were no studies on which information could be found where there could be comparisons of the present results with those in previous studies. In addition, seasonal differences in sperm membrane and acrosome integrity were observed in our study, and in particular a lesser AIS was observed in summer and a greater MIS was detected in the summer and autumn. A seasonal influence on sperm membrane integrity has been reported in donkeys (Contri et al., 2010).

The majority of the variables evaluated in the present study were significantly affected by the jackass, which is in agreement with previous studies (Carluccio et al., 2013; Contri et al., 2010; Dorado et al., 2013). Due to this fact, the male should be considered the main source of variability in quality of donkey semen rather than season, which could mask any effect of the season on donkey semen characteristics.

5. Conclusions

The present study, under climatic and management conditions that existed, clearly showed a seasonal influence on the majority of characteristics of Andalusian donkey semen. As all the ejaculates were of acceptable quality, these seasonal differences did not seem to influence semen quality throughout the year. Moreover, use of Gent® A resulted in maintenance of sperm motility characteristics after 72 h of cold storage to a greater extent than INRA 96® suggesting that Gent® A extender is more appropriate to preserve donkey spermatozoa. The addition of glutamine, proline, and taurine improves the outcome of preservation of Andalusian donkey spermatozoa because CASA-derived variables were more desirable in semen samples cooled in Gent® A supplemented with different concentrations of amino acids than in Gent® A with no supplementation. However, a lesser increase was noticed when these amino acids were added at a concentration of 20 mM. Although it remains to be determined if the greater motility of donkey spermatozoa in diluents containing glutamine, proline, or taurine will be reflected in enhanced fertility when used for inseminations, this would allow a more efficient use of cooled donkey semen for insemination. Further studies are needed to find more appropriate amino acids for semen extender supplementations and to demonstrate whether any positive additive effects can be derived from combining several amino acids.

Conflict of interest statement

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgments

This study was supported by Grant RZ2009-00006-00-00 (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Ministerio de Ciencia e Innovación, Spain). None of the authors of this manuscript has a financial or personal relationship with either of the two commercial enterprises from which the two commercial extenders

were procured that could inappropriately influence or bias the content of the paper. The authors are extremely grateful to Fundación Casa del Burro (Rute, Cordoba, Spain) for providing the animals. Part of this study has been previously presented at the 11th International Congress of the Spanish Association of Animal Reproduction (AERA), June 13–16, 2012, Cordoba, Spain.

References

Alvarez, J.G., Storey, B.T., 1983a. Role of superoxide-dismutase in protecting rabbit spermatozoa from O_2 toxicity due to lipid-peroxidation. Biol. Reprod. 28, 1129–1136.

Alvarez, J.G., Storey, B.T., 1983b. Taurine, hypotaurine, epinephrine and albumin inhibit lipid-peroxidation in rabbit spermatozoa and protect against loss of motility. Biol. Reprod. 29, 548–555.

Anchordoguy, T., Carpenter, J.F., Loomis, S.H., Crowe, J.H., 1988. Mechanisms of interaction of amino-acids with phospholipid-bilayers during freezing. Biochim. Biophys. Acta 946, 299–306.

Aurich, C., 2008. Recent advances in cooled-semen technology. Anim. Reprod. Sci. 107, 268–275.

Baran, A., Demir, K., Sahin, B.E., Evecen, M., Bacinoglu, S., Alkan, S., Ileri, I.K., 2009. Short-term chilled storage of cat semen extended with and without taurine containing milk extenders. J. Anim. Vet. Adv. 8, 1367–1371.

Batellier, F., Duchamp, G., Vidament, M., Arnaud, G., Palmer, E., Magistrini, M., 1998. Delayed insemination is successful with a new extender for storing fresh equine semen at 15 °C under aerobic conditions. Theriogenology 50, 229–236.

Batellier, F., Vidament, M., Fauquant, J., Duchamp, G., Arnaud, G., Yvon, J.M., Magistrini, M., 2001. Advances in cooled semen technology. Anim. Reprod. Sci. 68, 181–190.

Bedford, S.J., Graham, J.K., Amann, R.P., Squires, E.L., Pickett, B.W., 1995. Use of two freezing extenders to cool stallion spermatozoa to 5 °C with and without seminal plasma. Theriogenology 43, 939–953.

Brito, L.F.C., 2007. Evaluation of stallion sperm morphology. Clin. Tech. Equine Pract. 6, 249–264.

Bucak, M.N., Atessahin, A., Varisli, O., Yüce, A., Tekin, N., Akcay, A., 2007. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen Microscopic and oxidative stress parameters after freeze-thawing process. Theriogenology 67, 1060–1067.

Bucak, M.N., Sarioezkan, S., Tuncer, P.B., Sakin, F., Atessahin, A., Kulaksiz, R., Cevik, M., 2010. The effect of antioxidants on post-thawed Angora goat (*Capra hircus ancryrensis*) sperm parameters, lipid peroxidation and antioxidant activities. Small Rumin. Res. 89, 24–30.

Buff, S., Donze, A., Guerin, P., Guillaud, J., Fontbonne, A., Menezo, Y., 2001. Taurine and hypotaurine in spermatozoa and epididymal fluid of cats. J. Reprod. Fertil. Suppl. 57, 93–95.

Cabrita, E., Ma, S., Diogo, P., Martinez-Paramo, S., Sarasquete, C., Dinis, M.T., 2011. The influence of certain aminoacids and vitamins on post-thaw fish sperm motility, viability and DNA fragmentation. Anim. Reprod. Sci. 125, 189–195.

Carluccio, A., Panzani, S., Contri, A., Bronzo, V., Robbe, D., Veronesi, M.C., 2013. Influence of season on testicular morphometry and semen characteristics in Martina Franca jackasses. Theriogenology 79, 502–507.

Contri, A., De Amicis, I., Veronesi, M.C., Faustini, M., Robbe, D., Carluccio, A., 2010. Efficiency of different extenders on cooled semen collected during long and short day length seasons in Martina Franca donkey. Anim. Reprod. Sci. 120, 136–141.

Cortes-Gutierrez, E.I., Crespo, F., Gosalvez, A., Davila-Rodriguez, M.I., Lopez-Fernandez, C., Gosalvez, J., 2008. DNA fragmentation in frozen sperm of *Equus asinus*: Zamorano-Leones, a breed at risk of extinction. Theriogenology 69, 1022–1032.

Cottorello, A.C.P., Amancio, R.C., Henry, M., Borges, I., 2002. Effect of storage temperature and extenders on in vitro activity of donkey spermatozoa. Theriogenology 58, 325–328.

Cheng, F.P., Fazeli, A., Voorhout, W.F., Marks, A., Bevers, M.M., Colenbrander, B., 1996. Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. J. Androl. 17, 674–682.

Dorado, J., Acha, D., Gálvez, M.J., Ortiz, I., Carrasco, J.J., Díaz, B., Gómez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2013. Sperm motility patterns in Andalusian donkey (*Equus asinus*) semen: effects of body weight, age, and semen quality. Theriogenology 79, 1100–1109.

Dorado, J., Rodríguez, I., Hidalgo, M., 2007. Cryopreservation of goat spermatozoa: comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. Theriogenology 68, 168–177.

Dowsett, K.F., Knott, L.M., 1996. The influence of age and breed on stallion semen. Theriogenology 46, 397–412.

England, G.C.W., Ponzio, P., 1996. Comparison of the quality of frozen–thawed and cooled-rewarmed dog semen. Theriogenology 46, 165–171.

Funahashi, H., Sano, T., 2005. Select antioxidants improve the function of extended boar semen stored at 10 °C. Theriogenology 63, 1605–1616.

Gastal, M.O., Henry, M., Beker, A.R., Gastal, E.L., 1997. Effect of ejaculation frequency and season on donkey jack semen. Theriogenology 47, 627–638.

Gastal, M.O., Henry, M., Beker, A.R., Gastal, E.L., Gonçalves, A., 1996. Sexual behavior of donkey jacks: influence of ejaculatory frequency and season. Theriogenology 46, 593–603.

Gomendio, M., Roldan, E.R.S., 2008. Implications of diversity in sperm size and function for sperm competition and fertility. Int. J. Dev. Biol. 52, 439–447.

Gomez-Cuetara, C., Alvarez, A.L., Rodriguez, A., Santiago, I., Mateos, E., Serres, C., 2004. Refrigeration of Zamorano-Leones donkey semen collected with an open-ended artificial vagina. Reprod. Domest. Anim. 39, 267.

Heinz, K.A., Glofcheski, D.J., Lepock, J.R., Kruuv, J., 1990. Mechanism of freeze–thaw damage to liver alcohol-dehydrogenase and protection by cryoprotectants and amino acids. Cryobiology 27, 521–538.

Henry, M., Lodi, L.D., Gastal, M.M.F.O., 1998. Sexual behaviour of domesticated donkeys (*Equus asinus*) breeding under controlled or free range management systems. Appl. Anim. Behav. Sci. 60, 263–276.

Ijaz, A., Ducharme, R., 1995. Effect of various extenders and taurine on survival of stallion sperm cooled to 5 °C. Theriogenology 44, 1039–1050.

Janett, F., Sacher, K., Hassig, M., Thun, R., 2012. Quality of raw and of cold-stored semen in Icelandic stallions. J. Equine Vet. Sci. 32, 590–595.

Janett, F., Thun, R., Bettschen, S., Burger, D., Hassig, M., 2003. Seasonal changes of semen quality and freezability in Franches–Montagnes stallions. Anim. Reprod. Sci. 77, 213–221.

Jasko, D.J., Moran, D.M., Farlin, M.E., Squires, E.L., 1991. Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. Theriogenology 35, 1059–1067.

Kenney, R.M., Bergman, R.V., Cooper, W.L., Morse, G.W., 1975. Minimal contamination techniques for breeding mares: techniques and preliminary findings. In: Proccedings 21st Annual Convention of the American Association of Equine Practitioners, Lexington, p. 327.

Khlifaoui, M., Battut, I., Bruyas, J.F., Chatagnon, G., Trimeche, A., Tainturier, D., 2005. Effects of glutamine on post-thaw motility of stallion spermatozoa: an approach of the mechanism of action at spermatozoa level. Theriogenology 63, 138–149.

Kruuv, J., Glofcheski, D.J., 1990. Survival of mammalian-cells following multiple freeze–thaw cycles. II. Independence of cryoprotection using glutamine with dimethyl-sulfoxide, hydroxyethyl starch, propyleneglycol or glycerol. Cryo-Letters 11, 215–226.

Lalonde, R.J., Lepock, J.R., Kruuv, J., 1991. Site of freeze–thaw damage and cryoprotection by amino acids of the calcium ATPase of sarcoplasmic reticulum. Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. 1079, 128–138.

Magistrini, M., Chanteloube, P., Palmer, E., 1987. Influence of season and frequency of ejaculation on production of stallion semen for freezing. J. Reprod. Fertil. Suppl. 35, 127–133.

Marsh, D.C., Belzer, F.O., Southard, J.H., 1990. Hypothermic preservation of hepatocytes. II. Importance of Ca^2 and amino acids. Cryobiology 27, 1–8.

Martinez-Pastor, F., Johannisson, A., Gil, J., Kaabi, M., Anel, L., Paz, P., Rodriguez-Martinez, H., 2004. Use of chromatin stability assay, mitochondrial stain JC-1, and fluorometric assessment of plasma membrane to evaluate frozen–thawed ram semen. Anim. Reprod. Sci. 84, 121–133.

Meizel, S., Lui, C.W., Working, P.K., Mrsny, R.J., 1980. Taurine and hypotaurine: their effects on motility, capacitation and the acrosome reaction of hamster sperm in vitro and their presence in sperm and reproductive tract fluids of several mammals. Dev. Growth Differ. 22, 483–494.

Mello, S.L.V., Henry, M., Souza, M.C., Oliveira, S.M.P., 2000. Effect of split ejaculation and seminal extenders on longevity of donkey semen preserved at 5 °C. Arq. Bras. Med. Vet. Zootec. 52, 372–378.

Michael, A.J., Alexopoulos, C., Pontiki, E.A., Hadjipavlou-Litina, D.J., Saratsis, P., Ververidis, H.N., Boscos, C.M., 2009. Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. Anim. Reprod. Sci. 112, 119–135.

Miro, J., Lobo, V., Quintero-Moreno, A., Medrano, A., Pena, A., Rigaua, T., 2005. Sperm motility patterns and metabolism in Catalonian donkey semen. Theriogenology 63, 1706–1716.

Miro, J., Taberner, E., Rivera, M., Pena, A., Medrano, A., Rigau, T., Penalba, A., 2009. Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen. Theriogenology 72, 1017–1022.

Phetudomsinsuk, K., Sirinarumitr, K., Choothesa, A., Suthanmapinunt, P., Kornkaewrat, K., Laikul, A., Amornsak, S., Pinyopummin, A., 2009. Freezability of Thai native crossbred horse semen in different extenders. Thai J. Vet. Med. 39, 105–114.

Pickett, B.W., Sullivan, J.J., Byers, W.W., Pace, M.M., Remmenga, E.E., 1975. Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. Fertil. Steril. 26, 167–174.

Pillet, E., Batellier, F., Duchamp, G., Furstoss, V., Le Vern, Y., Kerboeuf, D., Desherces, S., Schmitt, E., Magistrini, M., 2008. High fertility rates with stallion sperm cryopreserved in INRA96® based extender are not predicted by in vitro parameters. Anim. Reprod. Sci. 107, 339–340.

Quartuccio, M., Marino, G., Zanghì, A., Garufi, G., Cristarella, S., 2011. Testicular volume and daily sperm output in Ragusano donkeys. J. Equine Vet. Sci. 31, 143–146.

Rota, A., Magelli, C., Panzani, D., Camillo, F., 2008. Effect of extender, centrifugation and removal of seminal plasma on cooled-preserved Amiata donkey spermatozoa. Theriogenology 69, 176–185.

Rota, A., Panzani, D., Sabatini, C., Camillo, F., 2012. Donkey jack (*Equus asinus*) semen cryopreservation: studies of seminal parameters, post breeding inflammatory response, and fertility in donkey jennies. Theriogenology 78, 1846–1854.

Rudolph, A.S., Crowe, J.H., 1985. Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. Cryobiology 22, 367–377.

Sanchez Partida, L.G., Maxwell, W.M.C., Paleg, L.G., Setchell, B.P., 1992. Proline and glycine betaine in cryoprotective diluents for ram spermatozoa. Reprod. Fertil. Dev. 4, 113–118.

Santos, G.F., Henry, M., Sampaio, I.B.M., Gastal, E.L., 1995. Effect of cooling system and rate of cooling on sperm quality of donkey semen preserved at 5 °C. Biol. Reprod. Monogr. 1, 761–767.

Serres, C., Rodriguez, A., Alvarez, A.L., Santiago, I., Gabriel, J., Gomez-Cuetara, C., Mateos, E., 2002. Effect of centrifugation and temperature on the motility and plasma membrane integrity of Zamorano-Leones donkey semen. Theriogenology 58, 329–332.

Storey, K.B., Storey, J.M., 1990. Frozen and alive. Sci. Am. 263, 92–97.

Trimeche, A., Renard, P., LeLannou, D., Barriere, P., Tainturier, D., 1996. Improvement of motility of post-thaw Poitou jackass sperm using glutamine. Theriogenology 45, 1015–1027.

Trimeche, A., Yvon, J.M., Vidament, M., Palmer, E., Magistrini, M., 1999. Effects of glutamine, proline, histidine and betaine on post-thaw motility of stallion spermatozoa. Theriogenology 52, 181–191.

Vidament, M., Vincent, P., Martin, F.X., Magistrini, M., Blesbois, E., 2009. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. Anim. Reprod. Sci. 112, 22–35.

Capítulo IV / Chapter IV

"Freezability of Andalusian donkey (Equus asinus) spermatozoa: effect of extenders and permeating cryoprotectants".

D. Acha, M. Hidalgo, M.J. Gálvez, I. Ortiz, J.J. Carrasco, V. Gómez-Arrones, J. Dorado. Reproduction, Fertility and Development 2015, accepted 4 June 2015.

CSIRO PUBLISHING

Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD14449

Freezability of Andalusian donkey (*Equus asinus*) spermatozoa: effect of extenders and permeating cryoprotectants

D. Acha[A], *M. Hidalgo*[A], *I. Ortiz*[A], *M. J. Gálvez*[A], *J. J. Carrasco*[B], *V. Gómez-Arrones*[B] *and J. Dorado*[A,C]

[A]Veterinary Reproduction Group, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Cordoba, Campus de Rabanales (Edif. Hospital Clínico Veterinario), Ctra. Madrid-Cádiz, km 396, 14071 Córdoba, Spain.
[B]Equine Reproduction Center, Centro de Selección y Reproducción Animal, (CENSYRA-Extremadura Government), Camino Santa Engracia, S/N (Estación Pecuaria), 06007 Badajoz, Spain.
[C]Corresponding author. Email: jdorado@uco.es

Abstract. The aim of this study was to compare the effect of two semen extenders and four permeating cryoprotectants on post-thaw sperm quality of Andalusian donkeys. First, 32 ejaculates were pooled, split and frozen in either Gent B or INRA 96 with egg yolk and glycerol. Second, 12 pooled semen samples were simultaneously frozen in Gent B (glycerol) or Gent A containing ethylene glycol (EG; 1 or 1.5%) or dimethyl sulfoxide (DMSO; 1.5 or 2%). Finally, nine pooled samples were simultaneously cryopreserved in Gent A containing 1% EG (as control), dimethylformamide (DMFA; 1 or 2.5%) or a combination of 1% EG and 1.5% DMFA. Gent B yielded a higher ($P < 0.01$) post-thaw sperm motility than modified INRA96. EG 1% increased the sperm membrane integrity ($P < 0.001$), whereas DMSO affected sperm motility and membrane integrity ($P < 0.001$). DMFA 2.5% yielded higher ($P < 0.001$) values for sperm motility and membrane integrity. We concluded that Gent B improves *in vitro* post-thaw sperm quality of donkey spermatozoa, but the replacement of glycerol with 1% EG or 2.5% DMFA increased sperm protection against cryodamage. The use of DMSO for freezing donkey semen was unsuccessful and a toxic effect is suspected. These extenders should be included in the pre-freeze test for each donkey.

Additional keywords: freezing extender, frozen-thawed semen, sperm quality.

Received 18 November 2014, accepted 4 June 2015, published online xx xxxxx xxxx

Introduction

According to the last census of the Food and Agriculture Organisation of the United Nations (FAO) conducted in 2013, the population of the Andalusian donkey accounted for 749 animals, of which only 94 individuals are considered as breeding males (DAD-IS-FAO 2013). This breed was declared at risk of extinction by the Spanish government (Real Decreto 2129/2008, regulation of the National Catalogue of Endangered Species). In such situations, the creation of genetic banks based on cryopreserved semen samples becomes necessary for the *ex situ* management of this endangered donkey (*Equus asinus*) breed (Andrabi and Maxwell 2007; Smits *et al.* 2012).

To date, there are several studies on donkey semen cryopreservation in which the effect of the freezing–thawing procedure was assessed (Trimeche *et al.* 1998; Canisso *et al.* 2008; Cortés-Gutiérrez *et al.* 2008; Flores *et al.* 2008; Contri *et al.* 2012*b*) and direct comparisons between extenders (Serres *et al.* 2004*a*; Canisso *et al.* 2011) and cryoprotectants (CPAs; Alvarez *et al.* 2004; Serres *et al.* 2004*a*; Álvarez *et al.* 2006; Oliveira *et al.* 2006; Rota *et al.* 2012; Madison *et al.* 2013) were also performed. However, artificial insemination (AI) with cryopreserved donkey semen has given disappointing results (Vidament *et al.* 2005, 2009; Rota *et al.* 2012; Serres *et al.* 2014). For this reason, a suitable method for the cryopreservation of semen from Andalusian donkeys is needed in order to use successfully these frozen donkey semen doses in AI programs. However, to our knowledge, there are no previous studies evaluating semen cryopreservation in this donkey breed.

Cryopreservation induces structural and functional damage to the spermatozoa that reduces their fertilising ability (Watson 1995). The addition of CPAs to semen freezing media prevents sperm cryodamage (Oldenhof *et al.* 2010). Recent research has demonstrated, however, that another source of damage to stallion spermatozoa is the CPA used (Glazar *et al.* 2009).

Over the past 50 years, glycerol (GLY) has been the CPA most commonly used for stallion (*Equus caballus*) semen cryopreservation (Hoffmann *et al.* 2011), which has also been successfully used for freezing donkey semen (Trimeche *et al.* 1998; Serres *et al.* 2004*a*; Oliveira *et al.* 2006; Vidament *et al.* 2009; Rota *et al.* 2012; Ortiz *et al.* 2015). However, GLY permeates the sperm membrane more slowly than water and other CPAs (Glazar *et al.* 2009) because of its low permeability (Ball and Vo 2001), thus inducing osmotic stress on stallion spermatozoa during cryopreservation (Peña *et al.* 2011). More recently, Macías García *et al.* (2012) demonstrated that the toxic effect of GLY on stallion spermatozoa is produced at concentrations above 3.5%. Moreover, a greater sensitivity of donkey spermatozoa to GLY has also been suggested (Alvarenga *et al.* 2005; Vidament *et al.* 2009). However, the higher pregnancy rates observed in mares compared with jennies after frozen–thawed donkey semen AI support the hypothesis that GLY could also exert a negative effect on the jenny genital tract (Vidament *et al.* 2009), which has not been clarified yet. In any case, pregnancy rates in jennies with frozen–thawed donkey semen were similar using both GLY or other CPAs (Oliveira *et al.* 2006; Rota *et al.* 2012).

The use of low-molecular-weight CPAs such as ethylene glycol (EG, 62.07 g mol^{-1}), dimethyl sulfoxide (DMSO, 73.09 g mol^{-1}) or dimethylformamide (DMFA, 78.13 g mol^{-1}), instead of GLY (92.09 g mol^{-1}), reduces the osmotic damage on stallion spermatozoa (Glazar *et al.* 2009) and could subsequently improve the *in vitro* characteristics of Andalusian donkey spermatozoa after thawing. In the last decade, several authors have demonstrated the higher efficacy of EG (Alvarenga *et al.* 2000*b*; Ball and Vo 2001; Henry *et al.* 2002; Mantovani *et al.* 2002; Squires *et al.* 2004), DMFA (Alvarenga *et al.* 2000*a*, 2005; Gomes *et al.* 2002; Medeiros *et al.* 2002) and DMSO (Chenier *et al.* 1998) to preserve stallion spermatozoa during the freezing–thawing process in comparison to GLY. In donkeys, direct comparisons between CPAs have also been performed (Alvarez *et al.* 2004; Álvarez *et al.* 2005; Oliveira *et al.* 2006; Vidament *et al.* 2009; Rota *et al.* 2012); however, no direct comparisons between GLY and these three alternative CPAs (EG, DMSO and DMFA) for donkey semen cryopreservation were found in the literature.

Few studies have evaluated the effect of different extenders on post-thaw donkey semen quality (Serres 2003; Serres *et al.* 2004*a*; Canisso *et al.* 2011). The extenders Gent B (Minitüb GmbH, Tiefenbach, Germany) and INRA 96 (IMV Technologies, L'Aigle, France) supplemented with 2% clarified egg yolk and 2.5% glycerol (INRA96-EYG) have been successfully used in stallions (Morillo Rodríguez *et al.* 2011) and donkeys (Flores *et al.* 2008; Rota *et al.* 2012; Ortiz *et al.* 2015). However, no direct comparisons between those two extenders have been performed to date.

The aims of the present study were therefore to: (1) compare the efficacy of two stallion semen extenders contain GLY in their composition (Gent B and INRA96-EYG) for the cryopreservation of Andalusian donkey semen and (2) assess the effect of four permeating CPAs (GLY, EG, DMSO and DMFA) on sperm quality of frozen–thawed Andalusian donkey semen.

Materials and methods

Animals

The study was performed at the Equine Center for Assisted Reproduction of the Centro de Selección y Reproducción Animal – CENSYRA (Badajoz, Spain) between June 2011 and November 2012. Ten healthy mature Andalusian donkeys of proven fertility (4–15 years of age) were used as semen donors. The animals were fed daily with hay and grain, and water was freely available.

Sperm collection and evaluation

In order to deplete the extragonadal sperm reserves, an initial semen collection was performed in sexually rested donkeys but the sample was not evaluated. As recommended by Contri *et al.* (2012*a*), 5 days later semen collections were performed on a regular basis (two collections per week) and semen samples evaluated. Collections were made using a Missouri-model artificial vagina (Minitüb GmbH) with an in-line gel filter (Minitüb GmbH). A jenny in natural or prostaglandin-induced oestrus (Luprostiol, 7.5 mg intramuscular; Prosolvin, Intervet International B.V., Boxmeer, The Netherlands) was used to induce copulatory activity.

After collection, the gel-free volume was measured in a graduated tube. Sperm concentration was calculated with a photometer (SpermaCue; Minitüb GmbH) as described by Vidament *et al.* (2009) and pH was determined using a pH meter (Hanna Instruments Inc., Woonsocket, RI, USA; Miró *et al.* 2009).

Sperm morphology was examined by light microscopy evaluation (Olympus BH-2; Olympus Optical Co. Ltd, Tokyo, Japan) on smears stained with Diff-Quick (Medion Diagnostics AG, Düdingen, Switzerland) staining (Brito 2007). At least 200 spermatozoa per slide were counted to determine the percentage of spermatozoa with normal morphology (NSM, %).

Motion characteristics of the samples were assessed using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyser; Microptic SL, Barcelona, Spain), as described by Miró *et al.* (2005) for donkey semen. Prior to the assessment of movement, semen aliquots were diluted with skimmed milk–glucose extender (Kenney *et al.* 1975) to reach a sperm concentration of $\sim 25 \times 10^6$ spermatozoa mL^{-1} and incubated at 37°C for 5 min. For each evaluation, three 5-μL drops of each semen sample were then evaluated using a phase-contrast microscope (Eclipse 50i; Nikon, Tokyo, Japan) with a warm stage at 37°C at 100× magnification. Two microscopic fields per drop were randomly filmed, including a minimum of 200 spermatozoa. Objects incorrectly identified as spermatozoa were minimised using the playback function. With respect to setting the parameters of the program, spermatozoa with a mean average path velocity (VAP) <10 μm s^{-1} were considered to be immotile. Spermatozoa with VAP >90 μm s^{-1} were considered as rapid, while spermatozoa deviating $<25\%$ from a straight line were designated as linear motile. The measured parameters of sperm motion were total motility (MOT, %), progressive motility (PMOT, %), curvilinear velocity (VCL, μm s^{-1}), straight-line velocity (VSL, μm s^{-1}); average path velocity (VAP, μm s^{-1}), linearity (LIN, as VSL/VCL, %), straightness

(STR, as VSL/VAP, %), wobble (WOB, as VAP/VCL, %), beat cross frequency (BCF, Hz) and amplitude of lateral head displacement (ALH, μm).

Sperm membrane integrity was assessed using the double stain propidium iodide (PI) with acridine orange (AO) from the Vital-Test kit (Halotech DNA SL, Madrid, Spain), as previously described by Dorado *et al.* (2014). At least 200 spermatozoa were counted, considering green spermatozoa as membrane-intact spermatozoa (MIS, %).

To evaluate the sperm acrosomes, we used the PI with peanut agglutinin–fluorescein isothiocyanate (FITC–PNA) double stain (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), as described by Dorado *et al.* (2014). Values were expressed as percentages of acrosome-intact spermatozoa (AIS, %) and acrosome-reacted spermatozoa (ARS, %).

Semen cryopreservation

Semen freezing was performed following the protocol previously described by Serres (2003) for the Zamorano–Leonés donkey semen with modifications. After semen evaluation, a 3-mL aliquot of gel-free semen was immediately diluted 1 : 1 (v : v, semen : extender) with a commercial extender without antibiotics (EquiPro A; Minitüb GmbH), in sterile 15-mL Falcon centrifuge tubes (BDFalcon Tubes; BD Biosciences, Erembodegem, Belgium) and pooled (two ejaculates per pool) in order to avoid introduction of uncontrolled male-to-male variation (Dorado *et al.* 2014). Pooled semen samples were divided into aliquots (as many as number of treatments in each experiment) then centrifuged at 400*g* for 7 min at 22°C. Once the supernatant was removed, the pellet was re-suspended in the tested extenders (see Experimental design section below) to reach the concentration of 200×10^6 spermatozoa mL^{-1}. Extended semen was maintained at room temperature (~22°C) for 10 min. Vials were slowly cooled (0.3°C min^{-1}) in an Equitainer I (Hamilton Research, Inc., Danvers, MA, USA) to 5°C for 120 min. Finally, the spermatozoa were loaded into 0.5-mL plastic straws at 5°C, frozen horizontally in ranks 2.5 cm above the surface of liquid nitrogen (LN_2) for 5 min and then placed into LN_2. After 24–48 h of storage, straws were thawed in a heater bath (Incudigit horizontal; Instrumentación Científica y Técnica SL, Lardero, Spain) at 37°C for 30 s, diluted to 25×10^6 spermatozoa mL^{-1} with the appropriate extender (see Experimental design section below) and evaluated considering sperm morphology, motility, membrane integrity and acrosome integrity as described previously.

Experimental design

Experiment 1: comparison of the effectiveness of INRA96-EYG and Gent B extenders for cryopreservation of Andalusian donkey semen

The effects of two different equine semen extenders (Gent B and INRA96-EYG) on the quality of frozen–thawed donkey semen were evaluated. Gent B has been successfully used for freezing stallion (Morillo Rodríguez *et al.* 2011) and donkey spermatozoa (Flores *et al.* 2008; Ortiz *et al.* 2015), whereas INRA96-EYG, a modified INRA 96, has also been used in horses (Morillo Rodríguez *et al.* 2011) and donkeys (Rota *et al.* 2012). To this end, 32 ejaculates from eight of the 10 donkeys (four ejaculates per donkey) were collected and pooled (two ejaculates per pool; four replicates). Each collection day, pooled semen samples were divided into two aliquots and re-suspended in either Gent B or INRA96-EYG. Extended semen was then frozen and stored in LN_2 until analysis. Semen quality was evaluated before freezing and after thawing. Osmolality and pH of the extenders were determined (INRA96-EYG, 1059 mOSm kg^{-1}, pH 7.03; Gent B, 1001 mOSm kg^{-1}, pH 6.8). All osmolalities were checked using a freezing-point digital micro-osmometer Type 6 (Löser Messtechnik, Berlin, Germany).

Experiment 2: effect of glycerol, ethylene glycol and dimethyl sulfoxide on the quality of frozen–thawed Andalusian donkey semen

Four ejaculates were collected from six of the 10 donkeys ($n = 24$) to evaluate the effect of three different permeating CPAs (glycerol (GLY), ethylene glycol (EG) and dimethyl sulfoxide (DMSO)) on the quality of frozen–thawed semen samples of Andalusian donkeys. The methodology was similar to that used in Experiment 1. Pooled semen samples (two ejaculates per pool; four replicates) were split into five aliquots and frozen in the tested extenders. Based on the results obtained in Experiment 1, the Gent B extender, which contains GLY as CPA, was chosen as control and compared with Gent A, which has the same composition as Gent B but without GLY, supplemented with 1 or 1.5% EG (Scharlan Chemie SA, Barcelona, Spain) or 1.5 or 2% DMSO (Carlo Erba Reagenti SpA, Rodano, Italy). Semen quality was evaluated before freezing and after thawing. Osmolality and pH of the extenders are shown in Table 1.

Experiment 3: effect of dimethylformamide on the quality of frozen–thawed Andalusian donkey semen

To evaluate the effect of dimethylformamide (DMFA) on the quality of frozen–thawed semen samples of Andalusian donkeys, three ejaculates were collected from six of the 10 donkeys (total

Table 1. Osmolality (mOsm kg^{-1}) and pH values in the extenders used in Experiments 2 and 3

DMSO 1.5, Gent A extender supplemented with 1.5% dimethyl sulfoxide; DMSO 2, Gent A extender supplemented with 2% dimethyl sulfoxide; EG 1, Gent A extender supplemented with 1% ethylene glycol; EG 1.5, Gent A extender supplemented with 1.5% ethylene glycol; DMFA 1, Gent A extender supplemented with 1% dimethylformamide; DMFA 2.5, Gent A extender supplemented with 2.5% dimethylformamide; DMFA 1.5 + EG 1, Gent A extender supplemented with a combination of 1.5% dimethylformamide and 1% ethylene glycol

Extender	Osmolality (mOsm kg^{-1})	pH
DMSO 1.5	549	6.81
DMSO 2	603	6.83
EG 1	470	6.70
EG 1.5	549	6.62
DMFA 1	461	6.84
DMFA 2.5	658	6.86
DMFA 1.5 + EG 1	662	6.69

of 18). The methodology was similar to that used in previous experiments. The Gent A extender supplemented with 1% EG was chosen (as control) for this experiment based on the results obtained in Experiment 2. Nine pooled semen samples (two ejaculates per pool; three replicates) were split into four aliquots and frozen in Gent A extender supplemented with 1 or 2.5% DMFA (Carlo Erba Reagenti SpA) only or in a combination of 1% EG and 1.5% DMFA. Semen quality was evaluated before freezing and after thawing. Table 1 shows the osmolality and pH of the extenders.

Statistical analysis

Results are expressed as mean ± s.e.m. Data were analysed by univariate, repeated-measures analysis of variance using the general linear model (GLM) procedure of the SAS (Version 9.0; SAS Institute Inc., Cary, NC, USA). Extender treatment was considered as fixed factor, whereas different collections were considered to be random. Dependent variables expressed as percentages were arcsine-transformed before the analysis. Differences between mean values were analysed by the Duncan method. The level of significance was set at $P < 0.05$.

Results

Experiment 1: comparison of the effectiveness of INRA96-EYG and Gent B extenders for cryopreservation of Andalusian donkey semen

Data for comparisons of semen quality analysis are provided in Table 2. Cryopreservation significantly ($P < 0.01$) affected all the sperm parameters assessed regardless of the extender used, with the exception of BCF, NSM and AIS. Most CASA-derived parameters (MOT, PMOT, VCL and ALH) were significantly ($P < 0.01$) higher in fresh semen than in frozen–thawed samples, whereas the opposite was found for LIN, STR and WOB ($P < 0.001$). Sperm motility measurements differed between extenders (Table 2). The use of Gent B resulted in greater ($P < 0.01$) values of MOT, PMOT, VCL and VSL compared with INRA96-EYG. Cryopreservation had an adverse effect ($P < 0.001$) on mean MIS values, but there were no differences ($P > 0.05$) between extenders used for this sperm quality variable (Table 2).

Experiment 2: effect of glycerol, ethylene glycol and dimethyl sulfoxide on the quality of frozen–thawed Andalusian donkey semen

Data obtained for comparisons of semen quality analysis using GLY, DMSO or EG as CPAs are provided in Table 3. As found in Experiment 1, most semen quality parameters evaluated were significantly ($P < 0.001$) higher in fresh semen than in frozen–thawed samples, with the exception of LIN, STR and WOB. No significant ($P > 0.05$) differences were found in mean BCF values between fresh semen and frozen–thawed samples regardless of the extender used. Sperm motility parameters differed between CPAs, except for LIN, STR, WOB and BCF (Table 3). The results of this experiment showed that treatment with Gent A supplemented with DMSO at a concentration of 1.5 or 2% provided the least desirable results ($P < 0.001$) for the majority of CASA-derived motility variables. There were no significant ($P > 0.05$) differences for MOT, PMOT, VSL, VAP and ALH between EG 1% and GLY. EG at 1% yielded a significant ($P < 0.05$) increase in mean NSM, AIS and MIS values compared with DMSO and GLY.

Table 2. Mean values (± s.e.m.) of Andalusian donkey sperm quality in fresh semen and after freezing–thawing using two different semen extenders for freezing equine spermatozoa (Gent B or INRA96-EYG)

MOT, total motility; PMOT, progressive motility; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; NSM, normal sperm morphology; AIS, acrosome-intact spermatozoa; MIS, membrane-intact spermatozoa; Gent B, Gent B (Minitüb, Tiefenbach, Germany); INRA96-EYG, INRA 96 (IMV Technologies, L'Aigle, France) containing 2% clarified egg yolk and 2.5% glycerol. [a–c]Different superscript letters within a row indicate significant differences ($P < 0.05$)

Parameter	Fresh semen	Frozen–thawed samples	
		Gent B	INRA96-EYG
MOT (%)	96.33 ± 0.43^{a}	60.54 ± 5.61^{b}	49.26 ± 5.42^{c}
PMOT (%)	75.31 ± 2.68^{a}	48.76 ± 4.91^{b}	38.08 ± 4.25^{c}
VCL (μm s^{-1})	149.95 ± 0.51^{a}	120.57 ± 6.82^{b}	102.08 ± 6.92^{c}
VSL (μm s^{-1})	93.30 ± 4.48^{ab}	99.40 ± 5.13^{a}	85.20 ± 5.45^{b}
VAP (μm s^{-1})	126.35 ± 6.62^{a}	113.50 ± 6.65^{ab}	94.95 ± 6.73^{b}
LIN (%)	60.37 ± 0.93^{b}	77.25 ± 1.88^{a}	77.24 ± 2.36^{a}
STR (%)	70.82 ± 0.68^{b}	83.05 ± 1.58^{a}	84.47 ± 1.74^{a}
WOB (%)	82.54 ± 0.57^{b}	90.44 ± 1.09^{a}	88.46 ± 1.55^{a}
ALH (μm)	3.59 ± 0.18^{a}	2.02 ± 0.07^{b}	1.91 ± 0.09^{b}
BCF (Hz)	7.49 ± 0.16^{a}	7.36 ± 0.15^{a}	7.46 ± 0.26^{a}
NSM (%)	88.82 ± 1.26^{a}	84.23 ± 1.63^{a}	84.26 ± 1.94^{a}
AIS (%)	92.05 ± 1.54^{a}	86.28 ± 2.81^{a}	85.88 ± 2.80^{a}
MIS (%)	82.43 ± 1.68^{a}	47.59 ± 4.20^{b}	45.71 ± 4.25^{b}

Experiment 3: effect of dimethylformamide on the quality of frozen–thawed Andalusian donkey semen

Table 4 shows data for comparisons of semen quality analysis using EG, DMFA or their combination as CPAs. As in Experiment 2, most semen quality parameters evaluated were significantly ($P < 0.001$) higher in fresh semen than in frozen–thawed semen samples regardless of the extender used, except LIN, STR and WOB. No significant ($P > 0.05$) differences were found in mean BCF or NSM values between fresh semen and frozen–thawed samples. The use of DMFA at concentration of 2.5% resulted in greater ($P < 0.001$) values of MOT, PMOT and MIS, but there were no differences ($P > 0.05$) between CPAs used for VAP, LIN, STR, WOB, ALH, BCF or NSM (Table 4). The lowest ($P < 0.001$) mean values of VCL and VSL were obtained when using DMFA at 1%. In addition, no significant ($P > 0.05$) differences were found in mean VCL and VSL values between treatments with 1% EG, 2.5% DMFA and the combination of 1.5% DMFA + 1% EG.

Discussion

In the present study, cryopreservation provoked a reduction in most of the sperm quality parameters of frozen samples regardless of the extender used; however, the average values we

Table 3. Mean values (± s.e.m.) of Andalusian donkey sperm quality in fresh semen and after freezing–thawing using either glycerol (Gent B), dimethyl sulfoxide (1.5 or 2%) or ethylene glycol (1 or 1.5%) as cryoprotectants

MOT, total motility; PMOT, progressive motility; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; NSM, normal sperm morphology; AIS, acrosome-intact spermatozoa; MIS, membrane-intact spermatozoa; DMSO 1.5, Gent A extender supplemented with 1.5% dimethyl sulfoxide; DMSO 2, Gent A extender supplemented with 2% dimethyl sulfoxide; EG 1, Gent A extender supplemented with 1% ethylene glycol; EG 1.5, Gent A extender supplemented with 1.5% ethylene glycol; GLY, Gent B extender. $^{a–d}$Different superscript letters within a row indicate significant differences ($P < 0.05$)

Parameters	Fresh semen	Frozen–thawed samples				
		GLY	DMSO 1.5	DMSO 2	EG 1	EG 1.5
MOT (%)	96.58 ± 0.45^{a}	61.45 ± 2.45^{bc}	36.85 ± 2.36^{d}	37.48 ± 1.39^{d}	65.09 ± 1.50^{b}	57.30 ± 2.24^{c}
PMOT (%)	86.13 ± 1.05^{a}	51.09 ± 2.56^{bc}	27.27 ± 1.74^{d}	29.03 ± 1.91^{d}	54.66 ± 2.43^{b}	47.43 ± 1.25^{c}
VCL (μm s^{-1})	186.10 ± 4.57^{a}	137.32 ± 6.96^{b}	103.33 ± 6.09^{c}	105.20 ± 4.73^{c}	130.00 ± 5.56^{b}	132.25 ± 7.36^{b}
VSL (μm s^{-1})	117.31 ± 3.56^{a}	107.65 ± 6.80^{a}	85.63 ± 5.68^{b}	87.99 ± 4.50^{b}	108.49 ± 5.73^{a}	113.02 ± 7.22^{a}
VAP (μm s^{-1})	157.57 ± 4.20^{a}	128.60 ± 6.67^{b}	96.21 ± 5.94^{c}	98.29 ± 4.67^{c}	122.25 ± 5.48^{b}	125.04 ± 7.47^{b}
LIN (%)	59.48 ± 1.16^{b}	71.80 ± 2.14^{a}	73.97 ± 2.23^{a}	75.92 ± 2.22^{a}	77.02 ± 2.23^{a}	77.41 ± 2.70^{a}
STR (%)	70.11 ± 0.72^{c}	78.16 ± 2.09^{b}	82.11 ± 1.65^{ab}	83.48 ± 1.85^{ab}	83.61 ± 1.90^{ab}	84.30 ± 2.16^{a}
WOB (%)	81.61 ± 0.89^{b}	88.99 ± 1.33^{a}	86.36 ± 1.83^{a}	87.74 ± 1.54^{a}	89.29 ± 1.33^{a}	88.55 ± 1.79^{a}
ALH (μm)	4.15 ± 0.19^{a}	2.30 ± 0.09^{b}	1.93 ± 0.08^{c}	1.96 ± 0.07^{c}	2.15 ± 0.07^{bc}	2.06 ± 0.06^{bc}
BCF (Hz)	7.52 ± 0.28^{a}	8.26 ± 0.60^{a}	7.95 ± 0.50^{a}	8.34 ± 0.44^{a}	8.54 ± 0.39^{a}	8.71 ± 0.51^{a}
NSM (%)	90.23 ± 1.20^{ab}	83.96 ± 1.38^{c}	86.49 ± 1.55^{bc}	84.47 ± 1.83^{c}	92.25 ± 1.55^{a}	89.52 ± 1.42^{ab}
AIS (%)	88.05 ± 2.22^{ab}	81.50 ± 2.06^{b}	81.74 ± 1.82^{b}	83.34 ± 2.02^{b}	90.18 ± 1.59^{a}	87.84 ± 1.65^{ab}
MIS (%)	83.32 ± 1.51^{a}	44.09 ± 3.23^{c}	37.49 ± 1.58^{d}	41.18 ± 2.10^{cd}	56.73 ± 2.09^{b}	52.99 ± 1.95^{b}

Table 4. Mean values (± s.e.m.) of Andalusian donkey sperm quality in fresh semen and after freezing–thawing using different cryoprotectants: ethylene glycol (1%), dimethylformamide (1 or 2.5%) or a combination of 1.5% dimethylformamide and 1% ethylene glycol

MOT, total motility; PMOT, progressive motility; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; NSM, normal sperm morphology; AIS, acrosome-intact spermatozoa; MIS, membrane-intact spermatozoa; EG 1, Gent A extender supplemented with 1% ethylene glycol; DMFA 1, Gent A extender supplemented with 1% dimethylformamide; DMFA 2.5, Gent A extender supplemented with 2.5% dimethylformamide; DMFA 1.5 + EG 1, Gent A extender supplemented with a combination of 1.5% dimethylformamide and 1% ethylene glycol. $^{a–d}$Different superscript letters within a row indicate significant differences ($P < 0.05$)

Parameters	Fresh semen	Frozen–thawed samples			
		EG 1	DMFA 1	DMFA 2.5	DMFA 1.5 + EG 1
MOT (%)	95.91 ± 1.05^{a}	53.76 ± 2.27^{cd}	50.96 ± 1.84^{d}	66.21 ± 2.60^{b}	58.03 ± 2.44^{c}
PMOT (%)	85.46 ± 2.52^{a}	42.54 ± 1.99^{cd}	39.51 ± 1.39^{d}	57.09 ± 2.99^{b}	48.51 ± 2.25^{c}
VCL (μm s^{-1})	188.20 ± 5.81^{a}	95.08 ± 7.14^{bc}	94.11 ± 7.96^{c}	109.54 ± 7.28^{b}	101.27 ± 6.85^{b}
VSL (μm s^{-1})	113.06 ± 5.81^{a}	80.64 ± 5.75^{bc}	76.96 ± 5.48^{c}	89.64 ± 5.32^{b}	86.31 ± 5.26^{b}
VAP (μm s^{-1})	155.48 ± 5.80^{a}	88.48 ± 6.65^{b}	86.62 ± 7.12^{b}	99.55 ± 6.37^{b}	94.54 ± 6.17^{b}
LIN (%)	56.92 ± 2.49^{b}	76.94 ± 1.73^{a}	75.91 ± 2.07^{a}	77.29 ± 2.11^{a}	79.40 ± 2.19^{a}
STR (%)	68.55 ± 1.73^{b}	84.86 ± 1.37^{a}	84.21 ± 1.55^{a}	85.81 ± 1.84^{a}	86.77 ± 1.60^{a}
WOB (%)	79.63 ± 1.72^{b}	86.72 ± 0.92^{a}	86.85 ± 1.22^{a}	87.00 ± 1.30^{a}	88.52 ± 1.26^{a}
ALH (μm)	4.45 ± 0.22^{a}	1.78 ± 0.11^{b}	1.88 ± 0.13^{b}	2.21 ± 0.17^{b}	1.85 ± 0.12^{b}
BCF (Hz)	7.67 ± 0.35^{a}	7.98 ± 0.38^{a}	8.19 ± 0.50^{a}	8.88 ± 0.51^{a}	8.49 ± 0.50^{a}
NSM (%)	83.29 ± 2.44^{a}	81.85 ± 4.35^{a}	81.58 ± 4.17^{a}	80.57 ± 4.16^{a}	81.25 ± 5.12^{a}
AIS (%)	88.43 ± 2.27^{a}	82.12 ± 3.50^{ab}	74.23 ± 5.47^{b}	84.57 ± 4.88^{ab}	79.22 ± 5.59^{ab}
MIS (%)	85.54 ± 1.95^{a}	53.89 ± 2.81^{c}	52.93 ± 2.75^{c}	64.95 ± 3.77^{b}	55.66 ± 3.39^{c}

obtained fall within the ranges other authors have taken to indicate that the freezing method has been successful (Álvarez *et al.* 2006; Flores *et al.* 2008; Contri *et al.* 2012*b*; Rota *et al.* 2012; Ortiz *et al.* 2015).

The chemical composition of freezing extenders plays a major role in sperm survival during cryopreservation (Pillet *et al.* 2008). Previous studies have demonstrated the usefulness of the extenders Gent B (Flores *et al.* 2008; Ortiz *et al.* 2015) and INRA96-EYG (Rota *et al.* 2012) for the cryopreservation of donkey spermatozoa; however, no direct comparison of these semen extenders has been performed in donkeys. This study compares, for the first time, the effectiveness of the extenders Gent B and INRA96-EYG for freezing semen from Andalusian donkeys. After thawing, some CASA-derived motility variables (MOT, PMOT, VCL and VSL) were greater for spermatozoa cryopreserved in Gent B than in INRA96-EYG. In addition, semen extended with Gent B had a 35.8% decrease in MOT and 26.6% decrease in PMOT. When using INRA96-EYG,

a reduction of more than 47% in MOT and more than 37% in PMOT occurred. In stallions, the use of Gent B had a beneficial effect on the percentage of linearly motile spermatozoa post-thawing compared with INRA96-EYG (Morillo Rodríguez *et al.* 2011). Furthermore, the use of Gent B resulted in greater sperm velocities (VCL and VSL) compared with INRA96-EYG, which is particularly interesting because the sperm velocities ascertained by CASA assessments have been previously correlated with *in vivo* fertility in the Andalusian donkey (Dorado *et al.* 2013). Conversely, sperm motility characteristics of semen cryopreserved with INRA96-EYG were consistent with data reported in previous studies (Rota *et al.* 2012). Since a discrepancy between good motility *in vitro* and low fertility obtained with frozen–thawed semen in donkeys has been recently described (Oliveira *et al.* 2006; Vidament *et al.* 2009; Rota *et al.* 2012), further studies are needed to better understand the changes in *in vitro* motility patterns of donkey spermatozoa caused by semen freezing extenders and the relationship with fertility.

No differences as a result of type of extender used were found, however, for sperm morphology, sperm membrane integrity and acrosome integrity. These results may be explained by the special formulation of INRA 96, which is thought to create physical protection for sperm membranes during preservation (Pillet *et al.* 2008).

To our knowledge, this is the first attempt to compare the effectiveness of four permeating CPAs (GLY, EG, DMSO and DMFA) in protecting Andalusian donkey spermatozoa during the cryopreservation process. In Experiment 2, EG and DMSO were used at different concentrations and compared with GLY (Gent B, as control). In agreement with previous findings (Rota *et al.* 2012), no differences were observed between semen frozen with GLY or EG in post-thaw motility. Similar findings have been reported in stallions (Alvarenga *et al.* 2000*a*; Álvarez *et al.* 2005). Our results show, however, that GLY did not improve the percentage of morphologically normal spermatozoa with intact membranes. Because low-molecular-weight CPAs such as EG permeate the plasma membrane more effectively than GLY (Moore *et al.* 2006), they could cause less osmotic damage to the spermatozoa (Hoffmann *et al.* 2011).

In donkeys, when GLY and EG were used at the same concentration (5%) motility was significantly lower with EG (Serres *et al.* 2004*b*). It is known that EG can cause damage to human (Alvarez and Storey 1993), dog (Martins-Bessa *et al.* 2006) and stallion (Mantovani *et al.* 2002) spermatozoa, and that this effect was concentration-dependent (humans, at 3%; dogs, at 8%; stallions, above 3%). In fact, these last authors observed that higher EG concentrations significantly reduced the total and progressive motility of stallion spermatozoa. In our experiment, a decrease in sperm motility (MOT and PMOT) was observed when 1.5% EG was added to the freezing medium. In this regard, it could be speculated that the toxic effect of EG at a concentration of 1.5% is more obvious for donkey than for stallion spermatozoa. Likewise, a greater sensitivity of donkey spermatozoa to GLY has been previously suggested (Alvarenga *et al.* 2005; Vidament *et al.* 2009). Although the toxic effect of EG could be influenced by the composition of the extender, our results suggest that Gent A containing 1% EG provide good cryoprotection for donkey spermatozoa. Nevertheless, previous studies demonstrated that pregnancy rates in jennies with frozen–thawed donkey semen were similar using both GLY and other CPAs (Oliveira *et al.* 2006; Rota *et al.* 2012).

The present study failed to elucidate the effect of DMSO on sperm motility and membrane integrity of cryopreserved donkey spermatozoa. Under our experimental conditions, DMSO was deleterious for some sperm parameters such as MOT, PMOT, VCL, VSL, VAP and MIS regardless of the concentration used (1.5 or 2%). Our results agree with those of previous studies in which stallion semen cryopreserved with DMSO had lower sperm motility and viability compared with GLY, EG or DMFA (Alvarenga *et al.* 2000*a*; Álvarez *et al.* 2005). Based on these results, a toxic effect of DMSO at concentrations of 1.5 and 2% on donkey spermatozoa is suspected.

In Experiment 3, a commercial equine semen extender (Gent A) with different combinations of CPAs (1% EG only, 1% DMFA only, 2.5% DMFA only, 1.5% DMFA + 1% EG) was used. The addition of DMFA at 2.5% had a positive influence on sperm motility (MOT and PMOT) and membrane integrity. In stallions, several authors reported, however, that EG provided similar or better post-thaw semen quality than DMFA (Alvarenga *et al.* 2000*a*; Squires *et al.* 2004; Álvarez *et al.* 2005; Moore *et al.* 2006). Although the exact mechanism of action of EG and DMFA during the sperm cryopreservation process is unknown (Hoffmann *et al.* 2011), the differences among CPAs activity have been recently attributed to the permeability coefficient and the structural model of the cryogenic agent (Snoeck *et al.* 2012). In line with this, differences in the membrane lipid composition of donkey and stallion spermatozoa have been reported (Cortés-Gutiérrez *et al.* 2008; Bucci *et al.* 2010), a fact which could explain, at least in part, our results. Indeed, a species-specific effect of EG has been suggested by Martins-Bessa *et al.* (2006).

It is known that values of osmotic pressure outside the optimal tolerance range of spermatozoa may cause cell volume changes and cell death (Ball and Vo 2001). In the present study, the osmolality of DMFA at 2.5% (658 mOsm kg^{-1}) was within the osmotic tolerance range (600–750 mOsm kg^{-1}) of stallion spermatozoa (Hoffmann *et al.* 2010). Hence, the percentage of membrane-intact spermatozoa was significantly higher in samples frozen in 2.5% DMFA than those frozen in the other CPAs and combinations. Our results corroborate those of Alvarez *et al.* (2004), who demonstrated that different amides, especially DMFA, improve the post-thaw quality of Zamorano–Leonés donkey semen.

It is noteworthy that the addition of DMFA to the Gent A extender failed to improve the quality of cryopreserved Andalusian donkey spermatozoa when a concentration of 1% was used, a finding which is in keeping with those of earlier studies (Vidament *et al.* 2009; Hoffmann *et al.* 2011). These studies demonstrated that post-thaw motility of stallion and donkey spermatozoa was significantly higher in 2–3% DMFA.

In order to minimise osmotic injury, the combination of DMFA with EG was used at low concentrations; however, no benefit of combining 1.5% DMFA with 1% EG was observed compared with 1% EG or 2.5% DMFA alone. Our findings could be due, at least in part, to the higher osmolality of this

freezing extender (662 mOsm kg^{-1}) compared with Gent A containing 1% EG or 2.5% DMFA (470 and 658 mOsm kg^{-1}, respectively) and consequently the protective effect of 1.5% DMFA + 1% EG could be negated by the sensitizing effect of the hypertonicity. According to our results, previous studies have demonstrated that stallion spermatozoa frozen with DMFA alone had greater motility than those frozen with other CPAs in combination with DMFA (Medeiros *et al.* 2002; Vidament *et al.* 2002). This effect has been attributed to the toxicity induced by a specific combination of CPAs (Molinia *et al.* 1994), which not only prevents the use of fully protective levels of these CPAs but also causes further damage to spermatozoa. In equine species, combining different amides (DMFA, dimethyl acetamide or methyl formamide) with GLY significantly enhanced the quality of cryopreserved stallion (Medeiros *et al.* 2002) and donkey (Oliveira *et al.* 2006) spermatozoa compared with GLY or dimethyl acetamide alone, suggesting that not all combinations of CPAs are toxic to spermatozoa.

Regardless of our *in vitro* results, it should be pointed out that the current methods available for freezing donkey semen have a negative effect on fertility of jennies with frozen–thawed donkey semen (pregnancy rates between 0 and 20%; Trimeche *et al.* 1998; Vidament *et al.* 2005, 2009; Oliveira *et al.* 2006; Rota *et al.* 2012), while acceptable pregnancy rates of at least 40% have been obtained when frozen–thawed donkey semen was employed for AI in mares (Oliveira *et al.* 2006; Vidament *et al.* 2009; Jepsen *et al.* 2010; Canisso *et al.* 2011). Therefore, the higher ability of mares to conceive after AI with frozen–thawed donkey semen, using different CPAs, than that of jennies, supports the hypothesis that the interaction between frozen semen and the jenny genital tract may result in reduced fertility due to an endometrial inflammation (Miró *et al.* 2011; Vilés *et al.* 2013). Further studies are necessary to optimise pregnancy rates in jennies.

In conclusion, the use of Gent B resulted in maintenance of sperm motility characteristics after thawing to a greater extent than INRA96-EYG, suggesting that Gent B extender is more appropriate to freeze donkey spermatozoa. However, the replacement of GLY by EG improved the outcome of cryopreservation of Andalusian donkey spermatozoa because it provided higher sperm quality after thawing. The use of DMSO for freezing donkey semen was unsuccessful and a toxic effect is suspected. DMFA at 2.5% seemed to offer the highest protection against cryoinjury during freezing and thawing, but no benefit was shown when DMFA was used in combination with EG. Although it remains to be determined if the better *in vitro* quality of Andalusian donkey spermatozoa in diluents containing different CPAs will be reflected in enhanced fertility when used for inseminations, this would allow a more efficient use of cryopreserved donkey semen for insemination. In any case, these extenders should be included in a freezability test to determine the best extender for each donkey.

Acknowledgements

We are extremely grateful to Fundación Casa del Burro (Rute, Cordoba, Spain) for supplying the animals. This work has been supported by Grant RZ2009–00006–00–00 (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Ministerio de Ciencia e Innovación, Spain). Part of this study has been previously presented at the 18th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR), September 10–13, 2014, Helsinki, Finland.

References

Alvarenga, M. A., Keith, S. L., Graham, J. K., Landim-Alvarenga, F. C., and Squires, E. L. (2000*a*). Alternative cryoprotectors for freezing stallion semen. In 'Proceedings of the 14th International Congress on Animal Reproduction and Artificial Insemination, Stockholm, Sweden'. (Ed(s) International Congress on Animal Reproduction (ICAR).) p. 73. (International Congress on Animal Reproduction (ICAR): Stockholm, Sweden.)

Alvarenga, M. A., Landim-Alvarenga, F. C., Moreira, R. M., and Cesarino, M. M. (2000*b*). Acrosomal ultrastructure of stallion spermatozoa cryopreserved with ethylene glycol using two packaging systems. *Equine Vet. J.* **32**(6), 541–545. doi:10.2746/042516400777584749

Alvarenga, M. A., Papa, F. O., Landim-Alvarenga, F. C., and Medeiros, A. S. L. (2005). Amides as cryoprotectants for freezing stallion semen: a review. *Anim. Reprod. Sci.* **89**(1–4), 105–113. doi:10.1016/J.ANIREPROSCI.2005.07.001

Alvarez, J. G., and Storey, B. T. (1993). Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in cryopreserved human spermatozoa: glycerol and other polyols as sole cryoprotectant. *J. Androl.* **14**(3), 199–209.

Alvarez, A. L., Serres, C., Crespo, F., Mateos, E., and Gómez-Cuétaral, C. (2004). Cryopreservation of Zamorano–Leonés donkey spermatozoa using different amides. *Reprod. Domest. Anim.* **39**, 267.

Álvarez, A. L., Gómez-Cuétara, C., and Graham, J. K. (2005). Effect of cholesterol-loaded cyclodextrin and cryoprotectants on the cryosurvival of stallion spermatozoa. *Reprod. Domest. Anim.* **40**(4), 375.

Álvarez, A. L., Serres, C., Torres, P., Crespo, F., Mateos, E., and Gómez-Cuétara, C. (2006). Effect of cholesterol-loaded cyclodextrin on the cryopreservation of donkey spermatozoa. *Anim. Reprod. Sci.* **94**(1–4), 89–91.

Andrabi, S. M. H., and Maxwell, W. M. C. (2007). A review on reproductive biotechnologies for conservation of endangered mammalian species. *Anim. Reprod. Sci.* **99**(3–4), 223–243. doi:10.1016/J.ANIREPROSCI.2006.07.002

Ball, B. A., and Vo, A. (2001). Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability and mitochondrial membrane potential. *J. Androl.* **22**(6), 1061–1069.

Brito, L. F. (2007). Evaluation of stallion sperm morphology. *Clinical Techniques in Equine Practice* **6**(4), 249–264. doi:10.1053/J.CTEP.2007.09.004

Bucci, D., Spinaci, M., Vallorani, C., Contri, A., Carluccio, A., Isani, G., Tamanini, C., and Galeati, G. (2010). Detection and localisation of GLUTs 1, 2, 3 and 5 in donkey spermatozoa. *Reprod. Domest. Anim.* **45**(5), e217–e220.

Canisso, I. F., Souza, F. A., and Ortigoza Escobar, J. M. (2008). Congelamiento de semen de burro (*Equus asinus*). *Rev. investig. vet. Perú* **19**(2), 113–125.

Canisso, I. F., Carvalho, G. R., Morel, M. D., Ker, P. G., Rodrigues, A. L., Silva, E. C., and Da Silva, M. A. C. (2011). Seminal parameters and field fertility of cryopreserved donkey jack semen after insemination of horse mares. *Equine Vet. J.* **43**(2), 179–183. doi:10.1111/J.2042-3306.2010.00130.X

Chenier, T., Merkies, K., Leibo, S., Plante, C., and Johnson, W. (1998). Evaluation of cryoprotective agents for use in the cryopreservation of equine spermatozoa. In 'American Association of Equine Practitioners Proceedings'. (Ed(s) American Association of Equine Practitioners – AAEP.) pp. 5–6. (American Association of Equine Practitioners – AAEP: Baltimore, MD.)

Contri, A., Gloria, A., Robbe, D., De Amicis, I., and Carluccio, A. (2012*a*). Characteristics of donkey spermatozoa along the length of the

epididymis. *Theriogenology* **77**(1), 166–173. doi:10.1016/J.THERIOGENOLOGY.2011.07.031

Contri, A., Gloria, A., Robbe, D., Sfirro, M. P., and Carluccio, A. (2012*b*). Effect of sperm concentration on characteristics of frozen–thawed semen in donkeys. *Anim. Reprod. Sci.* **136**(1–2), 74–80. doi:10.1016/J.ANIREPROSCI.2012.10.022

Cortés-Gutiérrez, E. I., Crespo, F., Gosálvez, A., Dávila-Rodríguez, M. I., López-Fernández, C., and Gósalvez, J. (2008). DNA fragmentation in frozen spermatozoa of *Equus asinus*: Zamorano–Leonés, a breed at risk of extinction. *Theriogenology* **69**(8), 1022–1032. doi:10.1016/J.THERIOGENOLOGY.2008.02.002

DAD-IS-FAO (2013) 'Information System Domestic Animal Diversity'. (Food and Agriculture Organization of the United Nations – FAO.)

Dorado, J., Acha, D., Ortiz, I., Gálvez, M. J., Carrasco, J. J., Díaz, B., Gómez-Arrones, V., Calero-Carretero, R., and Hidalgo, M. (2013). Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (*Equus asinus*). *Anim. Reprod. Sci.* **143**(1–4), 64–71. doi:10.1016/J.ANIREPROSCI.2013.10.003

Dorado, J., Acha, D., Ortiz, I., Gálvez, M., Carrasco, J., Gómez-Arrones, V., Calero-Carretero, R., and Hidalgo, M. (2014). Effect of extender and amino-acid supplementation on sperm quality of cooled–preserved Andalusian donkey (*Equus asinus*) spermatozoa. *Anim. Reprod. Sci.* **146**(1–2), 79–88. doi:10.1016/J.ANIREPROSCI.2014.02.009

Flores, E., Taberner, E., Rivera, M., Peña, A., Rigau, T., Miró, J., and Rodríguez-Gil, J. (2008). Effects of freezing–thawing on motile sperm subpopulations of boar and donkey ejaculates. *Theriogenology* **70**(6), 936–945. doi:10.1016/J.THERIOGENOLOGY.2008.05.056

Glazar, A. I., Mullen, S. F., Liu, J., Benson, J. D., Critser, J. K., Squires, E. L., and Graham, J. K. (2009). Osmotic tolerance limits and membrane permeability characteristics of stallion spermatozoa treated with cholesterol. *Cryobiology* **59**(2), 201–206. doi:10.1016/J.CRYOBIOL.2009.07.009

Gomes, G. M., Jacob, J. C. F., Medeiros, A. S. I., Papa, F. O., and Alvarenga, M. A. (2002). Improvement of stallion spermatozoa preservation with alternative cryoprotectants for the Mangalarga Marchador breed. *Theriogenology* **58**(2–4), 277–279.

Henry, M., Snoeck, P. P. N., and Cottorello, A. C. P. (2002). Post-thaw spermatozoa plasma membrane integrity and motility of stallion semen frozen with different cryoprotectants. *Theriogenology* **58**(2–4), 245–248.

Hoffmann, N., Morandini, C., and Sieme, H. (2010). Determination of the optimal concentration of cryoprotectant agent for freezing stallion spermatozoa. *Anim. Reprod. Sci.* **121**(1–2), S160–S162.

Hoffmann, N., Oldenhof, H., Morandini, C., Rohn, K., and Sieme, H. (2011). Optimal concentrations of cryoprotective agents for semen from stallions that are classified 'good' or 'poor' for freezing. *Anim. Reprod. Sci.* **125**(1–4), 112–118. doi:10.1016/J.ANIREPROSCI.2011.03.001

Jepsen, R. J., Evans, L. E., and Youngs, C. R. (2010). Use of direct thaw insemination to establish pregnancies with frozen–thawed semen from a standard jack. *J. Equine Vet. Sci.* **30**(11), 651–656. doi:10.1016/J.JEVS.2010.10.001

Kenney, R., Bergman, R., Cooper, W., and Morse, G. (1975). Minimal contamination techniques for breeding mares: techniques and preliminary findings. In 'Proceedings of the 21st AAEP Annual Convention'. (Ed(s) American Association of Equine Practitioners – AAEP) pp. 327–336. (American Association of Equine Practitioners – AAEP: Boston, MA.)

Macías García, B., Ortega Ferrusola, C., Aparicio, I. M., Miró-Morán, A., Morillo Rodriguez, A., Gallardo Bolaños, J. M., González Fernández, L., Balao da Silva, C. M., Rodríguez Martínez, H., Tapia, J. A., and Peña, F. J. (2012). Toxicity of glycerol for the stallion spermatozoa: effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. *Theriogenology* **77**(7), 1280–1289. doi:10.1016/J.THERIOGENOLOGY.2011.10.033

Madison, R. J., Evans, L. E., and Youngs, C. R. (2013). The effect of 2-hydroxypropyl-β-cyclodextrin on post-thaw parameters of cryopreserved jack and stallion semen. *J. Equine Vet. Sci.* **33**(4), 272–278. doi:10.1016/J.JEVS.2012.07.021

Mantovani, R., Rota, A., Falomo, M. E., Bailoni, L., and Vincenti, L. (2002). Comparison between glycerol and ethylene glycol for the cryopreservation of equine spermatozoa: semen quality assessment with standard analyses and with the hypo-osmotic swelling test. *Reprod. Nutr. Dev.* **42**(3), 217–226. doi:10.1051/RND:2002020

Martins-Bessa, A., Rocha, A., and Mayenco-Aguirre, A. (2006). Comparing ethylene glycol with glycerol for cryopreservation of canine semen in egg-yolk TRIS extenders. *Theriogenology* **66**(9), 2047–2055. doi:10.1016/J.THERIOGENOLOGY.2006.06.004

Medeiros, A., Gomes, G., Carmo, M., Papa, F., and Alvarenga, M. (2002). Cryopreservation of stallion spermatoza using different amides. *Theriogenology* **58**(2–4), 273–276.

Miró, J., Lobo, V., Quintero-Moreno, A., Medrano, A., Peña, A., and Rigau, T. (2005). Sperm motility patterns and metabolism in Catalonian donkey semen. *Theriogenology* **63**(6), 1706–1716. doi:10.1016/J.THERIOGENOLOGY.2004.07.022

Miró, J., Taberner, E., Rivera, M., Peña, A., Medrano, A., Rigau, T., and Peñalba, A. (2009). Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen. *Theriogenology* **72**(8), 1017–1022. doi:10.1016/J.THERIOGENOLOGY.2009.06.012

Miró, J., Vilés, K., Fernández, M., Fábregas, N., Soares, J., and García, W. (2011). Induced acute endometritis by frozen semen insemination in donkey. *Reprod. Domest. Anim.* **46**(s3), 130.

Molinia, F. C., Evans, G., and Maxwell, W. M. C. (1994). Incorporation of penetrating cryoprotectants in diluents for pellet-freezing ram spermatozoa. *Theriogenology* **42**(5), 849–858. doi:10.1016/0093-691X(94)90453-P

Moore, A. I., Squires, E. L., Bruemmer, J. E., and Graham, J. K. (2006). Effect of cooling rate and cryoprotectant on the cryosurvival of equine spermatozoa. *J. Equine Vet. Sci.* **26**(5), 215–218. doi:10.1016/J.JEVS.2006.03.003

Morillo Rodríguez, A., Ortega Ferrusola, C., Macías García, B., Morrell, J. M., Rodríguez Martínez, H., Tapia, J. A., and Peña, F. J. (2011). Freezing stallion semen with the new Cáceres extender improves post-thaw sperm quality and diminishes stallion-to-stallion variability. *Anim. Reprod. Sci.* **127**(1–2), 78. doi:10.1016/J.ANIREPROSCI.2011.07.009

Oldenhof, H., Friedel, K., Sieme, H., Glasmacher, B., and Wolkers, W. F. (2010). Membrane permeability parameters for freezing of stallion spermatozoa as determined by Fourier transform infrared spectroscopy. *Cryobiology* **61**(1), 115–122. doi:10.1016/J.CRYOBIOL.2010.06.002

Oliveira, J. V., Alvarenga, M. A., Melo, C. M., Macedo, L. M., DellÁqua, J. A., Jr, and Papa, F. O. (2006). Effect of cryoprotectant on donkey semen freezability and fertility. *Anim. Reprod. Sci.* **94**(1–4), 82–84.

Ortiz, I., Dorado, J., Acha, D., Gálvez, M. J., Urbano, M., and Hidalgo, M. (2015). Colloid single-layer centrifugation improves post-thaw donkey (*Equus asinus*) sperm quality and is related to ejaculate freezability. *Reprod. Fertil. Dev.* **27**(2), 332–340. doi:10.1071/RD13246

Peña, F. J., Macías García, B., Samper, J. C., Aparicio, I. M., Tapia, J. A., and Ortega Ferrusola, C. (2011). Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? *Theriogenology* **76**(7), 1177–1186. doi:10.1016/J.THERIOGENOLOGY.2011.06.023

Pillet, E., Batellier, F., Duchamp, G., Furstoss, V., Le Vern, Y., Kerboeuf, D., Desherces, S., Schmitt, E., and Magistrini, M. (2008). High fertility rates with stallion spermatozoa cryopreserved in INRA96-based

extender are not predicted by *in vitro* parameters. *Anim. Reprod. Sci.* **107** (3–4), 339–340. doi:10.1016/J.ANIREPROSCI.2008.05.116

Rota, A., Panzani, D., Sabatini, C., and Camillo, F. (2012). Donkey jack (*Equus asinus*) semen cryopreservation: studies of seminal parameters, post breeding inflammatory response and fertility in donkey jennies. *Theriogenology* **78**, 1846–1854. doi:10.1016/J.THERIOGENOLOGY.2012.07.015

Serres, C. (2003). Evaluación y Conservación del semen en el asno Zamorano-Leonés. Doctoral Thesis, Universidad Complutense de Madrid.

Serres, C., Álvarez, A., Rodriguez, A., Santiago, I., Mateos, E., and Gómez-Cuétaral, C. (2004*a*). Effect of extender and glycerol concentration on cryopreservation of semen from the Zamorano–Leonés donkey. *Reprod. Domest. Anim.* **39**, 267.

Serres, C., Alvarez, A. L., Rodriguez, A., Crespo, F., Mateos, E., and Gomez-Cuetara, C. (2004*b*). Use of ethylene glycol in the cryopreservation of Zamorano–Leones donkey semen. In 'Proceedings of the 15th International Congress on Animal Reproduction, Porto Seguro, Brazil'. (Ed(s) International Congress on Animal Reproduction – ICAR.) p. 512. (International Congress on Animal Reproduction – ICAR: Porto Seguro, Brazil.)

Serres, C., Gutiérrez-Cepeda, L., Gero, S., Gómez, V., Blázquez, J. C., and Crespo, F. (2014). Fertility rates in jennies by new semen-processing techniques. *J. Equine Vet. Sci.* **34**(1), 193–194. doi:10.1016/J.JEVS.2013.10.142

Smits, K., Hoogewijs, M., Woelders, H., Daels, P., and Van Soom, A. (2012). Breeding or assisted reproduction? Relevance of the horse model applied to the conservation of endangered equids. *Reprod. Domest. Anim.* **47**, 239–248. doi:10.1111/J.1439-0531.2012.02082.X

Snoeck, P., Cottorello, A., and Henry, M. (2012). Viability and fertility of stallion semen frozen with ethylene glycol and acetamide as a cryogenic agent. *Anim. Reprod.* **9**(1), 33–39.

Squires, E. L., Keith, S. L., and Graham, J. K. (2004). Evaluation of alternative cryoprotectants for preserving stallion spermatozoa. *Theriogenology* **62**(6), 1056–1065. doi:10.1016/J.THERIOGENOLOGY.2003.12.024

Trimeche, A., Renard, P., and Tainturier, D. (1998). A procedure for Poitou jackass sperm cryopreservation. *Theriogenology* **50**(5), 793–806. doi:10.1016/S0093-691X(98)00184-8

Vidament, M., Daire, C., Yvon, J. M., Doligez, P., Bruneau, B., Magistrini, M., and Ecot, P. (2002). Motility and fertility of stallion semen frozen with glycerol and/or dimethyl formamide. *Theriogenology* **58**(2–4), 249–251.

Vidament, M., Vincent, P., Yvon, J., Bruneau, B., and Martin, F. (2005). Glycerol in semen extender is a limiting factor in the fertility in asine and equine species. *Anim. Reprod. Sci.* **89**(1–4), 302.

Vidament, M., Vincent, P., Martin, F., Magistrini, M., and Blesbois, E. (2009). Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. *Anim. Reprod. Sci.* **112**(1–2), 22–35. doi:10.1016/J.ANIREPROSCI.2008.03.016

Vilés, K., Rabanal, R., Rodríguez-Prado, M., and Miró, J. (2013). Effect of ketoprofen treatment on the uterine inflammatory response after AI of jennies with frozen semen. *Theriogenology* **79**(7), 1019–1026. doi:10.1016/J.THERIOGENOLOGY.2013.01.006

Watson, P. F. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod. Fertil. Dev.* **7**(4), 871–891. doi:10.1071/RD9950871

Conclusiones

Conclusiones

De acuerdo a los resultados obtenidos en la presente Tesis Doctoral, se pueden extraer las conclusiones que se enumeran a continuación:

- Primera Publicación: ***Sperm motility patterns in Andalusian donkey (Equus asinus) semen: Effects of body weight, age, and semen quality.*** *J. Dorado, D. Acha, M.J. Gálvez, I. Ortiz, J.J. Carrasco, B. Díaz, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Theriogenology 2013, 79: 1100-1109.

1. Los resultados del presente estudio mostraron la existencia de cuatro subpoblaciones espermáticas con patrones cinéticos específicos en el semen fresco del asno andaluz, observándose una gran heterogeneidad en la distribución de estas subpoblaciones dentro del eyaculado del garañón.

2. La relación existente entre la distribución de las diferentes subpoblaciones espermáticas y el individuo valorado, así como con los parámetros de calidad seminal como el movimiento total y la concentración espermática demuestran que los espermatozoides de cada subpoblación tienen patrones cinéticos específicos.

3. La distribución de las subpoblaciones espermáticas presentes en el eyaculado no se vieron afectados por el peso o la edad del individuo.

4. Por primera vez se estableció una correlación significativa entre el peso corporal del individuo y determinados parámetros de calidad espermática como el movimiento y la morfología espermática, así como con el pH del eyaculado. Por el contrario, ninguna de las características espermáticas se vio influenciada por la edad del asno.

5. Por último, el estudio de las diferentes subpoblaciones espermáticas proporcionó un aumento de la información obtenida durante el análisis del semen de asno.

- Segunda Publicación: ***Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (Equus asinus).*** *J. Dorado, D. Acha, I. Ortiz, M.J. Gálvez, J.J. Carrasco, B. Díaz, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Animal Reproduction Science 2013, 143: 64-71.

1. Se evidenció la relación existente entre las características espermáticas evaluadas y la fertilidad *in vivo*. Entre los parámetros cinéticos obtenidos con el sistema CASA, se evidenciaron altos valores de velocidad curvilínea (VCL) y velocidad media (VAP) así como una alta frecuencia de cruzamiento (BCF) en aquellos asnos con porcentajes de preñez por ciclo ≥ al 60%. Así, el porcentaje de preñez por ciclo obtenido está relacionado con los parámetros de cinética espermática obtenidos con el sistema CASA.

2. Además, se relacionó la presencia en el eyaculado de determinadas subpoblaciones espermáticas con la fertilidad *in vivo*, presentándose porcentajes significativamente bajos de la subpoblación con espermatozoides rápidos y progresivos (sP4) en aquellos eyaculados de asnos con baja tasa de fertilidad.

3. Por último, nuestros resultados demostraron la importancia del empleo de las técnicas CASA para detectar diferencias sutiles de movimiento espermático entre asnos "fértiles" y "subfértiles". Asimismo, el estudio de las diferentes subpoblaciones espermáticas podría aportar información adicional en la detección de la subfertilidad.

- Tercera Publicación: ***Effect of extender and amino acid supplementation on sperm quality of cooled-preserved Andalusian donkey (Equus asinus) spermatozoa****. J. Dorado, D. Acha, I. Ortiz, M.J. Gálvez, J.J. Carrasco, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Animal Reproduction Science 2014, 146: 79-88.

1. El presente estudió mostró la existencia de una influencia de la estación sobre la mayoría de las características seminales del eyaculado del asno andaluz. El empleo de eyaculados con alta calidad espermática evitó la influencia de dicha estacionalidad en la calidad seminal de los eyaculados obtenidos a lo largo del año.

2. El diluyente Gent® A proporcionó valores de movimiento espermático tras 72 horas de refrigeración significativamente superiores en comparación a los obtenidos con el diluyente INRA 96®, sugiriendo así que el Gent® A es más apropiado para preservar espermatozoides de asno a 5ºC.

3. La adición de glutamina, prolina y taurina al diluyente de refrigeración mejoró la preservación de los espermatozoides de asno andaluz, obteniéndose valores superiores para la mayoría de variables de movimiento espermático analizadas con el sistema CASA en aquellas muestras seminales refrigeradas con el diluyente Gent® A suplementado con diferentes concentraciones de estos tres aminoácidos en comparación con aquellas muestras refrigeradas con Gent® A sin suplementar. Esta mejoría fue menos pronunciada cuando los aminoácidos se añadieron a concentraciones de 20 mM.

4. Los resultados obtenidos nos permiten afirmar que la adición de estos aminoácidos al diluyente de refrigeración daría lugar a un uso más eficaz del semen refrigerado de asno para la IA. Sin embargo, son necesarios futuros estudios para determinar si la mejora del movimiento espermático tras la refrigeración obtenida en aquellas muestras seminales suplementadas con glutamina, prolina y taurina se ve reflejada en un aumento de la fertilidad tras la IA.

- Cuarta Publicación: ***Freezability of Andalusian donkey (Equus asinus) spermatozoa: effect of extenders and permeating cryoprotectants.*** *D. Acha, M. Hidalgo, M.J. Gálvez, I. Ortiz, J.J. Carrasco, V. Gómez-Arrones, J. Dorado.* Reproduction, Fertility and Development 2015, accepted 4 June 2015**.**

1. Los resultados del presente estudio demostraron que el diluyente comercial Gent® B proporciona valores de motilidad espermática significativamente superiores a los obtenidos con el INRA96-EYG tras la descongelación, sugiriendo así que el diluyente Gent® B es más eficaz para congelar semen de asno.

2. La sustitución del glicerol por otros crioprotectores de bajo peso molecular, como el etilenglicol, mejoró la criopreservación del espermatozoide de asno andaluz, proporcionando una mayor calidad espermática a la descongelación.

3. El empleo del dimetilsulfóxido como crioprotector para congelar muestras seminales de asno fue insatisfactorio, por lo que sospechamos pueda resultar tóxico para esta especie.

4. La dimetilformamida 2,5% fue el crioprotector que mejor conservó la calidad espermática durante el proceso de congelación y descongelación.

5. La combinación de etilenglicol y dimetilformamida a bajas concentraciones no proporcionó buenos resultados de calidad seminal tras el proceso de congelación y descongelación.

6. Por último, aunque debe de ser determinado si una mejor calidad *in vitro* del espermatozoide de asno andaluz tras el proceso de criopreservación en diluyentes que contienen diferentes crioprotectores proporciona una mejoría en la fertilidad *in vivo* tras la inseminación artificial, ello permitiría un uso más eficiente del semen criopreservado de asno para la inseminación. En cualquier caso, los diluyentes de congelación evaluados en el presente estudio deberían ser incluidos en futuros estudios en los que se determine el diluyente de congelación más adecuado para cada asno.

Conclusions

Conclusions

- First Paper: ***Sperm motility patterns in Andalusian donkey (Equus asinus) semen: Effects of body weight, age, and semen quality.*** *J. Dorado, D. Acha, M.J. Gálvez, I. Ortiz, J.J. Carrasco, B. Díaz, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Theriogenology 2013, 79: 1100-1109.

1. Our results confirm that four sperm subpopulations with specific motility patterns are present in ejaculates from this group of Andalusian donkeys, and suggest a high heterogeneity in the ejaculate structure in donkey.

2. Therefore, the relationship between the distribution of the sperm subpopulations and individual donkey, total motility, and sperm concentration shows that the spermatozoa of each have different motility patterns.

3. However, the proportions of sperm subpopulations in the ejaculates did not vary with age and body weight.

4. Moreover, for the first time, this study highlights that body weight in the donkey are significantly correlated with sperm motility, morphology, and pH, whereas none of the evaluated sperm characteristics were correlated with the age of the donkey.

5. Finally, the study of discrete subpopulations of motile spermatozoa could lead to a substantial increase in information acquired during donkey semen analysis.

- Second Paper: ***Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (Equus asinus).*** *J. Dorado, D. Acha, I. Ortiz, M.J. Gálvez, J.J. Carrasco, B. Díaz, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Animal Reproduction Science 2013, 143: 64-71.

1. Relationships between the evaluated sperm characteristics and fertility of this group of donkeys have been evidenced. Among CASA-derived sperm motion measures, mean VCL, VAP, and BCF values were higher in donkeys achieving a percent pregnant per cycle ≥ 60%. Per cycle pregnancy rate seemed to be related to computer-assisted measurements of sperm motility.

2. In addition, the subpopulation characterized by rapid and progressive spermatozoa was significantly lower in donkeys with poor in vivo fertility results. Moreover, fertility was related to the subpopulations identified.

3. Our results stress the importance of using the CASA techniques to detect subtle differences in sperm motility between "fertile" and "sub-fertile" donkeys. In addition, the study of discrete subpopulations of motile spermatozoa could add additional insight into the detection of sub-fertility.

- Third Paper: ***Effect of extender and amino acid supplementation on sperm quality of cooled-preserved Andalusian donkey (Equus asinus) spermatozoa****. J. Dorado, D. Acha, I. Ortiz, M.J. Gálvez, J.J. Carrasco, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Animal Reproduction Science 2014, 146: 79-88.

1. The present study, under climatic and management conditions that existed, clearly showed a seasonal influence on the majority of characteristics of Andalusian donkey semen. As all the ejaculates were of acceptable quality, these seasonal differences did not seem to influence semen quality throughout the year.

2. Moreover, use of Gent® A resulted in maintenance of sperm motility characteristics after 72 h of cold storage to a greater extent than INRA 96® suggesting that Gent® A extender is more appropriate to preserve donkey spermatozoa.

3. The addition of glutamine, proline, and taurine improves the outcome of preservation of Andalusian donkey spermatozoa because CASA-derived variables were more desirable in semen samples cooled in Gent® A supplemented with different concentrations of amino acids than in Gent® A with no supplementation. However, a lesser increase was noticed when these aminoacids were added at a concentration of 20 mM.

4. Although it remains to be determined if the greater motility of donkey spermatozoa in diluents containing glutamine, proline, or taurine will be reflected in enhanced fertility when used for inseminations, this would allow a more efficient use of cooled donkey semen for insemination.

- Fourth Paper: ***Freezability of Andalusian donkey (Equus asinus) spermatozoa: effect of extenders and permeating cryoprotectants.*** *D. Acha, M. Hidalgo, M.J. Gálvez, I. Ortiz, J.J. Carrasco, V. Gómez-Arrones, J. Dorado.* Reproduction, Fertility and Development 2015, accepted 4 June 2015**.**

1. The use of Gent B resulted in maintenance of sperm motility characteristics after thawing to a greater extent than INRA96-EYG, suggesting that Gent B extender is more appropriate to freeze donkey spermatozoa.

2. However, the replacement of GLY by EG improved the outcome of cryopreservation of Andalusian donkey spermatozoa because it provided higher sperm quality after thawing.

3. The use of DMSO for freezing donkey semen was unsuccessful and a toxic effect is suspected.

4. DMFA at 2.5% seemed to offer the highest protection against cryoinjury during freezing and thawing. no benefit was shown when DMFA was used in combination with EG.

5. No benefit was shown when DMFA was used in combination with EG.

6. Although it remains to be determined if the better in vitro quality of Andalusian donkey spermatozoa in diluents containing different CPAs will be reflected in enhanced fertility when used for inseminations, this would allow a more efficient use of cryopreserved donkey semen for insemination. In any case, these extenders should be included in a freezability test to determine the best extender for each donkey.

Referencias Bibliográficas

Referencias Bibliográficas

Abaigar, T., Cano, M., Pickard, A.R., Holt, W.V., 2001. Use of computer-assisted sperm motility assessment and multivariate pattern analysis to characterize ejaculate quality in Mohor gazelles (*Gazella dama mhorr*): effects of body weight, electroejaculation technique and short-term semen storage. Reproduction 122, 265-273.

Abaigar, T., Holt, W.V., Harrison, R.A.P., Del Barrio, G., 1999. Sperm subpopulations in Boar (*Sus scrofa*) and Gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol. Reprod. 60, 32-41.

Ahmad, E., Ahmad, N., Naseer, Z., Aleem, M., Khan, M.S., Ashiq, M., et al., 2011. Relationship of age to body weight, scrotal circumference, testicular ultrasonograms, and semen quality in Sahiwal bulls. Trop. Anim. Health Prod. 43, 159-164.

Alvarenga, M., Papa, F., Landim-Alvarenga, F., Medeiros, A.S.L., 2005. Amides as cryoprotectants for freezing stallion semen: a review. Anim. Reprod. Sci. 89, 105-113.

Alvarenga, M.A., Keith, S.L., Graham, J.K., Landim-Alvarenga, F.C., Squires, E.L., 2000a. Alternative cryoprotectors for freezing stallion semen. In: Proc. 14th. Int. Cong. Anim. Reprod., Stockholm, Sweden, p. 73.

Alvarenga, M.A., Landim-Alvarenga, F.C., Moreira, R.M., Cesarino, M.M., 2000b. Acrosomal ultrastructure of stallion spermatozoa cryopreserved with ethylene glycol using two packaging systems. Equine Vet. J. 32, 541-545.

Alvarez, A., Serres, C., Crespo, F., Mateos, E., Gómez-Cuétaral, C., 2004. Cryopreservation of Zamorano-Leonés donkey sperm using different amides. Reprod. Domest. Anim. 39, 267.

Álvarez, A.L., Gómez-Cuétara, C., Graham, J.K., 2005. Effect of cholesterol loaded cyclodextrin and cryoprotectants on the cryosurvival of stallion sperm. Reprod. Domest. Anim. 40 (4), 375.

Álvarez, A.L., Serres, C., Torres, P., Crespo, F., Mateos, E., Gómez-Cuétara, C., 2006. Effect of cholesterol-loaded cyclodextrin on the cryopreservation of donkey spermatozoa. Anim. Reprod. Sci. 94, 89-91.

Alvarez, J.G., Storey, B.T., 1983a. Role of superoxide-dismutase in protecting rabbit spermatozoa from O_2 toxicity due to lipid-peroxidation. Biol. Reprod. 28, 1129-1136.

Alvarez, J.G., Storey, B.T., 1983b. Taurine, hypotaurine, epinephrine and albumin inhibit lipid-peroxidation in rabbit spermatozoa and protect against loss of motility. Biol. Reprod. 29, 548-555.

Alvarez, J.G., Storey, B.T., 1993. Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in cryopreserved human sperm: glycerol and other polyols as sole cryoprotectant. J. Androl. 14, 199-209.

Anchordoguy, T., Carpenter, J.F., Loomis, S.H., Crowe, J.H., 1988. Mechanisms of interaction of amino-acids with phospholipid-bilayers during freezing. Biochim. Biophys. Acta 946, 299-306.

Andrabi, S.M.H., Maxwell, W.M.C., 2007. A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim. Reprod. Sci. 99 (3), 223-243.

Argo, C.M., Smith, J.S., Kay, R.N.B., 1999. Seasonal changes of metabolism and appetite in Soay rams. Anim. Sci. 69, 191-202.

Aurich, C., 2008. Recent advances in cooled-semen technology. Anim. Reprod. Sci. 107, 268-275.

Bailey, J.L., Buhr, M.M., Robertson, L., 1994. Relationships among in vivo fertility, computer-analysed motility and in vitro Ca^{2+} flux in bovine spermatozoa. Can. J. Anim. Sci. 74, 53-58.

Ball, B.A., Vo, A., 2001. Osmotic Tolerance of Equine Spermatozoa and the Effects of Soluble Cryoprotectants on Equine Sperm Motility, Viability, and Mitochondrial Membrane Potential. J. Androl. 22, 1061-1069.

Baran, A., Demir, K., Sahin, B.E., Evecen, M., Bacinoglu, S., Alkan, S., Ileri, I.K., 2009. Short-term chilled storage of cat semen extended with and without taurine containing milk extenders. J. Anim. Vet. Adv. 8, 1367-1371.

Barlow, P., Delvigne, A., Van Dromme, J., Van Hoeck, J., Vandenbosch, K., Leroy, F., 1991. Predictive value of classical and automated sperm analysis for in-vitro fertilization. Hum. Reprod. 6, 1119-1124.

Batellier, F., Duchamp, G., Vidament, M., Arnaud, G., Palmer, E., Magistrini, M., 1998. Delayed insemination is successful with a new extender for storing fresh equine semen at 15 degrees C under aerobic conditions. Theriogenology 50, 229-236.

Batellier, F., Vidament, M., Fauquant, J., Duchamp, G., Arnaud, G., Yvon, J.M., Magistrini, M., 2001. Advances in cooled semen technology. Anim. Reprod. Sci. 68, 181-190.

Bedford, S.J., Graham, J.K., Amann, R.P., Squires, E.L., Pickett, B.W., 1995. Use of two freezing extenders to cool stallion spermatozoa to 5ºC with and without seminal plasma. Theriogenology 43, 939-953.

Bowling, E.R., Froman, D.P., Davis, A.J., Wilson, J.L., 2003. Attributes of broiler breeder males characterized by low and high sperm mobility. Poultry Sci. 82, 1796-1801.

Bravo, J.A., Montanero, J., Calero, R., Roy, T.J., 2011. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Ile de France rams. Anim. Reprod. Sci. 129, 22-29.

Brito, L.F.C., 2007. Evaluation of stallion sperm morphology. Clin. Tech. Equine Pract. 6, 249-264.

Brun, J.M., Theau-Clément, M., Bolet, G., 2002. The relationship between rabbit semen characteristics and reproductive performance after artificial insemination. Anim. Reprod. Sci. 70, 139-149.

Bucak, M.N., Atessahin, A., Varisli, O., Yüce, A., Tekin, N., Akcay, A., 2007. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen Microscopic and oxidative stress parameters after freeze-thawing process. Theriogenology 67, 1060-1067.

Bucak, M.N., Sarioezkan, S., Tuncer, P.B., Sakin, F., Atessahin, A., Kulaksiz, R., Cevik, M., 2010. The effect of antioxidants on post-thawed Angora goat (*Capra hircus ancryrensis*) sperm parameters, lipid peroxidation and antioxidant activities. Small Rumin. Res. 89, 24-30.

Bucci, D., Spinaci, M., Vallorani, C., Contri, A., Carluccio, A., Isani, G., Tamanini, C., Galeati, G., 2010. Detection and localization of GLUTs 1, 2, 3 and 5 in donkey spermatozoa. Reprod. Domest. Anim. 45, e217-e220.

Buff, S., Donze, A., Guerin, P., Guillaud, J., Fontbonne, A., Menezo, Y., 2001. Taurine and hypotaurine in spermatozoa and epididymal fluid of cats. J. Reprod. Fertil. Suppl. 57, 93-95.

Cabrita, E., Ma, S., Diogo, P., Martinez-Paramo, S., Sarasquete, C., Dinis, M.T., 2011. The influence of certain aminoacids and vitamins on post-thaw fish sperm motility, viability and DNA fragmentation. Anim. Reprod. Sci. 125, 189-195.

Canisso, I.F., Carvalho, G.R., Morel, M.D., Ker, P.G, Rodrigues, A.L, Silva, E.C, Da Silva, M.A.C., 2011. Seminal parameters and field fertility of cryopreserved donkey jack semen after insemination of horse mares. Equine Veterinary Journal 43, 179-183.

Canisso, I.F., Souza, F.A., and Ortigoza Escobar, J.M., 2008. Congelamiento de semen de burro (Equus asinus). Rev. investig. vet. Perú 19, 113-125.

Carluccio, A., Panzani, S., Contri, A., Bronzo, V., Robbe, D., Veronesi, M.C., 2013. Influence of season on testicular morphometry and semen characteristics in Martina Franca jackasses. Theriogenology 79, 502-507.

Casey, P.J., Gravance, C.G., Davis, R.O., Chabot, D.D., Liu, I.K.M., 1997. Morphometric differences in sperm head dimensions of fertile and subfertile stallions. Theriogenology 47, 575-582.

Cheng, F.P., Fazeli, A., Voorhout, W.F., Marks, A., Bevers, M.M., Colenbrander, B., 1996. Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. J. Androl. 17, 674-682.

Chenier, T., Merkies, K., Leibo, S., Plante, C., Johnson, W., 1998. Evaluation of cryoprotective agents for use in the cryopreservation of equine spermatozoa. In: AAEP Proc., p. 5-6.

Colenbrander, B., Gadella, B.M., Stout, T.A.E., 2003. The predictive value of semen analysis in the evaluation of stallion fertility. Reprod. Domest. Anim. 38, 305-311.

Contri, A., De Amicis, I., Veronesi, M.C., Faustini, M., Robbe, D., Carluccio, A., 2010. Efficiency of different extenders on cooled semen collected during long and short day length seasons in Martina Franca donkey. Anim. Reprod. Sci. 120, 136-141.

Contri, A., Gloria, A., Robbe, D., De Amicis, I., Carluccio, A., 2012a. Characteristics of donkey spermatozoa along the length of the epididymis. Theriogenology 77, 166-173.

Contri, A., Gloria, A., Robbe, D., Sfirro, M.P., Carluccio, A., 2012b. Effect of sperm concentration on characteristics of frozen-thawed semen in donkeys. Anim. Reprod. Sci. 136, 74-80.

Cortés-Gutiérrez, E., Crespo, F., Gosálvez, A., Dávila-Rodríguez, M., López-Fernández, C., Gósalvez, J., 2008. DNA fragmentation in frozen sperm of *Equus asinus*: Zamorano-Leonés, a breed at risk of extinction. Theriogenology 69, 1022-1032.

Cottorello, A.C.P., Amancio, R.C., Henry, M., Borges, I., 2002. Effect of storage temperature and extenders on "in vitro" activity of donkey spermatozoa. Theriogenology 58, 325-328.

Cremades, T., Roca, J., Rodriguez-Martinez, H., Abaigar, T., Vazquez, J.M., Martinez, E.A., 2005. Kinematic changes during the cryopreservation of boar spermatozoa. J. Androl. 26, 610-618.

DAD-IS-FAO, 2013. Information System Domestic Animal Diversity. FAO.

De Geyter, C., De Geyter, M., Koppers, B., Nieschlag, E., 1998. Diagnostic accuracy of computer-assisted sperm motion analysis. Hum. Reprod. 13, 2512-2520.

Domestic Animal Diversity Information Service (DAD-IS). http://dad.fao.org/. Accessed March 8, 2012 (Search Form. Link: Breeds; Browsing Options: Population structure and imbreeding (F) for more than one year; Choose a breed: Countries - Spain, Species - Ass, Breeds - Andaluza / Spain; Choose a year: 2011).

Dorado, J., Acha, D., Gálvez, M.J., Ortiz, I., Carrasco, J.J., Díaz, B., Gómez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2013. Sperm motility patterns in Andalusian donkey (*Equus asinus*) semen: effects of body weight, age, and semen quality. Theriogenology 79, 1100-1109.

Dorado, J., Acha, D., Ortiz, I., Gálvez, M., Carrasco, J., Gómez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2014. Effect of extender and amino acid supplementation on sperm quality of cooled-preserved Andalusian donkey (*Equus asinus*) spermatozoa. Anim. Reprod. Sci. 146, 79-88.

Dorado, J., Acha, D., Ortiz, I., Gálvez, M.J., Carrasco, J.J., Díaz, B., Gómez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2013. Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (Equus asinus). Anim. Reprod. Sci. 143(1), 64-71.

Dorado, J., Gálvez M.J., Murabito, M.R., Muñoz-Serrano, A., Hidalgo, M., 2011. Identification of sperm subpopulations in canine ejaculates: effects of cold storage and egg yolk concentration. Anim. Reprod. Sci. 127, 106-113.

Dorado, J., Hidalgo, M., Muñoz, A., Rodríguez, I., 2009. Assessment of goat semen freezability according to the spermatozoa characteristics from fresh and frozen samples. Anim. Reprod. Sci. 112, 150-157.

Dorado, J., Molina, I., Muñoz-Serrano, A., Hidalgo, M., 2010a. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Florida goats. Theriogenology 74, 795-804.

Dorado, J., Muñoz-Serrano, A., Hidalgo, M., 2010b. The effect of cryopreservation on goat semen characteristics related to sperm freezability. Anim. Reprod. Sci. 121, 115-123.

Dorado, J., Rodríguez, I., Hidalgo, M., 2007. Cryopreservation of goat spermatozoa: Comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. Theriogenology 68, 168-177.

Dowsett, K.F., Knott, L.M., 1996. The influence of age and breed on stallion semen. Theriogenology 46, 397-412.

England, G.C.W., Ponzio, P., 1996. Comparison of the quality of frozen-thawed and cooled-rewarmed dog semen. Theriogenology 46, 165-171.

Farrell, P.B., Presicce, G.A., Brockett, C.C., Foote, R.H., 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology 49, 871-879.

Flores, E., Fernández-Novell, J.M., Peña, A., Rodríguez-Gil, J.E., 2009. The degree of resistance to freezing-thawing is related to specific changes in the structures of motile sperm subpopulations and mitochondrial activity in boar spermatozoa. Theriogenology 72, 784-797.

Flores, E., Taberner, E., Rivera, M.M., Peña, A., Rigau, T., Miró, J., Rodríguez-Gil, J.E., 2008. Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates. Theriogenology 70, 936-945.

Froman, D.P., Kirby, J.D., 2005. Sperm mobility: Phenotype in roosters (Gallus domesticus) determined by mitochondrial function. Biol. Reprod. 72, 562-567.

Funahashi, H., Sano, T., 2005. Select antioxidants improve the function of extended boar semen stored at 10 ºC. Theriogenology 63, 1605-1616.

Gadea, J., 2005. Sperm factors related to in vitro and in vivo porcine fertility. Theriogenology 63, 431-444.

Gallon, F., Marchetti, C., Jouy, N., Marchetti, P., 2006. The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. Fertil. Steril. 86, 1526-1530.

Gastal, M.O., Henry, M., Beker, A.R., Gastal, E.L., 1997. Effect of ejaculation frequency and season on donkey jack semen. Theriogenology 47, 627-638.

Gastal, M.O., Henry, M., Beker, A.R., Gastal, E.L., Gonçalves, A., 1996. Sexual behavior of donkey jacks: influence of ejaculatory frequency and season. Theriogenology 46, 593-603.

Gil, M.A., Roca, J., Cremades, T., Hernández, M., Vázquez, J.M., Rodríguez-Martínez, H., Martínez, E.A., 2005. Does multivariate analysis of post-thaw sperm characteristics accurately estimate in vitro fertility of boar individual ejaculates? Theriogenology 64, 305-316.

Gillan, L., Kroetsch, T., Chis Maxwell, W.M., Evans, G., 2008. Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. Anim. Reprod. Sci. 103, 201-214.

Glazar, A.I., Mullen, S.F., Liu, J., Benson, J.D., Critser, J.K., Squires, E.L., Graham, J.K., 2009. Osmotic tolerance limits and membrane permeability characteristics of stallion spermatozoa treated with cholesterol. Cryobiology 59, 201-206.

Gloria, A., Contri, A., De Amicis, I., Robbe, D., Carluccio, A., 2011. Differences between epididymal and ejaculated sperm characteristics in donkey. Anim. Reprod. Sci. 128, 117-122.

Gomendio, M., Roldan, E.R.S., 2008. Implications of diversity in sperm size and function for sperm competition and fertility. Int. J. Dev. Biol. 52, 439-447.

Gomes, G., Jacob, J., Medeiros, A., Papa, F., Alvarenga, M., 2002. Improvement of stallion spermatozoa preservation with alternative cryoprotectants for the Mangalarga Marchador breed. Theriogenology 58, 277-279.

Gomez-Cuetara, C., Alvarez, A.L., Rodriguez, A., Santiago, I., Mateos, E., Serres, C., 2004. Refrigeration of Zamorano-Leones donkey semen collected with an open-ended artificial vagina. Reprod. Domest. Anim. 39, 267.

Goodson, S.G., Zhang, Z., Tsuruta, J.K., Wang, W., O'Brien, D.A., 2011. Classification of mouse sperm motility patterns using an automated multiclass support vector machines model. Biol. Reprod. 84, 1207-1215.

Harald, S., 2009. Semen evaluation, in: Samper, J.C. (Eds.), Equine breeding management and artificial insemination, Saunders Elsevier, St. Louis, USA, pp. 57-74.

Harrison, R.A.P., 1996. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. Reprod. Fertil. Dev. 8, 581-594.

Heinz, K.A., Glofcheski, D.J., Lepock, J.R., Kruuv, J., 1990. Mechanism of freeze-thaw damage to liver alcohol-dehydrogenase and protection by cryoprotectants and amino acids. Cryobiology 27, 521-538.

Henry, M., Lodi, L.D., Gastal, M.M.F.O., 1998. Sexual behaviour of domesticated donkeys (*Equus asinus*) breeding under controlled or free range management systems. Appl. Anim. Behav. Sci. 60, 263-276.

Henry, M., Snoeck, P., Cottorello, A., 2002. Post-thaw spermatozoa plasma membrane integrity and motility of stallion semen frozen with different cryoprotectants. Theriogenology 58, 245-248.

Hoffmann, N., Morandini, C., Sieme, H., 2010. Determination of the optimal concentration of cryoprotectant agent for freezing stallion spermatozoa. Anim. Reprod. Sci. 121, S160-S162.

Hoffmann, N., Oldenhof, H., Morandini, C., Rohn, K., Sieme, H., 2011. Optimal concentrations of cryoprotective agents for semen from stallions that are classified 'good' or 'poor' for freezing. Anim. Reprod. Sci. 125, 112-118.

Hoflack, G., Van Soom, A., Maes, D., de Kruif, A., Opsomer, G., Duchateau, L., 2006. Breeding soundness and libido examination of Belgian Blue and Holstein Friesian artificial insemination bulls in Belgium and The Netherlands. Theriogenology 66, 207-216.

Holt, C., Holt, W.V., Moore, H.D.M., Reed, H.C.B., Curnock, R.M., 1997. Objectivily measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. J. Androl. 18, 312-323.

Holt, W.V., 1996. Can we predict fertility rates? Making sense of sperm motility. Reprod. Domest. Anim. 31, 17-24.

Holt, W.V., Pickard A.R., 1999. Role of reproductive technologies and genetic resource banks in animal conservation. Rev. Reprod. 4, 143-150.

Iguer-ouada, M., Verstegen, J.P., 2001. Evaluation of the "Hamilton thorn computer-based automated system" for dog semen analysis. Theriogenology 55, 733-749.

Ijaz, A., Ducharme, R., 1995. Effect of various extenders and taurine on survival of stallion sperm cooled to 5ºC. Theriogenology 44, 1039-1050.

Janett, F., Sacher, K., Hassig, M., Thun, R., 2012. Quality of raw and of cold-stored semen in icelandic stallions. J. Equine Vet. Sci. 32, 590-595.

Janett, F., Thun, R., Bettschen, S., Burger, D., Hassig, M., 2003. Seasonal changes of semen quality and freezability in Franches–Montagnes stallions. Anim. Reprod. Sci. 77, 213-221.

Jasko, D.J., Lein, D.H., Foote, R.H., 1990. Determination of the relationship between sperm morphologic classifications and fertility in stallions: 66 cases (1987-1988). J. Am. Vet. Med. Assoc. 197, 389-394.

Jasko, D.J., Little, T.V., Lein, D.H., Foote, R.H., 1992. Comparison of spermatozoal movement and semen characteristics with fertility in stallions: 64 cases (1987-1988). J. Am. Vet. Med. Assoc. 200, 979-985.

Jasko, D.J., Moran, D.M., Farlin, M.E., Squires, E.L., 1991. Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. Theriogenology 35, 1059-1067.

Jepsen, R.J., Evans, L.E., Youngs, C.R., 2010. Use of direct thaw Insemination to establish pregnancies with frozen-thawed semen from a standard jack. J. Equine Vet. Sci. 30, 651-656.

Kanuga, M.K., Drew, R.E., Wilson-Leedy, J.G., Ingermann, R.L., 2012. Subpopulation distribution of motile sperm relative to activation medium in steelhead (Oncorhynchus mykiss). Theriogenology 77, 916-925.

Kastelic, J.P., Thundathil, J.C., 2008. Breeding soundness evaluation and semen analysis for predicting bull fertility. Reprod. Domest. Anim. 43, 368-373.

Kenney, R.M., Bergman, R.V., Cooper, W.L., Morse, G.W., 1975. Minimal contamination techniques for breeding mares: techniques and preliminary findings. In: Proc. 21st Annu. Conv. Am. Assoc. Equine Pract., Lexington, p. 327.

Khlifaoui, M., Battut, I., Bruyas, J.F., Chatagnon, G., Trimeche, A., Tainturier, D., 2005. Effects of glutamine on post-thaw motility of stallion spermatozoa: an approach of the mechanism of action at spermatozoa level. Theriogenology 63, 138-149.

Kruuv, J., Glofcheski, D.J., 1990. Survival of mammalian-cells following multiple freeze-thaw cycles. II. Independence of cryoprotection using glutamine with dimethyl-sulfoxide, hydroxyethyl starch, propylene-glycol or glycerol. Cryo-Letters 11, 215-226.

Kuisma, P., Andersson, M., Koskinen, E., Katila, T., 2006. Fertility of frozen-thawed stallion semen cannot be predicted by the currently used laboratory methods. Acta Vet. Scand. 48.

Lahnsteiner, F., Radner, M., 2010. Lysozyme activities and immunoglobulin concentrations in seminal plasma and spermatozoa of different teleost species and indications on its significance for sperm function. Theriogenology 74, 246-254.

Lalonde, R.J., Lepock, J.R., Kruuv, J., 1991. Site of freeze-thaw damage and cryoprotection by amino acids of the calcium ATPase of sarcoplasmic reticulum. Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. 1079, 128-138.

Larsen, L., Scheike, T., Jensen, T.K., Bonde, J.P., Ernst, E., Hjollund, N.H., Zhou, Y., Skakkebæk, N.E., Giwercman, A., Bonde, J.P.E., Hjollund, N.H.I., Henriksen, T.B., Kolstad, H.A., Anderson, A.M., Olsen, J., 2000. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. Hum. Reprod. 15, 1562-1567.

Love, C.C., 2011. Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology 76, 547-557.

Love, C.C., Varner, D.D., Thompson, J.A., 2000. Intra- and inter-stallion variation in sperm morphology and their relationship with fertility. J. Reprod. Fertil. Suppl., 93-100.

Macías García, B., Ortega Ferrusola, C., Aparicio, I.M., Miró-Morán, A., Morillo Rodriguez, A., Gallardo Bolaños, J.M., González Fernández, L., Balao da Silva, C.M., Rodríguez Martínez, H., Tapia, J.A., Peña, F.J., 2012. Toxicity of glycerol for the stallion spermatozoa: effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. Theriogenology 77, 1280-1289.

Macleod, I.C., Irvine, D.S., 1995. Andrology: The predictive value of computer-assisted semen analysis in the context of a donor insemination programme. Hum. Reprod. 10, 580-586.

Madison, R.J., Evans, L.E., Youngs, C.R., 2013. The effect of 2-Hydroxypropyl-β-Cyclodextrin on post-thaw parameters of cryopreserved jack and stallion semen. J. Equine Vet. Sci. 33, 272-278.

Magistrini, M., Chanteloube, P., Palmer, E., 1987. Influence of season and frequency of ejaculation on production of stallion semen for freezing. J. Reprod. Fertil. Suppl. 35, 127-133.

Malmgren, L., 1992. Sperm morphology in stallions in relation to fertility. Acta Vet. Scand. Suppl. 88, 39-47.

Mantovani, R., Rota, A., Falomo, M.E., Bailoni, L., Vincenti, L., 2002. Comparison between glycerol and ethylene glycol for the cryopreservation of equine spermatozoa: semen quality assessment with standard analyses and with the hypoosmotic swelling test. Reprod. Nutr. Dev. 42, 217-226.

Marsh, D.C., Belzer, F.O., Southard, J.H., 1990. Hypothermic preservation of hepatocytes. II. Importance of Ca^2 and amino acids. Cryobiology 27, 1-8.

Martinez-Pastor, F., Garcia-Macias, V., Alvarez, M., Herraez, P., Anel, L., De Paz, P., 2005. Sperm subpopulations in Iberian red deer epididymal sperm and their changes through the cryopreservation process. Biol. Reprod. 72, 316-327.

Martinez-Pastor, F., Johannisson, A., Gil, J., Kaabi, M., Anel, L., Paz, P., Rodriguez-Martinez, H., 2004. Use of chromatin stability assay, mitochondrial stain JC-1, and

fluorometric assessment of plasma membrane to evaluate frozen-thawed ram semen. Anim. Reprod. Sci. 84, 121-133.

Martínez-Pastor, F., Tizado, E.J., Garde, J.J., Anel, L., de Paz, P., 2011. Statistical series: opportunities and challenges of sperm motility subpopulation analysis. Theriogenology 75, 783-795.

Martins-Bessa, A., Rocha, A., Mayenco-Aguirre, A., 2006. Comparing ethylene glycol with glycerol for cryopreservation of canine semen in egg-yolk TRIS extenders. Theriogenology 66, 2047-2055.

Medeiros, A., Gomes, G., Carmo, M., Papa, F., Alvarenga, M., 2002. Cryopreservation of stallion sperm using different amides. Theriogenology 58, 273-276.

Meizel, S., Lui, C.W., Working, P.K., Mrsny, R.J., 1980. Taurine and hypotaurine: their effects on motility, capacitation and the acrosome reaction of hamster sperm invitro and their presence in sperm and reproductive tract fluids of several mammals. Dev. Growth Differ. 22, 483-494.

Mello, S.L.V., Henry, M., Souza, M.C., Oliveira, S.M.P., 2000. Effect of split ejaculation and seminal extenders on longevity of donkey semen preserved at 5ºC. Arq. Bras. Med. Vet. Zootec. 52, 372-378.

Michael, A.J., Alexopoulos, C., Pontiki, E.A., Hadjipavlou-Litina, D.J., Saratsis, P., Ververidis, H.N., Boscos, C.M., 2009. Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. Anim. Reprod. Sci. 112, 119-135.

Miró, J., Lobo, V., Quintero-Moreno, A., Medrano, A., Peña, A., Rigau, T., 2005. Sperm motility patterns and metabolism in Catalonian donkey semen. Theriogenology 63, 1706-1716.

Miró, J., Taberner, E., Rivera, M., Peña, A., Medrano, A., Rigau, T., Peñalba, A., 2009. Effects of dilution and centrifugation on the survival of spermatozoa and the structure of

motile sperm cell subpopulations in refrigerated Catalonian donkey semen. Theriogenology 72, 1017-1022.

Miró, J., Vilés, K., Fernández, M., Fábregas, N., Soares, J., García, W., 2011. Induced acute endometritis by frozen semen insemination in donkey. Reprod. Domest. Anim. 46, 130.

Molinia, F., Evans, G., Maxwell, W., 1994. Incorporation of penetrating cryoprotectants in diluents for pellet-freezing ram spermatozoa. Theriogenology 42, 849-858.

Moore, A., Squires, E., Bruemmer, J., Graham, J., 2006. Effect of cooling rate and cryoprotectant on the cryosurvival of equine spermatozoa. J. Equine Vet. Sci. 26, 215-218.

Moore, H.D.M., Akhondi, M.A., 1996. Fertilizing capacity of rat spermatozoa is correlated with decline in straight-line velocity measured by continuous computer-aided sperm analysis: epididymal rat spermatozoa from the proximal cauda have a greater fertilizing capacity in vitro than those from the distal cauda or vas deferens. J. Androl. 17, 50-60.

Morillo Rodríguez, A., Ortega Ferrusola, C., Macias García, B., Morrell, J., Rodriguez Martinez, H., Tapia, J., Peña, F., 2011. Freezing stallion semen with the new Cáceres extender improves post thaw sperm quality and diminishes stallion-to-stallion variability. Anim. Reprod. Sci. 127, 78.

Morrell, J.M., Johannisson, A., Dalin, A.M., Hammar, L., Sandebert, T., Rodriguez-Martinez, H., 2008. Sperm morphology and chromatin integrity in Swedish warmblood stallions and their relationship to pregnancy rates. Acta Vet. Scand., 50.

Muiño, R., Peña, A.I., Rodríguez, A., Tamargo, C., Hidalgo, C.O., 2009. Effects of cryopreservation on the motile sperm subpopulations in semen from Asturiana de los Valles bulls. Theriogenology 72, 860-868.

Muiño, R., Tamargo, C., Hidalgo, C.O., Peña, A.I., 2008. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: effects of cryopreservation and between-bull variation. Anim. Reprod. Sci. 109, 27-39.

Nie, G.J., Wenzel, J.G.W., Johnson, K.E., 2002. Comparison of pregnancy outcome in mares among methods used to evaluate and select spermatozoa for insemination. Anim. Reprod. Sci. 69, 211-222.

Novak, S., Smith, T.A., Paradis, F., Burwash, L., Dyck, M.K., Foxcroft, G.R., Dixon, W.T., 2010. Biomarkers of in vivo fertility in sperm and seminal plasma of fertile stallions. Theriogenology 74, 956-967.

Núñez-Martínez, I., Moran, J.M., Peña, F.J., 2006a. A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: changes after cryopreservation. Reprod. Domest. Anim. 41, 408-415.

Núñez-Martínez, I., Morán, J.M., Peña, F.J., 2006b. Two-step cluster procedure after principal component analysis identifies sperm subpopulations in canine ejaculates and its relation to cryoresistance. J. Androl. 27, 596-603.

O' Meara, C.M., Hanrahan, J.P., Prathalingam, N.S., Owen, J.S., Donovan, A., Fair, S., Ward, F., Wade, M., Evans, A.C.O., Lonergan, P., 2008. Relationship between in vitro sperm functional tests and in vivo fertility of rams following cervical artificial insemination of ewes with frozen-thawed semen. Theriogenology 69, 513-522.

Oldenhof, H., Friedel, K., Sieme, H., Glasmacher, B., Wolkers, W.F., 2010. Membrane permeability parameters for freezing of stallion sperm as determined by Fourier transform infrared spectroscopy. Cryobiology 61, 115-122.

Olds-Clarke, P., 1996. How does poor motility alter sperm fertilizing ability? J. Androl. 17, 183-186.

Oliveira, J.V., Alvarenga, M.A., Melo, C.M., Macedo, L.M., DellÁqua Jr., J.A., Papa, F.O., 2006. Effect of cryoprotectant on donkey semen freezability and fertility. Anim. Reprod. Sci. 94, 82-84.

Ortiz, I., Dorado, J., Acha, D., Gálvez, M.J., Urbano, M., Hidalgo, M., 2015. Colloid single-layer centrifugation improves post-thaw donkey (*Equus asinus*) sperm quality and is related to ejaculate freezability. Reprod. Fertil. Dev. 27 (2), 332-340.

Parlevliet, J.M., Colenbrander, B., 1999. Prediction of first season stallion fertility of 3-year-old Dutch Warmbloods with prebreeding assessment of percentage of morphologically normal live sperm. Equine Vet. J. 31, 248-251.

Peña Martínez, A.I., 2004. Canine fresh and cryopreserved semen evaluation. Anim. Reprod. Sci. 82-83, 209-224.

Peña, F., Macías García, B., Samper, J., Aparicio, I., Tapia, J., Ortega Ferrusola, C., 2011. Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? Theriogenology 76, 1177-1186.

Petrunkina, A.M., Waberski, D., Günzel-Apel, A.R., Töpfer-Petersen, E., 2007. Determinants of sperm quality and fertility in domestic species. Reproduction 134, 3-17.

Phetudomsinsuk, K., Sirinarumitr, K., Choothesa, A., Suthanmapinunt, P., Kornkaewrat, K., Laikul, A., Amornsak, S., Pinyopummin, A., 2009. Freezability of Thai native crossbred horse semen in different extenders. Thai J. Vet. Med. 39, 105-114.

Pickett, B.W., Sullivan, J.J., Byers, W.W., Pace, M.M., Remmenga, E.E., 1975. Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. Fertil. Steril. 26, 167-174.

Pillet, E., Batellier, F., Duchamp, G., Furstoss, V., Le Vern, Y., Kerboeuf, D., Desherces, S., Schmitt, E., Magistrini, M., 2008. High fertility rates with stallion sperm cryopreserved in INRA96® based extender are not predicted by in vitro parameters. Anim. Reprod. Sci. 107, 339-340.

Quartuccio, M., Marino, G., Zanghì, A., Garufi, G., Cristarella, S., 2011. Testicular volume and daily sperm output in Ragusano donkeys. J. Equine Vet. Sci. 31, 143-146.

Quintero-Moreno, A., Miró, J., Teresa Rigau, A., Rodríguez-Gil, J.E., 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 59, 1973-1990.

Quintero-Moreno, A., Rigau, T., Rodríguez-Gil, J.E., 2004. Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. Theriogenology 61, 673-690.

Quintero-Moreno, A., Rigau, T., Rodríguez-Gil, J.E., 2007. Multivariate cluster analysis regression procedures as tools to identify motile sperm subpopulations in rabbit semen and to predict semen fertility and litter size. Reprod. Domest. Anim. 42, 312-319.

Ramón, M., Martínez-Pastor, F., García-Álvarez, O., Maroto-Morales, A., Soler, A.J., Jiménez-Rabadán, P., et al., 2012. Taking advantage of the use of supervised learning methods for characterization of sperm population structure related with freezability in the Iberian red deer. Theriogenology 77, 1661-1672.

Rigau, T., Farré, M., Ballester, J., Mogas, T., Pea, A., Rodríguez-Gil, J.E., 2001. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology 56, 801-815.

Rijsselaere, T., Maes, D., Hoflack, G., De Kruif, A., Van Soom, A., 2007. Effect of body weight, age and breeding history on canine sperm quality parameters measured by the Hamilton-Thorne analyser. Reprod. Domest. Anim. 42, 143-148.

Rivera, M.M., Quintero-Moreno, A., Barrera, X., Rigau, T., Rodríguez-Gil, J.E., 2006. Effects of constant, 9 and 16-h light cycles on sperm quality, semen storage ability and motile sperm subpopulations structure of boar semen. Reprod. Domest. Anim. 4, 386-393.

Rodríguez-Martínez, H., 2003. Laboratory semen assessment and prediction of fertility: still utopia? Reprod. Domest. Anim. 38, 312-318.

Rota, A., Magelli, C., Panzani, D., Camillo, F., 2008. Effect of extender, centrifugation and removal of seminal plasma on cooled-preserved Amiata donkey spermatozoa. Theriogenology 69, 176-185.

Rota, A., Panzani, D., Sabatini, C., Camillo, F., 2012. Donkey jack (*Equus asinus*) semen cryopreservation: Studies of seminal parameters, post breeding inflammatory response, and fertility in donkey jennies. Theriogenology 78, 1846-1854.

Rudolph, A.S., Crowe, J.H., 1985. Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. Cryobiology 22, 367-377.

Sanchez Partida, L.G., Maxwell, W.M.C., Paleg, L.G., Setchell, B.P., 1992. Proline and glycine betaine in cryoprotective diluents for ram spermatozoa. Reprod. Fertil. Dev. 4, 113-118.

Sánchez-Partida, L.G., Windsor, D.P., Eppleston, J., Setchell, B.P., Maxwell, W.M.C., 1999. Fertility and its relationship to motility characteristics of spermatozoa in ewes after cervical, transcervical, and intrauterine insemination with frozen-thawed ram semen. J. Androl. 20, 280-288.

Santos, G.F., Henry, M., Sampaio, I.B.M., Gastal, E.L., 1995. Effect of cooling system and rate of cooling on sperm quality of donkey semen preserved at 5 °C. Biol. Reprod. Monogr. 1, 761-767.

SAS, 2002. SAS/STAT TM Software. SAS Institute Inc., Cary, NC, USA.

Schwartz, D., Mayaux, M.J., Spira, A., 1983. Semen characteristics as a function of age in 833 fertile men. Fertil. Steril. 39, 530-535.

Scott, M.A., 2000. A glimpse at sperm function in vivo: Sperm transport and epithelial interaction in the female reproductive tract. Anim. Reprod. Sci. 60-61, 337-348.

Serres, 2003. Evaluación y Conservación del semen en el asno Zamorano-Leonés. Tesis Doctoral.

Serres, C., Álvarez, A., Rodriguez, A., Santiago, I., Mateos, E., Gómez-Cuétaral, C., 2004a. Effect of extender and glycerol concentration on cryopreservation of semen from the Zamorano-Leonés donkey. Reprod. Domest. Anim. 39, 267.

Serres, C., Alvarez, A.L., Rodriguez, A., Crespo, F., Mateos, E., Gomez-Cuetara, C., 2004b. Use of ethylene glycol in the cryopreservation of Zamorano-Leones donkey semen. In Proc. 15th Int. Cong. Anim. Reprod., Porto Seguro, Brazil, p. 512.

Serres, C., Gutiérrez-Cepeda, L., Gero, S., Gómez, V., Blázquez, J.C., Crespo, F., 2014. Fertility rates in jennies by new semen processing techniques. J. Equine Vet. Sci. 34 (1), 193-194.

Serres, C., Rodriguez, A., Alvarez, A.L., Santiago, I., Gabriel, J., Gomez-Cuetara, C., Mateos, E., 2002. Effect of centrifugation and temperature on the motility and plasma membrane integrity of Zamorano-Leones donkey semen. Theriogenology 58, 329-332.

Silva, A.R., Cardoso, R.d.C.S., Silva, L.D.M., Chirinéa, V.H., Lopes, M.D., Souza, F.F., 2006. Prognostic value of canine frozen-thawed semen parameters on in vitro sperm-oocyte interactions. Theriogenology 66, 456-462.

Smits, K., Hoogewijs, M., Woelders, H., Daels, P., Van Soom, A., 2012. Breeding or assisted reproduction? Relevance of the horse model applied to the conservation of endangered equids. Reprod. Domest. Anim. 47, 239-248.

Snoeck, P., Cottorello, A., Henry, M., 2012. Viability and fertility of stallion semen frozen with ethylene glycol and acetamide as a cryogenic agent. Anim. Reprod. Sci. 9, 00-00.

Squires, E.L., Keith, S.L., Graham, J.K., 2004. Evaluation of alternative cryoprotectants for preserving stallion spermatozoa. Theriogenology 62, 1056-1065.

Storey, K.B., Storey, J.M., 1990. Frozen and alive. Sci. Am. 263, 92-97.

Suarez, S.S., Katz, D.F., Owen, D.H., Andrew, J.B., Powell, R.L., 1991. Evidence for the function of hyperactivated motility in sperm. Biol. Reprod. 44, 375-381.

Trimeche, A., Renard, P., Tainturier, D., 1998. A procedure for Poitou jackass sperm cryopreservation. Theriogenology 50, 793-806.

Trimeche, A., Yvon, J.M., Vidament, M., Palmer, E., Magistrini, M., 1999. Effects of glutamine, proline, histidine and betaine on post-thaw motility of stallion spermatozoa. Theriogenology 52, 181-191.

Van den Bergh, M., Emiliani, S., Biramane, J., Vannin, A.S., Englert, Y., 1998. A first prospective study of the individual straight line velocity of the spermatozoon and its influences on the fertilization rate after intracytoplasmic sperm injection. Hum. Reprod. 13, 3103-3107.

Verstegen, J., Iguer-Ouada, M., Onclin, K., 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57, 149-179.

Vidament, M., Daire, C., Yvon, J., Doligez, P., Bruneau, B., Magistrini, M., Ecot, P., 2002. Motility and fertility of stallion semen frozen with glycerol and/or dimethyl formamide. Theriogenology 58, 249-251.

Vidament, M., Magistrini, M., Palmer, E., Clement, F., 2000. Equine artificial insemination in French National Studs. Reprod. Domest. Anim. 35, 61-66.

Vidament, M., Vincent, P., Martin, F., Magistrini, M., Blesbois, E., 2009. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. Anim. Reprod. Sci. 112, 22-35.

Vidament, M., Vincent, P., Yvon, J., Bruneau, B., Martin, F., 2005. Glycerol in semen extender is a limiting factor in the fertility in asine and equine species. Anim. Reprod. Sci. 89, 302.

Vilés, K., Rabanal, R., Rodríguez-Prado, M., Miró, J., 2013. Effect of ketoprofen treatment on the uterine inflammatory response after AI of jennies with frozen semen. Theriogenology 79, 1019-1026.

Voss, J.L., Pickett, B.W., Squires, E.L., 1981. Stallion spermatozoal morphology and motility and their relationships to fertility. J. Am. Vet. Med. Assoc. 178, 287-289.

Vulcano, G.J., Moses, D.F., Valcárcel, A., De Las Heras, M.A., 1998. A lineal equation for the classification of progressive and hyperactive spermatozoa. Math. Biosci. 149, 77-93.

Watson, P.F., 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fertil. Dev. 7, 871-891.

Wildt, D.E., Ellis, S., Howard, J.G., 2001. Linkage of reproductive sciences: from 'quick fix' to 'integrated' conservation. J. Reprod. Fertil. Suppl. 57, 295-307.

Lista de Publicaciones

Lista de Publicaciones

Informe con el factor de impacto y cuartil del Journal Citation Reports (SCI y/o SSCI) del área en el que se encuentran las publicaciones presentadas

Primera Publicación:

Dorado, J., Acha, D., Gálvez, M.J., Ortiz, I., Carrasco, J.J., Díaz, B., Gómez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2013. Sperm motility patterns in Andalusian donkey (*Equus asinus*) semen: Effects of body weight, age, and semen quality. *Theriogenology* 79, 1100-1109.

- Base de datos internacional en la que está indexada: Journal Citation Report (JCR)
- Área temática en la Base de datos de referencia: Veterinary Sciences
- Índice de impacto de la revista en el año de publicación del artículo: 1,845
- Lugar que ocupa / Nº de revistas del área temática:17/132

Segunda Publicación:

Dorado, J., Acha, D., Ortiz, I., Gálvez, M.J, Carrasco, J.J., Díaz, B., Gómez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2013. Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (*Equus asinus*). *Anim. Reprod. Sci.* 143, 64-71.

- Base de datos internacional en la que está indexada: Journal Citation Report (JCR)
- Área temática en la Base de datos de referencia: Agriculture, Dairy and Animal Science
- Índice de impacto de la revista en el año de publicación del artículo: 1,581
- Lugar que ocupa / Nº de revistas del área temática: 11/52

Tercera Publicación:

Dorado, J., Acha, D., Ortiz, I., Gálvez, M.J., Carrasco, J.J., Gómez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2014. Effect of extender and amino acid supplementation on sperm quality of cooled-preserved Andalusian donkey (*Equus asinus*) spermatozoa. *Anim. Reprod. Sci.* 146, 79-88.

- Base de datos internacional en la que está indexada: Journal Citation Report (JCR)
- Área temática en la Base de datos de referencia: Agriculture, Dairy and Animal Science
- Índice de impacto de la revista en el año de publicación del artículo: 1,511
- Lugar que ocupa / Nº de revistas del área temática: 11/55

Cuarta Publicación:

Acha, D., Hidalgo, M., Gálvez, M.J., Ortiz, I., Carrasco, J.J., Gómez-Arrones, V., Dorado, J., 2015. Freezability of Andalusian donkey (*Equus asinus*) spermatozoa: effect of extenders and permeating cryoprotectants. *Reprod. Fertil. Dev. accepted 4 June 2015*.

- Base de datos internacional en la que está indexada: Journal Citation Report (JCR)
- Área temática en la Base de datos de referencia: Zoology
- Índice de impacto de la revista en el año de publicación del artículo: 2,400
- Lugar que ocupa / Nº de revistas del área temática: 18/153

Producción Científica Derivada

Producción Científica Derivada

Otras aportaciones científicas derivadas directamente de la tesis doctoral

Otros publicaciones en revistas indexadas en el JCR:

Acha, D., Hidalgo, M., Ortiz, I., Demyda, S., Herrera, M., Calero, R., Carrasco, J., Díaz, B., Gómez-Arrones, V., Dorado, J., 2011. Sperm motility patterns in andalusian donkey semen. *Reprod. Domest. Anim.* 46 (s3), 79.

Acha, D., Hidalgo, M., Galvez, M.J., Ortiz, I., Carrasco, J., Gómez-Arrones, V., Calero, R., Urbano, M., Ramírez, L., Demyda-Peyras, S., Dorado, J., 2013. Effect of cooling rate on sperm quality of cryopreserved Andalusian donkey spermatozoa. *Reprod. Domest. Anim.* (s1), 72.

Acha, D., Hidalgo, M., Galvez, M.J., Ortiz, I., Herrera, M., Calero, R., Carrasco, J., Díaz, B., Gómez-Arrones, V., Dorado, J., 2012. Kinematic changes after cryopreservation of Andalusian donkey spermatozoa. *Reprod. Domest. Anim.* 47 (s3), 113.

Acha, D., Hidalgo, M., Galvez, M.J., Ortiz, I., Herrera, M., Calero, R., Carrasco, J., Díaz, B., Gómez-Arrones, V., Dorado, J., 2012. Effect of amino acids supplementation on cooled-preserved Andalusian donkey spermatozoa. *Reprod. Domest. Anim.* 47 (s3), 113.

Dorado, J., Ortiz, I., **Acha, D.**, Galvez, M.J., Carrasco, J.J., Gómez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2014. Dimethyl sulfoxide added to freezing extender does not improve the post-thaw quality of Andalusian donkey semen. *Reprod. Domest. Anim.* 49 (s3), 65-66.

Contribuciones a Congresos:

Dorado, J., Ortiz, I., **Acha, D.**, Galvez, M.J., Carrasco, J.J., Gómez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2014. Dimethyl sulfoxide added to freezing extender does not improve the post-thaw quality of Andalusian donkey semen. 18th Ann. Conf. Eur. Soc. Domest. Anim. Reprod. (ESDAR). Helsinki, Finland, September 10-13, 2014.

Acha, D., Hidalgo, M., Galvez, M.J., Ortiz, I., Herrera, M., Calero, R., Carrasco, J., Díaz, B., Gómez-Arrones, V., Dorado, J., 2012. Effect of amino acids supplementation on cooled-preserved Andalusian donkey spermatozoa. XI Cong. Int. Asoc. Esp. Reprod. Anim. Córdoba, España, 13-16 de Junio de 2012.

Acha, D., Hidalgo, M., Galvez, M.J., Ortiz, I., Carrasco, J., Gómez-Arrones, V., Calero, R., Urbano, M., Ramírez, L., Demyda-Peyras, S., Dorado, J., 2013. Effect of cooling rate on sperm quality of cryopreserved Andalusian donkey spermatozoa. 17th Ann. Conf. Eur. Soc. Domest. Anim. Reprod. (ESDAR). Bologna, Italy, September 12-14, 2013.

Acha, D., Hidalgo, M., Demyda, S., Ortiz, I., Calero, R., Carrasco, J.J., Diaz, B., Gómez-Arrones, V., Dorado, J., 2011. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Andalusian donkeys. 15th Ann. Conf. Eur. Soc. Domest. Anim. Reprod. (ESDAR). Belek-Antalya, Turkey, September, 15-17, 2011.

Acha, D., 2013. Influencia de la rampa de enfriamiento en la crioconservación del semen de asno andaluz: Resultados preliminares. III Cong. Inv. Univ. Córdoba. Córdoba, España, 9-10 de Abril de 2013.

Acha, D., Hidalgo, M., Galvez, M.J., Ortiz, I., Herrera, M., Calero, R., Carrasco, J., Díaz, B., Gómez-Arrones, V., Dorado, J., 2012. Kinematic changes after cryopreservation of Andalusian donkey spermatozoa. XI Cong. Int. Asoc. Esp. Reprod. Anim. Córdoba, España, 13-16 de Junio de 2012.

Acha, D., Hidalgo, M., Gálvez, M.J., Ortiz, I., Urbano, M., Alcaraz, L., Duarte, N., Portero, J.M., Dorado, J., 2011. Optimización de la metodología de congelación de esperma del asno Andaluz. Inv. Vet. y CYTA. Córdoba, España, 7-11 de Noviembre de 2011.