Tecnologies reproductives aplicades a la conservació del burro Català

Reproductive strategies applied for Catalonian donkey conservation

Ester Taberner Brugué
Director de Tesi: Jordi Miró Roig
Facultat de Veterinària
Departament de Medicina i Cirurgia Animals

Tesi doctoral / Doctoral thesis
Universitat Autònoma de Barcelona
2010

Jordi Miró Roig, professor titular del Departament de Medicina i Cirurgia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona

CERTIFICA

Que la tesi titulada "**Tecnologies reproductives aplicades a la conservació del burro Català**" presentada per la Ester Taberner Brugué per optar al grau de Doctor en Veterinària s'ha realitzat sota la meva direcció i, considerant-la acabada, autoritzo la seva presentació perquè sigui jutjada per la comissió corresponent.

I per tal que així consti als efectes que correspongui, firmo la present a Bellaterra (Cerdanyola del Vallès), el 15 de Maig del 2010.

Jordi Miró Roig

Aquesta Tesi Doctoral s'ha realitzat en el marc dels projectes RZ03-021 i RZ2007-00017-00-00 (*Instituto Nacional de Investigaciones Agrarias)* titulats: *" Conservación «ex situ» de semen y embriones del asno «Guarà Català» como estrategia para la conservación de la raza."* i *"Creación de un banco de semen congelado para las razas asnales Balear y Majorera a partir de la información genética molecular obtenida de sus reproductores."*

A vegades sentim que el que fem és només una gota en el mar,
però el mar seria menys si l'hi faltés una gota.

Mare Teresa de Calcuta *(1910-1997)*

Viure no és només existir,
sinó existir i crear,
saber gaudir i patir
i no dormir sense somiar.

Gregorio Marañon *(1887-1960)*

Tesi Doctoral com a compendi de publicacions

I. Taberner, E., Medrano, A., Peña, A., Rigau, T., Miró, J. 2008. Oestrus cycle characteristics and prediction of ovulation in Catalonian jennies. *Theriogenology* 70, 1489-1497.

II. 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.

III. Taberner, E., Peñalba, A., Peña, A., Miró, J. 2010. Effect of pre-centrifugation short-term conservation temperature on the survival and motility of Catalonian donkey spermatozoa. *Submitted for publication.*

IV. Flores, E., Taberner, E., Rivera, 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.

V. Taberner, E., Morató, R., Mogas, T., Miró, J. 2010. Ability of Catalonian donkey sperm to penetrate zona pellucida-free bovine oocytes matured *in vitro.* *Animal Reproduction Science* 118, 354-361.

Agraïments / Acknowledgements

Finalment ha arribat el moment d'agrair tota l'ajuda i el suport que he rebut tots aquests anys durant la realització de la Tesi, que no han sigut pas pocs… i hi ha tantes persones que m'han recolzat que no em voldria deixar a ningú!

Primer de tot, agrair al meu director de Tesi, Jordi Miró, per donar-me la possibilitat de realitzar aquesta Tesi en burros, per tota la seva ajuda i consells durant tot aquest temps, tant a nivell professional com personal. Per donar-me l'oportunitat de treballar en la reproducció de burros i cavalls. Moltíssimes gràcies!

A en Joan Enric Rodríguez, Teresa Mogas i Teresa Rigau per ajudar-me en tot el que he necessitat al llarg de tots aquests anys.

A tots els meus companys de la Unitat de Reproducció que heu estat al meu costat, compartint laboratori, pipetes i el que fes falta! Per tots els moments que hem viscut junts, tant els de bogeria com els de preocupacions. Els que encara esteu per la universitat i els que ja heu emprès el vol: Roser, Laura, Eva, Joan, Lina, Jorge, Alex, Xus, Montse, Sílvia, Diego, Bladimir, Dalia, Fanny, Antonio, Armando, José Luis. A tots els que m'heu acompanyat a les granges amb els cavalls o els burros, passant hores de calor i de fred, però sobretot gaudint de la vostra companyia: Olga, Sandra, Agustina, Anna, Macarena, Angelica, Jessica, Juan, Juleide, Núria, Catalina. Moltíssimes gràcies per la vostra ajuda i per la vostra amistat! Ha estat un plaer compartir aquests anys amb vosaltres.

A en Manel López per confiar en mi i oferir-me la oportunitat de continuar investigant en el món de la reproducció i recordar el món de l'anatomia, una mica oblidat… Moltes gràcies per tot!

A en Carlos López per donar-me la possibilitat d'ajudar-lo amb la realització de l'atlas d'anatomia, però sobretot, per preocupar-se i ser-hi en tot moment! Moltes gràcies per l'acollida i per tots els cafès!

A en Pedro Mayor, tot i que has arribat al final d'aquest procés moltes gràcies per ajudar-me en tot el que he necessitat i pels moments compartits.

A les meves companyes d'Anatomia: Mari, Laia, Tamara, Sharon i Alba. Tot i que heu arribat a la fase final d'aquesta Tesi també l'heu patit. Moltes gràcies per tot el suport i per ajudar-me en tot moment! Gràcies per preocupar-vos per mi i compartir més que un laboratori! Per fer-me riure i sentir-me com a casa. Moltíssimes gràcies!

I finalment, voldria donar infinites gràcies a la tota meva família i amics, sense els quals aquesta Tesi no hagués estat possible. Gràcies per ser al meu costat i ajudar-me en tot, sense esperar res a canvi. Gràcies per sentir-vos orgullosos de mi. Gràcies per animar-me en els moments difícils i riure amb mi en els feliços. No tinc suficients paraules per expressar-vos tota la meva gratitud, per tant us envio molts petons a tots!!! Moltíssimes gràcies: Papa, Mama, Xevi, Laura, Anna, Smith, Eva, Jordi, Anna A., Jordi A, Tios i Ties, Teresa, Marina, Sandra, Roser, Laura, nens de Girona, nenes del pis i amics de Fornells.

Moltíssimes gràcies a tots!!!

Continguts / Contents

Resum **3**

Abstract **5**

Resumen **7**

Introducció / Introduction **9**

1. Recursos genètics animals 11

1.1. Conservació dels recursos genètics animals 11

1.2. El Guarà Català 14

1.3. Estratègies de conservació 16

2. Característiques reproductives 17

2.1. Característiques reproductives de la burra 17

Peculiaritats anatòmiques 17

Comportament sexual 17

Estacionalitat 19

Característiques del cicle estral i l'ovulació 20

Predicció de l'ovulació 21

2.2. Característiques reproductives del burro 21

Peculiaritats anatòmiques 21

Comportament sexual 21

Característiques del semen 22

3. Tecnologies reproductives 23

3.1. Inseminació artificial 23

3.2. Dilució i centrifugació del semen 24

3.3. Refrigeració 26

3.4. Criopreservació 27

4. Avaluació de la qualitat del semen 29

4.1. Concentració espermàtica 29

4.2. Viabilitat i anomalies morfològiques 30

4.3. Motilitat espermàtica 31

4.4. Avaluació fecundació *in vitro* 34

Bibliografia 36

Objectius / Aims **45**

Capítols / Chapters **49**

Chapter I: Oestrus cycle characteristics and prediction of ovulation in Catalonian jennies. **51**

Chapter II: Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen. **63**

Chapter III: Effect of pre-centrifugation short-term conservation temperature on the survival and motility of Catalonian donkey spermatozoa. **71**

Chapter IV: Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates. **95**

Chapter V: Ability of Catalonian donkey sperm to penetrate zona pellucida-free bovine oocytes matured *in vitro*. **107**

Discussió / Discussion **117**

Conclusions generals / General conclusions **129**

Resum

L'objectiu general d'aquesta tesi és l'estudi de les característiques reproductives del mascle i de la femella del burro Català per aplicar tecnologies reproductives que potenciïn la seva cria i evitin l'extinció d'aquesta raça de burro.

El primer treball va estudiar el comportament sexual, les característiques del cicle estral i la predicció de l'ovulació en la burra Catalana. Els signes de zel més característics observats en les burres foren l'obertura i tancament de la boca junt amb l'extensió de les orelles enrere, orinar i vulvejar. Es va determinar que la durada del cicle estral era de 24.9 ± 0.3 dies, on el zel tenia una durada de 5.6 ± 0.2 dies. Les ovulacions es produïen amb un diàmetre mig preovulatori de 44.9 ± 0.5 mm i es va observar un percentatge elevat d'ovulacions múltiples (44.3%). La combinació dels següents paràmetres: diàmetre fol·licular, textura fol·licular i comportament de zel van determinar-se com els millors predictors de l'ovulació.

El segon treball va estudiar l'efecte de la dilució i la centrifugació del semen (dos mètodes d'eliminació del plasma seminal) sobre la viabilitat, la motilitat i l'estructura subpoblacional mòtil del semen de burro Català refrigerat a 5ºC durant 24, 48 i 72h. Es va observar que la centrifugació o una ràtio de dilució elevada (1:10) eren els tractaments que mantenien més òptimament la viabilitat espermàtica durant les primeres 48h de refrigeració. L'estructura de 4 subpoblacions espermàtiques mòtils es mantenia amb la dilució o centrifugació, ara bé, només les mostres centrifugades després de 24h de refrigeració (5ºC) mantenien exactament la mateixa proporció d'espermatozoides en cada subpoblació que les mostres de semen fresc.

El tercer treball va avaluar els canvis en la viabilitat i la motilitat que pateixen els espermatozoides de ruc Català durant la refrigeració (2h) i posterior centrifugació a tres temperatures diferents (5, 15 i 20ºC). En tots els tractaments es van mantenir les 4 subpoblacions mòtils del semen del ruc, observant que la refrigeració i centrifugació a

20ºC era la que mantenia l'estructura subpoblacional espermàtica més similar al semen fresc de burro Català.

El quart estudi va valorar els efectes de la congelació-descongelació del semen de burro Català en comparació amb el semen de porc, sobre l'estructura subpoblacional mòtil i la viabilitat dels espermatozoides. Les característiques de motilitat dels espermatozoides de burro i de porc van respondre de manera molt diferent al procés de congelació-descongelació, mantenint però en els dos casos l'estructura de 4 subpoblacions mòtils. Els canvis en els valors mitjans de motilitat en les dues espècies estudiades es van deure sobretot, a canvis en les proporcions dels espermatozoides inclosos en cada subpoblació específica.

En l'últim treball es va desenvolupar un model per avaluar la funcionalitat espermàtica del semen fresc i congelat de burro Català mitjançant l'interacció dels espermatozoides amb oòcits bovins lliures de zona pel·lúcida. Els espermatozoides de burro van ser capaços de fusionar-se amb l'oolemma i descondensar-se i formar el pronucli masculí (85-94%). Es va observar que les mostres de semen fresc i descongelat amb una viabilitat elevada (>60%) tenien índex de penetració superiors a les mostres amb viabilitat baixa (<40%). Així doncs, es van obtenir correlacions positives significatives (P<0.01) entre el percentatge de fecundació i la viabilitat (r=0.84), i també amb alguns paràmetres de motilitat (VAP, r=0.56; VCL, r=0.61; and mean ALH, r=0.68).

En conclusió, una predicció adequada de l'ovulació de les burres, juntament amb l'utilització dels mètodes òptims de maneig i congelació del semen de ruc Català contribuiran positivament a l'aplicació de tecnologies reproductives en programes de cria que ajudin a evitar l'extinció d'aquesta raça.

Abstract

The aim of this work is the knowledge of Catalonian donkey reproductive features of its females and males in order to apply reproductive technologies to maximize the production of live foals and help conserve the breed.

In the first work we studied the reproductive behaviour, oestrous cycle characteristics and indicators that predict the moment of ovulation of the Catalonian donkey. The main signs of oestrus detected were mouth clapping, urinating and contraction and relaxation of the labia of the vulva with eversion of the clitoris. The length of the oestrus cycle was 24.9 ± 0.3 days, with oestrus itself lasting 5.6 ± 0.2 days. The mean diameter of the preovulatory follicle at day -1 was 44.9 ± 0.5 mm and we observed high incidence of multiple ovulations (44.3%). The combination of follicle size, follicle texture and oestrous behaviour were the best predictors of ovulation.

In the second study we evaluated the effects of dilution and centrifugation (two methods of reducing the influence of the seminal plasma) on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen at 24, 48 and 72h. At 48h, more surviving spermatozoa were seen in the more diluted (1:10) and in the centrifuged semen samples. The four donkey motile sperm subpopulations were maintained by refrigeration and centrifugation, moreover only centrifuged samples and only at 24h (5ºC) showed exactly the same motile sperm subpopulation proportions as recorded for fresh sperm.

In the third work we assessed the effect of three storage temperatures (5, 15, 20ºC) on the survival and motility of Catalonian donkey sperm during transport (2h) to the laboratory for centrifugation. All treatments maintained the four motile sperm subpopulation, but 2h of storage at 20ºC followed by centrifugation was the treatment that maintained subpopulation structure more similar than fresh samples.

In the fourth study we evaluated the influence of freeze/thawing on motile sperm subpopulations and sperm viability in ejaculates from two phylogenetically different mammalian species, boar and donkey. Boar and donkey sperm respond very differently in their mean motion characteristics to freezing/thawing, this process did not change the existence of a 4-subpopulations structure in the ejaculates. Motility changes induced by freezing/thawing protocol are especially linked to changes in the specific percentage of each of the motile sperm subpopulations.

In the last work we studied the interaction between fresh/frozen-thawed donkey spermatozoa and zona pellucida (ZP)-free bovine oocytes in an attempt to develop a model for assessing cryopreserved Catalonian donkey sperm function. The donkey spermatozoa were able to fuse with the oolema and even to decondense and form the male pronucleus (85-94%). Fresh or frozen-thawed high viability (>60%) spermatozoa had higher penetration percentage than fresh or frozen/thawed low viability (<40%) spermatozoa. A significant positive correlation (P<0.01) was detected between percentage fertilization and viability (r=0.84), and between percentage fertilization and certain CASA parameters (VAP, r=0.56; VCL, r=0.61; and mean ALH, r=0.68).

In conclusion, the exact moment prediction of ovulation in Catalonian jennies and adequate donkey semen manipulation and cryopreservation should have a positive impact upon the reproductive technologies application. Such knowledge should help maximize the production of live foals and help conserve the breed.

Resumen

El objetivo general de esta tesis es el conocimiento de las características reproductivas del macho y la hembra del burro Catalán con el fin de aplicar tecnologías reproductivas que potencien su cría y eviten la extinción de esta raza de burro.

El primer trabajo estudió el comportamiento sexual, las características del ciclo estral y la predicción de la ovulación de la burra Catalana. Los signos de celo más característicos observados en las burras fueron la masticación junto con la extensión de las orejas hacia atrás, orinar y vulveo. Se determinó que la duración del ciclo estral era de 24.9 ± 0.3 días, dónde el celo tuvo una duración de 5.6 ± 0.2 días. Las ovulaciones se producían con un diámetro medio preovulatorio de 44.9 ± 0.5 mm y se observó un porcentaje elevado de ovulaciones múltiples (44.3%). La combinación de los siguientes parámetros: diámetro folicular, textura folicular y comportamiento de celo se determinaron como los mejores predictores de la ovulación.

El segundo trabajo estudió el efecto de la dilución y la centrifugación del semen, (dos métodos de eliminación del plasma seminal) sobre la viabilidad, la motilidad y la estructura subpoblacional motil del semen de burro Catalán refrigerado a 5ºC durante 24, 48 i 72h. Se observó que la centrifugación o una ratio elevada de dilución (1:10) eran los tratamientos que mantenían más óptimamente la viabilidad espermática durante las primeras 48h de refrigeración. La estructura de 4 subpoblaciones espermáticas motiles se mantenía con la dilución o la centrifugación, sin embargo solo las muestras centrifugadas después de 24h de refrigeración (5ºC) mantenían exactamente la misma proporción de espermatozoides en cada subpoblación que las muestras frescas a las 24h.

El tercer trabajo evaluó los cambios en la viabilidad y la motilidad que sufren los espermatozoides de burro Catalán durante la refrigeración (2h) y la posterior centrifugación a tres temperaturas diferentes (5, 15, 20ºC). Todos los tratamientos mantuvieron las 4 subpoblaciones motiles del semen de burro, observándose que la

refrigeración y centrifugación a 20ºC era la que mantenía la estructura subpoblacional espermática más similar al semen fresco del burro Catalán.

El cuarto estudio valoró los efectos de la congelación-descongelación del semen de burro Catalán en comparación con el semen de cerdo, sobre la estructura subpoblacional motil y la viabilidad de los espermatozoides. Las características de la motilidad de los espermatozoides de burro y cerdo respondieron de manera muy diferente al proceso de congelación-descongelación, manteniendo en los dos casos la estructura de 4 subpoblaciones motiles. Los cambios en los valores medios de motilidad en las dos especies estudiadas se debieron sobretodo a cambios en las proporciones de los espermatozoides incluidos en cada subpoblación específica.

En el último trabajo se desarrollo un modelo para evaluar la funcionalidad espermática del semen fresco y congelado de burro Catalán mediante la interacción de los espermatozoides con ovocitos bovinos libres de zona pelúcida. Los espermatozoides de burro fueron capaces de fusionarse con el oolema y descondensarse y formar el pronúcleo masculino (85-94%). Se observó que las muestras de semen fresco y descongelado con una viabilidad elevada (>60%) tenían índices de penetración superiores a las muestras con viabilidad baja (<40%). Se obtuvieron correlaciones positivas significativas (P<0.01) entre el porcentaje de fecundación y la viabilidad (r=0.84), y también con algunos parámetros de motilidad (VAP, r=0.56; VCL, r=0.61; y mean ALH, r=0.68).

En conclusión, una predicción adecuada de la ovulación de las burras Catalanas, junto con la utilización de los métodos óptimos de manipulación y congelación del semen de burro Catalán contribuirán positivamente en la aplicación de las tecnologías reproductivas en programas de cría que ayuden a evitar la extinción de esta raza.

Introducció / Introduction

Introducció / Introduction

1. Recursos genètics animals

1.1. Conservació dels recursos genètics animals

La conservació és definida com l'administració de l'ús humà de la biosfera de tal manera que ella produeixi el major profit sostenible per les generacions actuals, mantenint sempre el potencial per satisfer les necessitats i les aspiracions de les generacions futures. La conservació té un sentit positiu, englobant la preservació, el manteniment, l'ús sostenible, la restauració i la millora de l'ambient natural (IUCN-UNEP-WWF i FAO-UNESCO, 1980).

Els recursos genètics animals comprenen les espècies, races i estirps que tenen interès econòmic, científic i cultural per l'agricultura, ara i en un futur. Les espècies més freqüents són ovelles, cabres, bovins, cavalls, porcs, búfals i aus de corral, però existeixen molts animals domesticats com camells, burros, elefants, rens, conills i espècies rosegadores que són importants per a diferents regions del món.

Així doncs, els animals domèstics satisfan com a mínim el 30% de les necessitats humanes d'alimentació i agricultura en forma de carn, llet, productes làctics, ous, fibra, fertilitzant pels cultius i potència de tracció. Aquesta aportació la realitzen aproximadament 4.500 races obtingudes a partir de 40 espècies d'animals domèstics. Aquestes races, desenvolupades al llarg de 12.000 anys i que representen el patrimoni restant de diversitat genètica a partir del qual s'hauran de cobrir les necessitats futures, s'estan extingint a una velocitat de 6 races al mes (DAD-IS, 1997)

La FAO (Food and Agricultural Organization of the United Nations) estima que el 32% de les races de bestiar estan en perill d'extinció i més de la meitat d'aquestes races es troben en països en desenvolupament. A Europa, la meitat de les races que existien a

principis de segle XX s'han extingit i el 41% de les 1.500 races restants (de les quals hi ha dades de població) corren perill d'extinció en els pròxims 20 anys (Fig. 1).

A nivell mundial, l'alta especialització de la producció pecuària moderna suposa una amenaça per la diversitat zoogenètica. La producció intensiva ha potenciat milers de generacions d'entrecreuament controlat en la majoria d'animals domèstics, que no ofereixen una reserva genètica suficient pel futur.

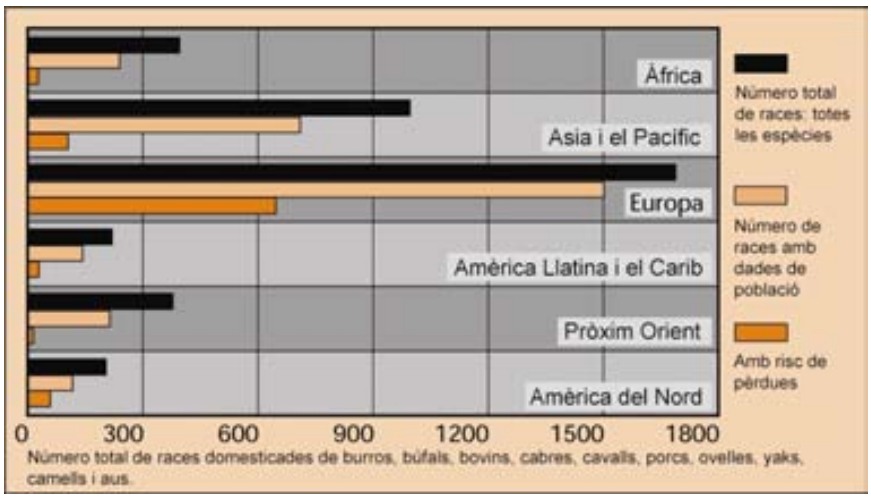

Figura 1. Número total de races, de races amb dades de població i de races en perill d'extinció segons les regions del món (Font: FAO, http://dad.fao.org).

Degut a aquesta disminució alarmant de la diversitat genètica animal, aquests últims anys, ha guanyat importància la conservació i l'ús sostenible d'aquests recursos irreemplaçables per prevenir, frenar i revertir la tendència a l'erosió de la biodiversitat. Mason i Hall amb les seves afirmacions, justifiquen de manera clara, qualsevol programa de conservació de les espècies i/o races en perill d'extinció: "Qualsevol extinció o desaparició d'una espècie o raça representa un irreemplaçable element de la diversitat de la vida que es perd" (Mason, 1974) i "Les races domèstiques són recursos genètics que han d'ésser protegits com a part de l'herència mundial de la biodiversitat" (Hall, 1993).

En aquest marc, la FAO ha establert un Programa Global pel Maneig dels Recursos Genètics dels Animals de Granja per coordinar els esforços regionals i nacionals per superar l'erosió d'aquests recursos. La Conferència de les Nacions Unides

sobre el Medi Ambient i el Desenvolupament i la Convenció sobre la Diversitat Biològica i el Programa 21 han actuat com un impuls suplementari i un mecanisme per l'identificació de la diversitat dels animals domèstics com un element autèntic i important de la biodiversitat global.

Les espècies de mamífers domèstics presents a la llista de la FAO (boví, búfal, yak, cabra, ovella, porc, burro, cavall, camell, dromedari, alpaca, guanac, llama, vicunya) representen 3.019 races, de les quals només es tenen dades de població de 2.191 (72.6%). Tot i la importància d'aquestes espècies pel manteniment de l'home, hi ha 364 (16.6%) espècies amb dades de població que es troben en alt risc d'extinció.

A Espanya, hi ha 6 races de burros reconegudes oficialment al catàleg de races autòctones d'animals domèstics (R.D. 1682/1997) que són les següents: la raça Andalusa, el burro de les Encartacions, la raça Catalana, la Mallorquina, la Majorera i la Zamorana-Lleonesa, dins l'apartat de Races de Protecció Especial (B.O.E. 1997). Aquestes races han vist reduïda la seva població, així com la seva diversitat genètica. Aquests factors predisposen a aquestes races a patir els efectes adversos de l'endogàmia. Segons les estadístiques de la FAO (DAD-IS, 1997), la majoria d'aquestes races es troben en situació crítica (Mallorquina, Majorera, Burro de les Encartacions, Andalusa) donat que el número efectiu de femelles reproductores no supera els 100 individus. La raça Catalana es troba en la categoria de races en perill d'extinció mantingudes, perquè té un cens global de 400 animals, i compta amb organitzacions públiques i privades encarregades de la seva conservació. La raça Zamorana-Lleonesa és l'única excepció, ja que presenta un cens global entre 1.000-10.000 animals amb un número efectiu de femelles reproductores de 994, classificant-se fora de perill segons el barem del Llistat Mundial de Vigilància per la Diversitat dels Animals Domèstics (DAD-IS, 1997).

1.2. El Guarà Català

El Guarà Català és una raça autòctona de Catalunya que prové del tronc ancestral *Equus asinus somaliensis* (Ase de Somàlia). Aquesta raça es caracteritza per un format hipermètric i perfil cranial subconcavilini amb tendència rectilínia. Són animals de gran talla, amb unes alçades de 145 a 160 cm en els mascles i 135 a 150 cm en les femelles, i pesos compresos entre 350-450 kg. De conformació harmònica i silueta esvelta, presenten extremitats robustes i ben conformades que els capaciten per realitzar treballs durs en temps i esforç. Són de caràcter dòcil, molt nobles i de gran vivacitat. La capa és fosca, denominada també "color de pansa". El pèl curt i brillant amb degradacions blanquinoses envolta el musell, les zones orbitals del ulls, la base de les orelles, el pit, el ventre, el braguer i les axil·les (Jordana i Folch, 1996) (Fig. 2).

Els burros Catalans han tingut una gran importància en la formació i la millora d'altres poblacions, principalment europees i americanes. El burro Català, en menor grau que la raça Zamorana-Lleonesa, es va utilitzar per millorar el Baudet de Poitou, per incrementar la seva talla i capacitat sexual (Romagosa, 1959; Parés i Vilaró, 1994). Ara bé, el guarà Català va influenciar de manera molt important en la millora de races italianes com la Pantesca, la Martina-Franca i la Siciliana o Ragusana (Aparicio, 1960; Epstein, 1984; Romagosa, 1959). Sotillo i Serrano (1985) també atribueixen certa influència sobre les races Xipriota i Maltesa. Però, on el burro Català va tenir una contribució directa i decisiva va ser en la formació de l'Ase de Kentucky o Mammoth. Aquest nom prové del millor semental fundador de la raça, "Imported Mammoth", que va arribar a Charleston l'any 1819 i va ser utilitzat als estats de Kentucky, Tennessee i Missouri (Briggs, 1971).

Figura 2. Exemplar de raça asinina Catalana.

Durant els anys 60 i 70, la mecanització del camp i l'èxode rural, va conduir a la raça Catalana a una situació límit. Malgrat tot, l'any 1978 es va fundar l'Associació del Foment de la Raça Asinina Catalana (AFRAC) per protegir, fomentar i seleccionar aquesta població. A finals de 1994, es va iniciar el Programa de Conservació i Manteniment de Recursos Genètics Animals en la Raça Asinina Catalana, promogut pel DARP (Departament d'Agricultura, Ramaderia i Pesca) de la Generalitat de Catalunya, en col·laboració amb l'AFRAC i l'Unitat de Genètica i Millora de la Facultat de Veterinària de Barcelona (UAB). A més, la Unitat de Reproducció de la Facultat de Veterinària de la UAB gràcies a dos projectes d'investigació finançats per l'INIA (Instituto Nacional de Investigación Agraria) ha estudiat des de l'any 2000, l'activitat seminal dels guarans Catalans i el cicle reproductiu de les burres Catalanes.

L'any 2002 es va crear i reglamentar el Llibre Genealògic de la Raça Asinina Catalana (Diari Oficial de la Generalitat de Catalunya (DOGC) núm. 3608, de 4 d'abril) que és gestionat per l'AFRAC. Actualment, la seva població està composada per 557 individus registrats al Llibre Genealògic de l'AFRAC (Associació del Foment de la Raça

Asinina Catalana), 291 dels quals són femelles (>3 anys), 129 són mascles (>3 anys) i 137 són pollins (<3 anys). Es troben distribuïts en diferents comarques prepirinenques i pirinenques de Catalunya (nord-est Espanya), especialment a les comarques del Berguedà, Osona, Ripollès, Garrotxa i Pla de l'Estany.

1.3. Estratègies de conservació

Existeixen dues vies de conservació dels recursos genètics animals, la conservació *in situ* i la *ex situ*. La conservació *in situ* són totes les mesures per mantenir els animals en l'hàbitat on estan adaptats, i la *ex situ* consisteix en treure els recursos genètics animals del seu medi ambient i conservar-los en bancs de genoma, com instal·lacions de crioconservació, granges o zoològics. Això, inclou la recollida i congelació de semen, oòcits o embrions i també la cria en captivitat d'espècies i animals domesticats en granges o zoològics.

Un dels principals problemes de la població de burro Català són els inevitables aparellaments entre individus emparentats, que comporta un increment de la consanguinitat i la conseqüent depressió consanguínia; una reducció del valors fenotípics dels caràcters productius i reproductius, i com a conseqüència dels problemes reproductius, la disminució i/o extinció de la població (Aranguren, 2002). Per aquest motiu, una de les prioritats del programa de conservació del burro Català és mantenir al màxim la diversitat genètica i reduir l'endogàmia utilitzant les tecnologies reproductives assistides (inseminació artificial, transferència embrionària i la fecundació *in vitro*) i la creació de bancs genètics (semen, oòcits i embrions). Aquestes accions ens ajudaran a garantir una distribució dels gens més eficient i, conseqüentment, una reducció de l'endogàmia. La aplicació d'aquestes tecnologies implica però el coneixement específic de les característiques reproductives de cada espècie, donat que extrapolar protocols de espècies properes no sempre aporta resultats satisfactoris (Pukazhenthi *et al.*, 2004). Per

tant, és necessari la realització de estudis per caracteritzar les peculiaritats reproductives del mascle i la femella del burro Català en àrees com l'anatomia, la morfologia i la fisiologia reproductiva de l'ase per millorar l'aplicació de la tecnologia.

2. Característiques reproductives

2.1. Característiques reproductives de la burra

Peculiaritats anatòmiques

Les races de burros presenten una gran variació en la seva talla, existint races de gran talla com el Baudet de Poitou o el burro Català i races miniatura com les africanes. La mida de la raça pot suposar un repte a l'hora de realitzar una palpació transrectal o una ecografia. Per aquesta raó, és recomanable adaptar tècniques utilitzades en races de cavalls miniatura a l'hora d'explorar races de burres miniatures o petites (Purdy, 2005). El cèrvix de la burra és més llarg i més estret que el de l'euga i protueix cap a la vagina de manera més pronunciada, fent més dificultós l'accés a l'úter. Aquesta conformació estreta del cèrvix de la burra, la predisposa a patir més laceracions durant el part, en cas de distòcia (Vendramini *et al*., 1998).

Comportament sexual

Freqüentment s'han assumit com a característiques reproductives de les burres, característiques pròpies de les eugues. Però, tot i ser dues espècies relacionades filogenèticament, presenten diferències tant en el comportament reproductiu com en la funció reproductiva. Així doncs, a diferència de les eugues, el signe més constant i útil com indicatiu de zel en les burres, és el moviment de masticació (Henry *et. al*., 1987; Henry *et. al*., 1991; Nishikawa i Yamazaki, 1949a; Vandeplassche *et. al*., 1981; Clayton

et. al., 1981; Nishikawa, 1959). Aquest comportament no es dóna normalment en eugues. En general, quan una burra és sexualment receptiva mostra signes d'acomodació i serenitat. Els signes de zel descrits en burres són mastegar, estirar les orelles cap enrere amb el coll estès, emetre ejeccions d'orina, aixecar la cua, i algunes vegades fer contraccions vulvars repetides descobrint el clítoris (Fig. 3) (Trimeche *et al*., 1995; Henry *et al*., 1998). Les burres també poden mostrar comportaments heterotípics (típics del mascle) que inclouen la munta, la persecució (herding/chasing), la recel.la i el Flehmen, i que són infreqüents en les eugues (Henry *et. al*., 1991).

Figura 3. Signes de zel en una burra Catalana: mastegar, estirar les orelles enrere i immobilitat en front el mascle.

Estacionalitat

L'estacionalitat de la burra és objecte de controvèrsia en diferents estudis. Pot estar influenciada pel fotoperíode, la nutrició o la temperatura. Alguns autors la descriuen com una femella polièstrica estacional (Nishikawa, 1959; Henry *et al*., 1987) i altres com una femella polièstrica amb una gran tendència a ciclar durant tot l'any (Ginther *et al*., 1987; Blanchard *et al*., 1999). Ginther *et al*. (1987) al sud de Wisconsin (43º N) van observar que el cicle estral de la burra estava menys influenciat per l'estació que en ponis

o cavalls. En el seu estudi un 64% ovulava al desembre i 82-100% durant els altres mesos. L'època anovulatòria es donava al hivern, era curta (39-72 dies) i acabava amb un estre perllongat (17-41 dies). En canvi, a Brasil (19ºS) les burres mostraven una marcada estacionalitat (54% presentaven anestre) amb una durada mitjana de l'anestre estacional de 166.3±63.2 dies (Henry *et al.*, 1987)

Característiques del cicle estral i l'ovulació

El cicle estral de la burra ha estat poc estudiat pels investigadors, i els estudis que s'han realitzat, tenen condicions ambientals i número d'animals molt diferents. Malgrat això, la majoria descriuen que la duració del cicle estral de la burra és major que el de les eugues: 23-25 dies en la burra vs 21-22 dies en l'euga (Taula 1). La durada de l'estre varia entre 6-9 dies (rang 2-12 dies) depenent de l'estudi i la durada del diestre és molt similar entre els diferents estudis, entre 17-18 dies.

La mitjana del diàmetre del fol·licle preovulatori el dia abans de l'ovulació és de aproximadament 36 mm en burres Poitou i burres Pega (Trimeche *et al.*, 1995; Meira *et al.*, 1995). En canvi, Miró *et al.* (2003), en burres Catalanes, van observar un diàmetre mig del fol·licle preovulatori superior (43 mm). Dadarwal *et al.* (2004) descriu un creixement mig diari durant l'estre de 2.7 mm/dia, també ovulant amb un diàmetre aproximat de 41 mm. L'ovulació és la culminació d'una sèrie d'esdeveniments que comporten un pic de LH i com a resultat el col·lapse del fol·licle preovulatori i l'expulsió de l'oòcit (Pierson, 1993). Alguns autors han relacionat l'ovulació amb l'inici del zel i han observat que es produïa l'ovulació entre 5.6-6.6 dies des de l'inici (Nishikawa *et al.*, 1949b; Vandeplassche *et al.*, 1981; Trimeche *et al.*, 1995).

L'incidència de les ovulacions múltiples en la burra varia entre 5%-38% (Vandeplassche *et al.*, 1981; Henry *et al.*, 1987; Miró *et al.* 2003). Henry *et al.* (1987) van observar una incidència d'ovulacions dobles, triples i quàdruples de 25.5%, 10.5% i 1.1%, respectivament. En burres Mammoth al sud-est de Texas, van observar una

incidència d'ovulacions múltiples molt més elevada del 61% (Blanchard *et al.*, 1999). El 75% de les ovulacions dobles són asincròniques i ovulen amb un interval mig de 3.1 dies (rang 1-11 dies). A més, s'ha observat una freqüència lleugerament més elevada d'ovulacions a l'ovari esquerre (61%) que en el dret (39%) en burres (Nishikawa i Yamazaki, 1949b; Henry *et al*., 1987). El cos luti té una vida mitjana d'entre 15 i 20 dies [19.3 ±0.6 en burres (Vandeplassche *et al.* 1981), 17.4 ± 2.6 dies en burres Mammoth (Blanchard *et al.*, 1999)].

Duració cicle estral (dies)	Duració estre (dies)	Duració diestre (dies)	Races	Referències
25.8±2.2[a]§ 26.4±2.4[b]§	7.5±1.2[a]§ 7.1±0.9[b]§		Boudet de Poitou	Trimeche *et al.* , 1995
24.2±3.2	6.3±2.17	17.95±2.04	Burres Pega i creuades	Meira *et al.,* 1995
25.49±2.7	7.9±2.5	18.2±2.3		Henry *et al.,* 1987
23.3±2.6	5.9±2.1	17.4±2.6	Burres Mammoth	Blanchard *et al.,* 1999
22.8±0.1	6.0±0.6			Nishikawa *et al.*,1949a
24±1.6	2-7	17-22	Burres Catalanes	Miró *et al.,* 2003
24.9±0.7	3-12	19.3±0.6		Vandeplassche *et al.*, 1981
21.7±3.5	6.5±2.6	14.9±2.8	Eugues	Ginther, 1992

Taula 1. Duració del cicle estral, el diestre i l'estre en diferents races de burres i en eugues. [a] a la primavera ; [b] a l'estiu; § SEM (error estàndard de la mitjana).

Predicció de l'ovulació

La predicció del dia de l'ovulació ha guanyat importància degut a l'interès creixent en el transport de semen, la congelació de semen i la transferència embrionària (Lindeberg *et al*., 1992). La majoria d'estudis realitzats fan referència a les eugues, en

canvi en burres els estudis són inexistents. Miró *et al.* (2004) en eugues de diverses races (Selles Franceses, Anglo-Àrabs, Espanyols i Pura Sang Anglesos) van analitzar paràmetres com el comportament sexual, la mida fol·licular, la textura del fol·licle, l'aparença ecogràfica del fol·licle, el to uterí i l'aparença ecogràfica de l'úter amb la finalitat de validar-los per predir l'ovulació. Van estudiar l'evolució d'aquests paràmetres des del dia -4 al dia -1 abans de l'ovulació. Mitjançant el procediment estadístic de selecció de variables "stepwise", van escollir la textura fol·licular i la mida fol·licular com els millors paràmetres per predir l'ovulació en les eugues. A través d'una regressió logística d'aquests dos paràmetres aconseguien probabilitats superiors al 70% que l'euga ovules en 24 hores i al 80% que l'euga ovules en 48 hores.

2.2. Característiques reproductives del burro

Peculiaritats anatòmiques

Existeixen moltes similituds reproductives entre el cavall i el burro. Tanmateix, els testicles i el penis del burro semblen ser més grans en comparació amb cavalls de la mateixa mida (Kreuchauf, 1984). Encara que el cavall i el burro tenen les mateixes glàndules accessòries, l'ampul·la és de major mida en el burro.

Comportament sexual

Els burros domèstics, la majoria d'ases salvatges i la zebra de Grevi (*E. grevyi*) són animals territorials, cada mascle guarda un territori i té accés per muntar les femelles que resideixen o passen pel territori (McDonnell, 2000; Klingel, 1977, 1974; Mohr, 1971). Aquesta territorialitat sembla ser una adaptació a hàbitats secs on els recursos no estan distribuïts uniformement. En aquestes condicions les femelles no estableixen grups

permanents, degut a la gran competència intraespecífica. Així doncs, els mascles defensen zones àmplies que contenen els recursos necessaris per les femelles. Hi ha diferències notables entre el comportament precopulatori dels cavalls i els burros reflectint les diferents organitzacions socials.

Una peculiaritat dels burros és el temps més perllongat per aconseguir l'erecció i l'ejaculació (5-30 min) en comparació amb els cavalls (10-11 min)(Kreuchauf, 1984). Durant l'interacció precòpula, el mascle efectua episodis de flehmen, té contacte naso-nasal amb la femella, mossega el cap, coll, genoll i laterals de la burra, munta sense erecció, exterioritza parcialment el penis, té episodis sobtats de desinterès i olora, sobretot la zona perineal de la burra. Finalment, el mascle té l'erecció a distància, simulant desinterès, i seguidament s'acosta i munta a la burra (Henry *et. al*., 1991; Lobo, 2003).

Característiques del semen

El volum de semen ejaculat varia segons la raça de burro. Així doncs, podem trobar volums de 25-50 ml en burros miniatura o burros del nord de l'Àfrica i volums de 25 i 250 ml en races de major talla (Tibary *et al.* 2006). Les característiques de l'ejaculat són: entre 5-25 x 10^9 de número total d'espermatozoides, 70-80% motilitat progressiva, 80-88% viabilitat, i un pH 7.6-7.7 (Kreuchauf *et al.* 1984; Gastal *et al.*, 1997). Les anomalies morfològiques dels espermatozoides són similars a les descrites en cavalls i l'unió abaxial de la cua de l'espermatozoide és normal tant en els burros com en el cavalls (Miró *et al.*, 2005). Diversos estudis han investigat l'efecte de l'estació sobre la producció i la qualitat del semen i la libido, obtenint resultats poc concloents. Alguns han descrit major producció i qualitat del semen durant l'època reproductiva, però en la majoria els efectes estacionals són poc marcats (Gastal *et al.*, 1996; Roy *et al.*, 2003).

3. Tecnologies reproductives

3.1 Inseminació artificial

La llegenda explica que el primer animal on es va realitzar una inseminació artificial (IA) amb èxit fou el cavall. En el 1322, es va recuperar semen de la vagina d'una euga que acabava de ser coberta per un semental àrab, diluint-lo amb llet de camell i inseminant una altra eugua d'una tribu àrab rival. Al cap d'un any s'obtingué un poltre, segons diuen excepcionalment preciós i sa (Allen, 2005).

Actualment, la IA és una tècnica molt utilitzada arreu del món en la cria de cavalls degut als nombrosos avantatges que suposa: màxima distribució dels gens, reducció del risc de contraure malalties de transmissió venèria o d'accidents per munta natural, múltiples IA per ejaculat i fàcil transport del semen. No obstant, per la seva aplicació és necessari el ple coneixement del cicle reproductiu, el moment de l'ovulació i la estacionalitat de les femelles, que han sigut àmpliament estudiats en les eugues.

En burres, els estudis del comportament reproductiu, cicle estral i ovulació són molt escassos. Alguns autors han descrit el cicle estral (Nishikawa i Yamasaki, 1949a, 1949b; Henry *et al.*, 1987a ; Meira *et al.*, 1995) i la dinàmica ovàrica i canvis hormonals de l'*Equus asinus* (Blanchard *et al.*, 1999). Tanmateix, existeix poca informació sobre les diferències existents entre races de burros.

L'ús de la inseminació artificial depèn també de la qualitat o fertilitat del semen del ruc. Podem inseminar amb semen fresc, refrigerat o congelat. La refrigeració del semen dels guarans Catalans pel transport i la posterior inseminació artificial és un mètode alternatiu pel maneig de la reproducció d'aquesta espècie. Després de l'ejaculació i durant tota la manipulació del semen, la membrana plasmàtica de l'espermatozoide està exposada a diversos factors ambientals que poden contribuir a la pèrdua de motilitat i de capacitat fecundant. La manipulació del semen per refrigerar inclou: addició del diluent, centrifugació, refrigeració i el temps de transport (Aurich, 2005).

3.2. Dilució i centrifugació del semen

La dilució del semen pur en un diluent adequat allarga la vida dels espermatozoides gràcies al manteniment de la integritat de la membrana plasmàtica, protegeix els espermatozoides de condicions ambientals desfavorables, disminueix els efectes perjudicials del plasma seminal, controla el pH i l'osmolaritat, aporta nutrients als espermatozoides i controla el creixement de microorganismes durant la refrigeració del semen (Katila, 1997).

Al llarg dels anys, s'ha estudiat una gran varietat de diluents amb combinacions de diferents components (sucres, electròlits, *buffers*, rovell d'ou i llet). El diluent a base de llet descremada i glucosa descrit per Kenney *et al.* (1975) és molt popular i utilitzat arreu del món. Aquest diluent és barat, fàcil de preparar i es pot conservar congelat. Es pot obtenir comercialment amb algunes modificacions (EZ-Mixin®, ARS, Chino, USA). Els diluents a base de rovell d'ou resulten més complicats de produir i no milloren molt més la qualitat del semen (Malmgren *et al.*, 1994). Actualment, s'han desenvolupat diluents amb composició més definida, per intentar disminuir components perjudicials de substàncies biològiques tant complexes, com la llet o el rovell d'ou, i obtenir un diluent amb una composició constant (Aurich, 2005). El fraccionament de la llet mitjançant diversos mètodes ha permès la preparació de fraccions purificades, descobrint que les proteïnes més beneficioses pel manteniment del semen eren el fosfocaseinat natiu i la β-lactoglobulina (Batellier *et al.*, 1998; 2001). A partir d'aquests estudis, es va desenvolupar un diluent a base d'aquests components específics de la llet (INRA96®, IMV, L'Aigle Cédex, França). S'han comercialitzat altres diluents amb composició definida a partir de la llet (Equipro®, Minitüb, Alemanya). I també s'han desenvolupat diluents a base de lecitina de soja substituint el rovell d'ou (AndroMed E®, Minitüb, Alemanya) (Aurich, 2005).

Diversos autors han estudiat la conservació del semen de burro en diferents tipus de diluents i amb diferents condicions. Mello *et al.* (2000) van observar una millor

conservació del semen de burro amb un diluent a base de rovell d'ou (Baken modificat amb 10% de rovell d'ou) que amb diluent Kenney (llet descremada i glucosa), mantenint més òptimament la motilitat i la morfologia espermàtica durant la refrigeració a 5ºC. Cottorello *et al.* (2002) descriuen també una major activitat *in vitro* del semen de ruc diluït en Baken al 3% (3% rovell d'ou) o Baken al 10% (10% rovell d'ou) en temperatures pròximes a 0ºC. Més recentment, Rota *et al.* (2008) van descriure millors resultats en la conservació de la motilitat de semen de ruc utililitzant INRA82-Y (2% rovell d'ou) en comparació amb l'INRA82 i l'INRA96 (IMV, L'Aigle Cédex, França). En canvi, Serres (2003) va obtenir millors resultats de conservació del semen de burro Zamorano-Leonés en els diluents a base de llet, com l'INRA82 o el Kenney, que en el diluent amb rovell d'ou (TRIS-rovell), independentment de la temperatura de refrigeració.

El plasma seminal està format per les secrecions de l'epidídim i de les glàndules accessòries de l'aparell reproductor. En la monta natural, el semen és dipositat a l'interior de l'úter de la femella i el contacte dels espermatozoides amb el plasma seminal és menor que en la recollida de semen mitjançant vagina artificial. En diversos estudis s'ha descrit que altes concentracions de plasma seminal produïen efectes adversos sobre la motilitat i la fertilitat del semen refrigerat de cavalls i toros (Pickett *et al.*, 1975). Aquests efectes perjudicials es poden reduïr amb l'extracció i dilució de la fracció rica de l'ejaculat (que conté el 75% dels espermatozoides) o bé, diluïnt amb ràtios elevades de diluent:semen abans de la refrigeració (Varner *et al.*, 1987; Jasko *et al.*, 1991).

Una alternativa per l'eliminació del plasma seminal i per aconseguir concentracions espermàtiques superiors en animals amb baixa concentració d'espermatozoides és la centrifugació. Tanmateix, aquest és un pas crític en la manipulació del semen perquè indueix la peroxidació de la membrana plasmàtica de l'espermatozoide (Parinaud *et al.*, 1997) i pot produir danys importants en els espermatozoides. Les condicions de centrifugació utilitzades normalment són 10-15 minuts a 400-600 x g (Aurich, 2008). La centrifugació es realitza de rutina en la

criopreservació espermàtica, com a pas previ abans d'incorporar el diluent que conté el crioprotector.

Existeixen diferents estudis sobre l'efecte del plasma seminal en la conservació del semen de ruc refrigerat. Ferreira *et al.* (1991) obtenien millor motilitat espermàtica a les 48h de refrigeració a 5ºC quan el semen era centrifugat i resuspès amb un diluent amb rovell d'ou. En canvi, Mello *et al.* (2000) no van observar diferències de longevitat dels espermatozoides entre ejaculats complets i la fracció rica de l'ejaculat (recollit en vagina oberta) diluits amb dos tipus de diluents (Kenney i Baken al 10% rovell d'ou) i mantinguts a 5ºC. Rota *et al.* (2008) tampoc van descriure una millora després d'eliminar el plasma seminal per centrifugació, en les característiques mòtils dels espermatozoides de burro de la raça Amiata.

3.3. Refrigeració

La refrigeració i conservació del semen diluït a curt termini és molt útil per la difusió del material genètic entre poblacions de burros aïllades perquè permet traslladar la dosi seminal fins al lloc on es troba la femella. La refrigeració del semen disminueix el metabolisme basal dels espermatozoides, redueix el creixement de microorganismes i manté la viabilitat del espermatozoides. En cavalls, normalment la temperatura de refrigeració utilitzada és entre 4-6ºC, temperatura òptima pel manteniment de la motilitat (Varner *et al.*, 1988, 1989; Moran *et al.*, 1992) i la fertilitat (Varner *et al.*, 1989). El pas més crític és el descens de temperatura dels espermatozoides de 37ºC a 8ºC, sobretot quan el ritme de refredament és >0,3ºC/min, perquè pot produir danys específics a la membrana plasmàtica de l'espermatozoide, anomenats xoc tèrmic (Amann i Pickett, 1987). Els espermatozoides que han sofert xoc tèrmic presenten una disminució del metabolisme, una pèrdua de components intracel·lulars, de motilitat i de capacitat fecundant (Amann i Graham, 1993; Storey, 1997). Per altra banda, alguns autors han

descrit que refrigerar el semen de cavall a temperatures superiors (15 i 20 ºC) mantené millor la motilitat i la fertilitat en les primeres 12h de conservació (Province *et al.*, 1985; Francl *et al.*, 1987). Batellier *et al.* (1997, 2001) han demostrat que el semen diluït amb INRA96 i mantingut a 15ºC millora la viabilitat i la fertilitat del semen de cavall en comparació amb altres diluents a base de llet.

En burro, existeixen alguns estudis que han avaluat diferents diluents i temperatures per refrigerar el semen i observar els efectes sobre la motilitat i la integritat de la membrana plasmàtica (Santos *et al.*, 1995; Cottorello *et al.*, 2002; Serres *et al.*, 2002; Gómez-Cuétara *et al.*, 2004). Santos *et al.* (1995) van estudiar diferents sistemes i velocitats de refrigeració del semen de ruc, concluent que velocitats de -0.6 i -1ºC/min eren més adequades que -3ºC/min per mantenir la motilitat del semen conservat a 5ºC en diluent de Kenney. Estudis realitzats amb el semen de ruc Zamorano-Leonés van observar que el semen diluït amb INRA82 i mantingut a 15ºC en condicions d'anaerobiosi era el que conservava millor la integritat de la membrana plasmàtica. Aquestes condicions mantenien la motilitat espermàtica per igual que la refrigeració a 4ºC (Serres *et al.*, 2002; Gómez-Cuétara *et al.*, 2004). En canvi, Cottorello *et al.* (2002) conclouent que utilitzant com a diluent Baken modificat (10% rovell d'ou) les temperatures més òptimes de refrigeració són 0 i 5ºC.

3.4. Criopreservació

La criopreservació espermàtica és un procediment que permet conservar els espermatozoides durant períodes de temps llargs, pràcticament il·limitats. Per això, l'ús de semen congelat en programes de inseminació artificial en espècies en perill d'extinció seria de gran ajuda. Permetria mantenir el material genètic d'animals d'alt valor genètic i poder reduir la endogàmia en determinades poblacions de rucs. Ara bé, el procés de congelació i descongelació de l'espermatozoide produeix estrès físic i químic a la

membrana plasmàtica reduïnt la seva viabilitat i capacitat fecundant. Per altra banda, s'ha descrit en cavall que la capacitat fecundant del semen congelat varia molt en funció dels sementals. En alguns sementals, el semen no es pot congelar perquè els seus espermatozoides no són capaços de resistir el procés de congelació/descongelació. (Katila, 2001).

La criopreservació de semen de ruc ha estat poc estudiada al llarg dels anys (Trimeche *et al.*, 1996, 1998; Silva *et al.*, 1997; Vidament *et al.*, 2008; Oliveira *et al.*, 2006; Álvarez *et al.*, 2004, 2006). Normalment els mètodes de criopreservació utilitzats són extrapolats dels mètodes de criopreservació de semen de cavall. Trimeche *et al.* (1998) va suggerir la utilització de glicerol, glutamina i rovell d'ou de perdiu per la congelació de semen de ruc de Poitou. No obstant, només va obtenir gestacions en burres quan rediluïa el semen descongelat abans de inseminar (8/21, gestacions/inseminades). Oliveira *et al.* (2006) van utilitzar diferents combinacions de crioprotectors (dimetilsulfòxid (DMSO), dimetilformamida (DMF), dimetilacetamida (DMA) i glicerol) obtenint bons resultats en el manteniment de la motilitat i la integritat de la membrana plasmàtica dels espermatozoides descongelats de burro. Malgrat tot, en el seu estudi no va aconseguir cap gestació en burres inseminades (53 cicles) amb semen congelat amb cap crioprotector (concentració total 2-5%). Álvarez *et al.* (2004) també van comparar diferents crioprotectors (metilformamida, DMA, metilacetamida i glicerol) obtenint resultats adequats de motilitat i viabilitat postdescongelació, especialment amb la DMA. Vidament *et al.* (2008) van estudiar la fertilitat obtinguda després de inseminar eugues i burres amb semen de ruc refrigerat i congelat (glicerol), observant fertilitats similars entre eugues i burres quan s'utilitzava semen refrigerat (45%), i fertilitats diferents quan s'inseminava amb semen congelat (eugues 36% vs burres 11%). En aquest mateix estudi, van intentar millorar la fertilitat del semen de burro congelat, eliminant el glicerol postdescongelació amb rentats o utilitzant la DMF com a crioprotector. No obstant, no van observar una millora de la fertilitat del semen congelat de ruc, fent necessaris més estudis sobre la

sensibilitat del semen de burro al procès de congelació i la resposta uterina de les burres en front a la inseminació artificial amb semen congelat amb diferents crioprotectors.

4. Avaluació de la qualitat del semen

4.1. Concentració espermàtica

L'avaluació de la concentració espermàtica és una valoració rutinària molt important per poder determinar el número d'espermatozoides totals d'un ejaculat i poder calcular el número de dosis que es poder produir de cada ejaculat. Normalment les eugues s'inseminen amb 500 x 10^6 d'espermatozoides mòtils progressius amb semen fresc i en semen refrigerat 24h a 5ºC (Brinsko, 2006). Aquesta dosi de inseminació és semblant a la utilitzada en les inseminacions de les burres (Vidament *et al.*, 2008)

Existeixen diferents mètodes per determinar la concentració espermàtica d'un ejaculat, les més utilitzades són les cambres de recompte cel·lular (Neubauer o Thoma) (Fig. 4) i els espectrefotòmetres. També existeixen comptadors cel·lulars electrònics, citometria de flux, colorimetria i programes informàtics d'anàlisi seminal, com l'ISAS (Integrated Semen Analysis System, PROISER), que permeten fer el recompte.

La cambra de recompte cel·lular determina el número d'espermatozoides per unitat de volum. El comptatge es realitza mitjançant un microscopi òptic (400x) de la mostra seminal diluïda amb una solució fixadora (citrat formulat). Aquest mètode és molt econòmic i senzill, tot i que com a inconvenient es requereix més temps per a realitzar-lo.

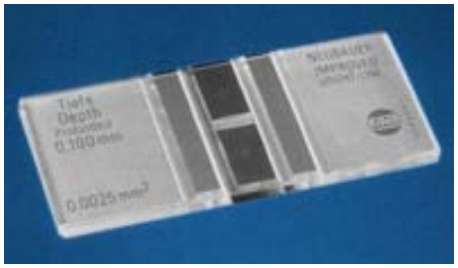

Figura 4. Cambre de recompte cel.lular Neubauer.

4.2. Viabilitat i anomalies morfològiques

La determinació de la integritat funcional de la membrana plasmàtica de l'espermatozoide ha sigut àmpliament estudiada. La pèrdua de permeabilitat selectiva de la membrana plasmàtica impedeix a l'espermatozoide de mantenir les concentracions necessàries d'ions i soluts que asseguren la motilitat i les funcions vitals de l'espermatozoide.

Existeixen diferents mètodes per avaluar la integritat de la membrana plasmàtica, entre ells la tinció de l'eosina-nigrosina (Bamba, 1988). Aquesta tinció és de membrana impermeable, on els espermatozoides que presenten una membrana plasmàtica estructuralment íntegra no permeten que la tinció penetri (viables). Aquesta tècnica tenyeix de color rosat els espermatozoides amb la membrana alterada (no viables), en canvi els espermatozoides vius s'observen sense tinció en un fons púrpura.

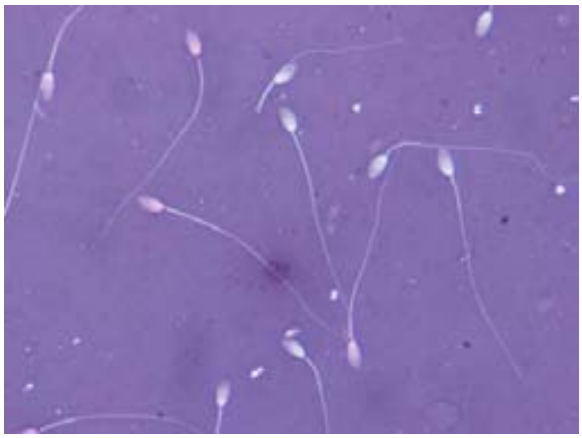

Figura 5. Tinció d'eosina-nigrosina per valorar la viabilitat i les anomalies espermàtiques. Els espermatozoides tenyits en rosa són morts, mentre que els no tenyits són vius.

L'avaluació de la morfologia espermàtica ens proporciona informació important sobre l'estat funcional de l'espermatogènesi i de la maduració epididimària. En cavalls, una reducció en el percentatge dels espermatozoides morfològicament normals és indicatiu d'una disminució de la fertilitat (Jasko *et al.*, 1990). Mitjançant la tinció vital de

l'eosina-nigrosina podem observar i categoritzar les anomalies morfològiques dels espermatozoides. Aquestes es classifiquen en primàries (malformacions durant l'espermatogènesi), secundàries (malformacions durant la maduració epididimària) i terciàries (conseqüència del mal maneig al laboratori).

4.3. Motilitat espermàtica

El percentatge d'espermatozoides mòtils d'una mostra seminal s'ha convertit en un anàlisi de laboratori rutinari per valorar la qualitat seminal. Aquesta valoració es pot realitzar mitjançant l'observació visual en microscòpia òptica o bé, mitjançant l'ajut d'un sistema d'anàlisi computeritzat (CASA). L'observació visual permet calcular el percentatge d'espermatozoides mòtils (motilitat total, MT), el percentatge d'espermatozoides amb motilitat progressiva (MP) i el vigor dels espermatozoides en una escala de 1-5. Aquest és un mètode poc objectiu, degut a les diferències de interpretació entre els individus observadors. Els sistemes CASA permeten reduir la variabilitat entre els anàlisis i realitzar una valoració més acurada de les característiques de moviment individual dels espermatozoides (Jasko, 1992; Verstegen *et al.*, 2002).

El sistema CASA es basa en la captura successiva de imatges a través d'un microscopi de contrast de fases que són digitalitzades. Cada espermatozoide observat és identificat en les successives imatges i es calcula a partir d'aquí la seva trajectòria. Aquesta trajectòria individual és processada i reflectida en una sèrie de paràmetres calculats matemàticament (Verstegen *et al.*, 2002). El sistema CASA està format per un microscopi de contrast de fases amb platina atemperada a 37ºC, una càmera de vídeo conectada amb una pantalla de ordinador i un software d'anàlisi de imatges per ordinador. El software s'encarrega de identificar i seguir els espermatozoides en les imatges de vídeo i realitzar els càlculs de les dades.

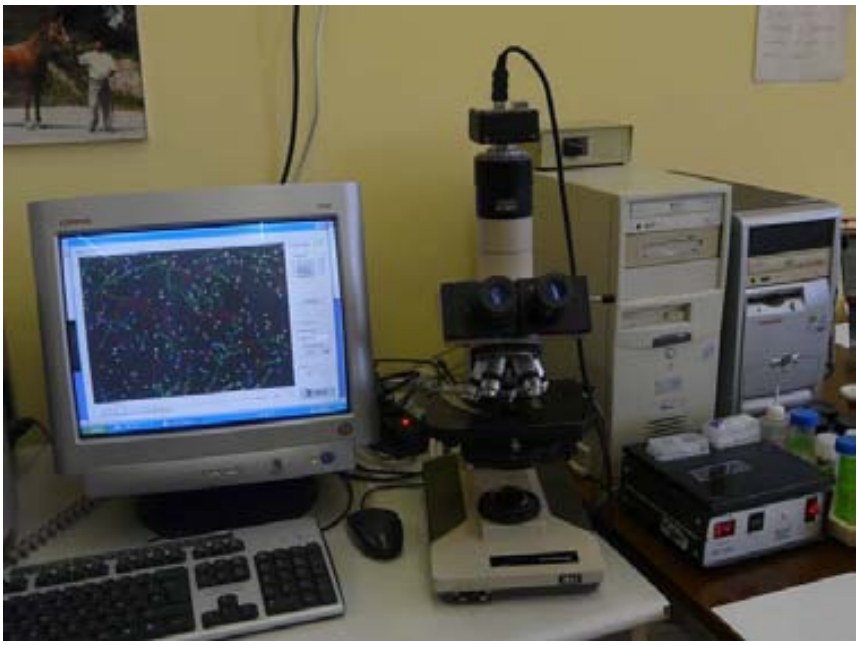

Figura 6. Avaluació de la motilitat espermàtica mitjançant el sistema ISAS (Integrated Semen Analysis System, PROISER).

Els paràmetres de motilitat determinats per la majoria de sistemes CASA són els següents:

Velocitat mitjana (VAP): velocitat de la trajectòria promig realitzada per l'espermatozoide (μm/s).

Velocitat curvilínia (VCL): distància transcorreguda per l'espermatozoide al llarg de la seva trajectòria real en funció del temps (μm/s).

Velocitat rectilínia (VSL): velocitat del recorregut en línia recta des del punt inicial al final (μm/s).

Índex de linealitat (LIN): relació percentual entre la VSL i VCL (%).

Índex de rectitud (STR): relació percentual entre la VSL i VAP (%).

Índex d'oscil·lació (WOB): relació percentual entre la VAP i VCL (%).

Amplitud lateral del cap (ALH): desplaçament lateral del cap dels espermatozoides mòtils (μm/s).

Freqüència de batuda (BCF): freqüència de batuda del flagel dels espermatozoides mótils (Hz).

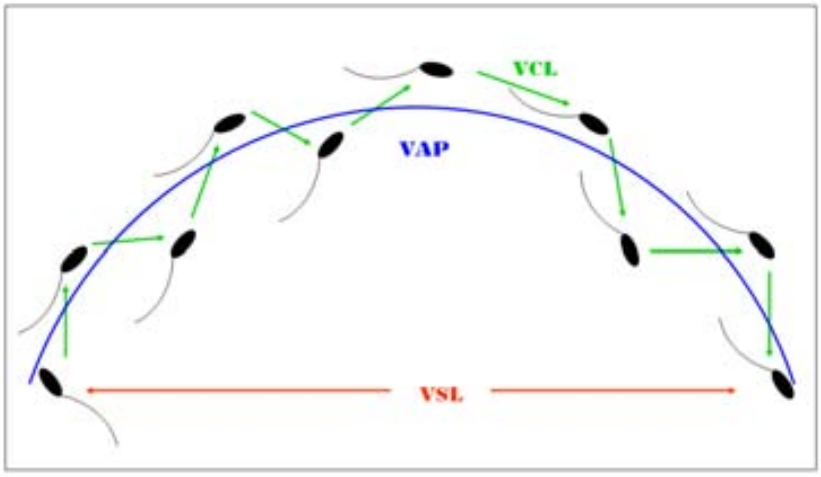

Figura 7. Representació gràfica d'alguns paràmetres de motilitat obtinguts pel sistema CASA.

La majoria d'estudis en burros realitzen l'anàlisi de la motilitat espermàtica mitjançant la visualització subjectiva en un microscopi òptic (Santos *et al.*, 1995; Gastal *et al.*, 1997; Henry *et al.*, 1999; Cottorello *et al.*, 2002). Trimeche *et al.* (1998), Miró *et al.* (2005) i Rota *et al.* (2008) utilitzen sistemes computeritzats per l'evaluació del semen de burro de Poitou, burro Català i burro Amiata (Taula 2).

Recentment, s'ha descobert que els ejaculats de diversos mamífers estan estructurats en subpoblacions espermàtiques ben definides i caracteritzades per valors precisos dels paràmetres de motilitat obtinguts gràcies al sistema CASA (Abaigar *et al.*, 1999; Rigau *et al.*, 2001; Quintero-Moreno *et al.*, 2003 i 2004; Martínez-Pastor *et al.*, 2005; Miró *et al.*, 2005; Quintero-Moreno *et al.*, 2007). La identificació de les subpoblacions espermàtiques en l'ejaculat dels mamífers ha esdevingut una qüestió de gran importància per l'avaluació d'aquest. L'aproximació clàssica de considerar tot l'ejaculat com una població homogènia i utilitzar els valors mitjans per classificar els ejaculats és considerat erroni (ESHRE Andrology Special Interest Group, 1998; Mortimer, 2000). Tot i aquest avenç, existeix una manca de informació per comprendre el paper fisiològic i la importància pràctica d'aquesta estructura de subpoblacions espermàtiques mòtils en l'ejaculat.

Paràmetres	Burro Poitou *Trimeche, 1996*	Burro Català *Miró, 2005*	Burro Amiata *Rota, 2008*
MT (%)	-	68.4±16.6	94.9±3.5
MP (%)	-	-	65.8±3.7
VCL (μm/s)	63.3±6.8	80.2±51.7	211.6±21.9
VSL (μm/s)	44.6±4.8	49.8±43.2	108.1±12.2
VAP (μm/s)	40.1±3.3	59.4±43.0	127.6±13.7
LIN (%)	77.5±7.0	60.5±28.8	51.0±3.9
STR (%)	-	79.2±24.5	82.8±2.8
ALH (Hz)	3.9±0.5	2.4±2.0	7.1±0.8

Taula 2. Paràmetres de motilitat de semen de burro Poitou, Català i Amiata. Número d'ejaculats evaluats en cada estudi són: Burro Poitou, 10 ejaculats x 4 individus; Burro Català, 78 ejaculats totals de 4 individus; Burro Amiata, 3 ejaculats x 4 individus.

4.4. Avaluació de la fecunditat *in vitro*

A fi de mantenir la màxima diversitat genètica i reduir l'endogàmia es poden utilitzar tecnologies reproductives com la inseminació artificial, la transferència embrionària i la fecundació *in vitro*. Per l'optimització d'aquestes tècniques és de gran importància la predicció de la capacitat fecundant del semen. Per la valoració *in vitro* del semen s'han utilitzat mètodes clàssics com la concentració espermàtica, les característiques de motilitat, el percentatge d'espermatozoides vius i l'estat de l'acrosoma. Tanmateix, aquests paràmetres són poc predictius de la capacitat fecundant del semen, només les mostres de molt poca qualitat seminal es poden detectar (Gadea, 2005; García-Alvarez *et al.* 2008).

Ara bé, existeixen tècniques *in vitro* que avaluen la capacitat dels espermatozoides per unir-se a la zona pel·lúcida dels oòcits o per fecundar oòcits homòlegs o heteròlegs i que poden ser d'utilitat per identificar canvis en la funció

espermàtica, que les proves estàndards no poden detectar. Yanagimachi *et al.* (1976) van descriure que els espermatozoides humans capacitats eren capaços de penetrar oòcits de hàmster en els quals se'ls havia eliminat la zona pel·lúcida. A partir d'aquest estudi, s'ha utilitzat el test de penetració heteròloga per avaluar la capacitat fecundant del semen humà. En animals de renda també s'han realitzat test de penetració heteròloga obtenint correlacions positives entre el test i la fecunditat *in vivo* (Bousquet i Brackett, 1982; Ramesha i Goswani, 1994). Landim-Alvarenga et *al.* (2001) recentment van utilitzar oòcits bovins sense zona pel·lúcida per valorar la penetració d'espermatozoides de cavalls.

El test de penetració heteròloga en oòcits sense zona pel·lúcida valora la capacitat de l'espermatozoide per unir-se a la membrana vitel·lina, penetrar l'oòcit i formar el pronucli. El valor del test és limitat a l'hora d'avaluar la capacitat de penetrar oòcits amb zona pel·lúcida (Brahmkshtri *et al.*, 1999). Tot i això, el test de penetració heteròloga en oòcits sense zona pel·lúcida monitoritza indirectament la presència d'espermatozoides que s'han capacitat i han sofert la reacció acrosòmica essent capaços de fusionar-se amb l'oòcit i formar pronuclis (Yanagimachi, 1984; Hanada *et al.*, 1976).

Bibliografia

Abaigar T, Holt WV, Harrison R, Barrio G. (1999) Sperm subpopulation 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.

Allen WR. (2005) The development and application of the modern reproductive technologies to horse breeding. Reprod Dom Anim 40: 310-329.

Álvarez AL, Serres C, Crespo F, Mateos E, Gómez-Cúetara C. (2004) Cryopreservation of Zamorano-Leonés donkey sperm using different amides. Reprod Dom Anim 39: 267 (Abstract).

Álvarez AL, Serres C, Torres P, Crespo F, Mateos E, Gomez-Cuetara C. (2006) Effect of cholesterol-loaded cyclodextrin on the cryopreservation of donkey spermatozoa. En: Evans, MJ. (Ed), Equine Reproduction IX. Anim Reprod Sci 94: 89-91.

Amann RP, Pickett BW (1987) Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. Eq Vet Sci 7: 145-173.

Amann RP, Graham JK (1993) Spermatozoal function. En: Mckinnon AO i Voss JL (Eds). Equine Reproduction. Lea & Febiger, Pennsylvania.

Aparicio G. (1960) Zootecnia Especial. Etnología Compendiada. Córdoba: Imprenta Moderna.

Aranguren JA. (2002) Caracterización y relaciones filogenéticas de cinco razas asnales españolas en peligro de extinción mediante la utilización de marcadores microsatélites: su importancia en los programas de conservación. Tesis Doctoral, Universitat Autònoma de Barcelona, Barcelona.

Aurich C. (2005) Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. Anim Reprod Sci 89: 65-75.

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

Bamba K. (1988) Evaluation of acrosomal integrity of boar spermatozoa by bright field microscope using an eosin-nigrosin stain. Theriogenology 29:1245-1251.

Batellier F, Magistrini M, Fauquant J, Palmer E. (1997) Effect of milk fractions on survival of equine spermatozoa. Theriogenology 48: 391-410.

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

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

Blanchard TL, Taylor TS, Love CL. (1999) Estrous cycle characteristics and response to estrus synchronization in mammoth asses (*Equus asinus americanus*). Theriogenology 52: 827-834.

Bousquet D, Brackett BG. (1982) Penetration of zona-free hamster ova as a test to assess fertilizing ability of bull sperm after frozen storage. Theriogenology 17: 199-213.

Brahmkshtri BP, Edwin MJ, John MC, Nainar AM, Krishnan AR. (1999) Relative efficacy of conventional sperm parameters and sperm penetration bioassay to assess bull fertility in vitro. Anim Reprod Sci 54: 159-168.

Briggs HM. (1971) Razas modernas de animales domésticos. Zaragoza: Acribia.

Brinsko SP. (2006) Insemination doses: How low can we go?. Theriogenology 66: 543-550.

Clayton HM, Lindsay FEF, Forbes AC, Hay LA. (1981) Some studies of comparative aspects of sexual behaviour in ponies and donkeys. Appl Anim Ethol 7: 169-174.

Cottorello ACP, Amancio RC, Henry M, Borges I. (2002) Effect of storage temperature and extenders on “in vitro” activity of donkey spermatozoa. En: Evans, MJ (Ed), Equine Reproduction VIII. Theriogenology 58: 325-328.

Dadarwal D, Tandon SN, Purohit GN, Pareek PK. (2004) Ultrasonographic evaluation of uterine involution and postpartum follicular dynamics in French jennies (*Equus asinus*). Theriogenology 62: 257-264.

DAD-IS. Information System Domestic Animal Diversity. FAO 1997 (http://dad.fao.org).

Epstein H. (1984) Ass, mule and onager. In: Mason IL (Ed). Evolution of Domesticated Animals. London and New York: Longman.

ESHRE Andrology Special Interest Group. (1998) Guidelines on the application of CASA technology in the analysis of spermatozoa. Huma Reprod 13: 2512-2520.

Ferreira MF, Jones DN, Gonçalves A. (1991) Effect of centrifugation and diluent on the storage of cooled donkey semen. IX congresso brasileiro de reproduçao animal, vol. II: 452.

Francl AT, Amann RP, Squires EL, Pickett BW. (1987) Motility and fertility of equine spermatozoa in a milk extender after 12 or 24 hours at 20ºC. Theriogenology 27:517-525.

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

García-Álvarez O, Maroto-Morales A, Martínez-Pastor F, Fernández-Santos MR, Esteso MC, Pérez-Guzmán MD, Soler AJ. (2008) Heterologous in vitro fertilization is a good procedure to assess the fertility of thawed ram spermatozoa. Theriogenology 71:643-650.

Gastal MO, Henry M, Beker AR, Gastal EL, Gonçalves A. (1996) Sexual behavior of donkey jacks: Influence of ejaculatory frecuency and season. Theriogenology 46: 593-603.

Gastal MO, Henry M, Beker AR, Gastal EL. (1997) Effect of ejaculation frequency and season on donkey jack semen. Theriogenology 47:627-638.

Ginther OJ. (1992) Reproductive Biology of the Mare: Basic and Aplied Aspects. Cross Plains, WI: Equiservices Publishing.

Ginther OJ, Scraba ST, Bergfelt DR. (1987) Reproductive seasonality of the jenney. Theriogenology 27:587-592.

Gómez-Cuétara C, Álvarez AL, Rodríguez A, Santiago I, Mateos E, Serres C. (2004) Refrigeration of Zamorano-Leonés donkey semen collected with an Open-ended artificial vagina. Reprod Dom Anim 39: 267 (Abstract).

Hall SJG. (1993) Strategies for genetic improvement of local livestock and their consequence for conservation. In: Cartwright A (Ed). World Agriculture. London: Sterling Publishing Group PLC, 75-7.

Hanada A, Chang MC. (1976) Penetration of hamster and rabbit zona-free eggs by rat and mouse spermatozoa with special reference to spermatozoa capacitation. J Reprod Fertil 46: 239-241.

Henry M, Figueiredo AEF, Palhares MS, Coryn M. (1987) Clinical and endocrine aspects of the oestrous cycle in donkeys (*Equus asinus*). J Reprod Fert Suppl 35:297-303.

Henry M, Lodi LD, Gastal MMFO. (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, McDonnell SM, Lodi LD, Gastal EL. (1991) Pasture mating behaviour of donkeys (*Equus asinus*) at natural and induced oestrus. J Reprod Fert Suppl 44:77-86.

Henry M, Silva KM, Souza MCN, Resille DN, Melo MIV, Becker ARCL. (1999) Seminal parameters of donkeys: influence of the season. Rev Bras Reprod Anim 23 (3):237-238.

IUCN/UNEP/WWF/FAO/UNESCO. (1980) World Conservation Strategy. Living resources conservation for sustainable development. IUCN, Switzerland.

Jasko DJ, Lein DH, Foote RH. (1990) Determination of the relationship between sperm morphologic classification and fertility in stallions: 66 cases (1987-1988). JAVMA 197 (3): 389-394.

Jasko DJ, Moran DM, Farlin ME, Squires EL. (1991) Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. Theriogenology 35: 1059-1067.

Jasko DJ. (1992) Evaluation of stallion semen. Vet Clin North Am [Equine Pract] 8:129–148.

Jordana J, Folch P. (1996) The endangered catalonian donkey breed: the main ancestor of the american ass or mammoth. J Eq Vet Sci 16(10):436-441.

Katila T. (1997) Procedures for handling fresh stallion semen. Theriogenology 48:1217-1227.

Katila T. (2001) In vitro evaluation of frozen-thawed stallion semen: a review. Acta Vet Scand 42: 199-217.

Kenney RM, Bergman RV, Cooper WL. (1975) Minimal contamination techniques for breeding mares: technique and preliminary findings. Proc Am Assoc Equine Pract 21: 327-336.

Klingel H. (1974) Soziale organisation und verhalten des Grevy-zebras (*Equus grevyi*). Z. Tierpsychol. 23:7-11.

Klingel H. (1977) Observations on social organization and bahaviour of African and Asiatic wild asses (*Equus africanus* and *E. hemionus*). Z Tierpsychol 44:323-331.

Kreuchauf A. (1984) Reproductive physiology in the jackass. Anim Res Dev 20:51-78.

Landim-Alvarenga FC, Alvarenga MA, Seidel GE, Squires EL, and Graham JK. (2001) Penetration of zona-free hamster, bovine and equine oocytes by stallion and bull spermatozoa pretreated with equine follicular fluid, dilauroylphosphatidyl-choline or calcium ionophore A23187. Theriogenology 56:937-953.

Lindeberg H, Kuntsi-Vaattovaara H, Katila T. (1992) Predicting ovulation in the mare. In: comunication, 12th International Congress on Animal Reproduction. The Hague, Netherlands.

Lobo VL. (2003) Comportamiento sexual y normalización de las características seminales del burro catalan. Universitat Autónoma de Barcelona. Facultat de Veterinària/Unitat de Reproduccció. Treball de Master 9-11.

Malmgren L, den Kamp BO, Wöckener A, Boyle M, Colenbrander B. (1994) Motility, velocity and acrosome integrity in equine spermatozoa stored under different conditions. Reprod Domest Anim. 29. 469-476.

Martínez-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.

Mason IL. (1974) Introduction to round table a: The conservation of animal genetic resources. Proc of the 1st World Congress of Applied Livestock Production 2:13-21.

McDonnell SM. (2000) Reproductive behavior of stallions and mares: comparison of free-running and domestic in-hand breeding. Anim Reprod Sci 60-61:211-219.

Meira C, Ferreira JCP, Papa FO, Tornero MTT, Bicudo SD. (1995) Study of the estrous cycle in donkeys (Equus asinus) using ultrasonography and plasma progesterone concentrations. Biol Reprod Mono 1:403-410.

Mello SL, Henry M, Souza MC, Oliveira SM. (2000) Effect of split ejaculation and seminal extenders on longevity of donkey semen preserved at 5ºC. Arq Bras Med Vet Zootec 52: 372-378.

Miró J, Lobo V, Medrano A, Rigau T. (2003) Dinamica folicular en la burra catalana. En: Comunicación, IV Congreso Ibérica de Reproducción Animal. Arucas (Las Palmas).

Miró J, Muñoz F, Piedrafita J, Quintero-Moreno A. (2004) Prediction of the day of ovulation in mares through physiological parameters measured during estrous. Revista Científica, FCV-LUZ XIV(1):54-58.

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.

Mohr E. (1971) The Asiatic Wild Horse. London: JA Allen and Co Ltd.

Moran DM, Jasko DJ, Squires EL, Amann RP. (1992) Determination of temperature and cooling rate which induce cold shock in stallion spermatozoa. Theriogenology 38: 999-1012.

Mortimer ST. (2000) CASA-practical aspects. J Androl 21: 514-524.

Nishikawa Y. (1959) Studies on Reproduction in Horses. Tokyo: Japan Racing Assoc 279-300.

Nishikawa Y, Yamazaki Y. (1949a) Studies on reproduction in asses I. Breeding season, oestrous cycle and length of heat. Jpn J Zootech Sci 19:119-124.

Nishikawa Y, Yamazaki Y. (1949b) Studies on reproduction in asses II. Growth of follicles in ovaries and ovulation. Jpn J Zootech Sci 20:29-32.

Oliveira JV, Alvarenga MA, Melo CM, Macedo LM, Dell'Aqua JA, Papa FO. (2006) Effect of cryoprotectant on donkey semen freezability and fertility. Anim Reprod Sci 94: 82-84.

Parés PM, Vilaró T. (1994) La Ramaderia. Quaderns de la Revista de Girona 52:68-69.

Parinaud J, Le Lannou D, Vieitenz G, Griveau J-F, Milhet P, Richoilley G. (1997) Enhancement of motility by treating spermatozoa with an antioxidant solution (Sperm-Fit©) following ejaculation. Hum. Reprod. 12: 2434-2436.

Pickett BW, Sullivan JJ, Byers MS, Pace MM, Remmenga EE. (1975) Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. Fert Steril 26: 167-174.

Pierson RA. (1993) Folliculogenesis and Ovulation. In: Angus O, Mckinnon J, Voss JL (Eds). Equine Reproduction. Philadelphia/London: Lea & Febiger 161-171.

Province CA, Squires EL, Pickett BW, Amann RP. (1985) Cooling rates, storage temperatures and fertility of extended equine spermatozoa. Theriogenology 23:925-934.

Pukazhenthi BS, Wildt DE. (2004) Which reproductive technologies are most relevant to studying, managing and conserving wildlife?. Reproduction, Fertility and Development 16: 33-46.

Purdy SR. (2005) Ultrasound examination of the female miniature donkey reproductive tract. In: Matthews NS and Taylor TS (Eds). Veterinary Care of Donkeys. International Veterinary Information Service, Ithaca NY (www.ivis.org).

Quintero-Moreno A, Miró J, Rigau T, Rodríguez-Gil JE. (2003) Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 59: 1973-1990.

Quintero-Moreno A, Rigau T, Rodríguez-Gil JE. (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 JE. (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 Dom Anim 42: 312-319.

Ramesha KP, Goswami SL. (1994) Zona-free hamster egg penetration test for assessing fertility of Karan-swiss and Karan-fries bulls. Indian Vet. J. 71, 47–50.

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

Romagosa JA. (1959) El garañón Catalán. Tesis Doctoral. Universidad de Madrid, Madrid.

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

Roy AK, Yadav MP, Sengar OPS. (2003) Effect of season on the physical characteristics of donkey (Equus asinus) semen. Indian J Anim Sci 73: 620-622.

Santos GF, Henry M, Sampaio IBM, Gastal EL. (1995) Effect of cooling system and rate of cooling on sperm quality of donkey semen preserved at 5ºC. Biol Reprod Monograph 1: 761-767.

SAS. SAS/STAC Software : SAS Inst. Inc. Carry, NC. USA 1996.

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

Serres C, Rodríguez A, Álvarez AL, Santiago I, Gabriel J, Gómez-Cúetara C, Mateos E. (2002) Effect of centrifugation and temperature on motility and plasma membrane integrity of Zamorano-Leonés donkey semen. Theriogenology 58: 329-332 (Abstract).

Silva SS, Henry M, Nunes SA, Mello SLV. (1997) Influencia do sistema de envasamento sobre a qualidade espermatica de jumentos (Equus asinus) avaliada “in-vitro” pos-descongelacao. Revista Brasileira de Reproducao Animal 21: 140-146.

Sotillo JL, Serrano V. (1985) Producción Animal 1. Etnología Zootécnica. Madrid:Tebar-Flores.

Storey BT. (1997) Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Mol Hum Reprod 3: 203-213.

Tibary A, Sghiri A, Bakkoury M, Fite C. (2006) Reproductive patterns in donkeys. In: Prooceedings of the 9th International Congress of World Equine Veterinary Association, Marrakech, Morocco 311-319.

Trimeche A, Tainturier D. (1995) Étude échographique de la cinétique folliculaire des baudettes du Poitou pendant l’oestrus au cours du printemps et de l’été. Revue Méd Vét 146(11):743-748.

Trimeche A, Renard P, Le Lannou D, Barrière P, Tainturier D. (1996) Improvement of motility of post-thaw Poitou jackass sperm using glutamine. Theriogenology 45: 1015-1027.

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

Vandeplassche M, Wesson JA, Ginther OJ. (1981) Behavioral, follicular and gonadotropin changes during the estroud cycle in donkeys. Theriogenology 16:239-249.

Varner DD, Blanchard TL, Love CL, Garcia MC, Kenney RM. (1987) Effects of semen fractionation and dilution ratio on equine spermatozoal motility parameters. Theriogenology 28: 709-718.

Varner DD, Blanchard TL, Love CL, Garcia MC, Kenney RM. (1988) Effects of cooling rate and storage temperature on equine spermatozoal motility parameters. Theriogenology 29: 1043-1054.

Varner DD, Blanchard TL, Meyers PJ, Meyers SA. (1989) Fertilizing capacity of equine spermatozoa stored for 24 hours at 5 or 20ºC. Theriogenology 32: 515-525.

Vendramini OM, Guintard C, Moreau J, Tainturier D. (1998) Cervix conformation: a first anatomical approach in Baudet du Poitou jenny asses. Animal Science 66:741-744.

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

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: 22-25.

Yanagimachi R, Yanagimachi H, Rogers BJ. (1976) The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of, human spermatozoa. Biol Reprod 15:471–6.

Yanagimachi R. (1984) Zona free hamster eggs: their use in assessing fertilizing capacity and examining chromosomes of human spermatozoa. Gamete Res 10: 187-232.

Objectius / Aims

Objectius / Aims

L'objectiu general d'aquesta tesi és el coneixement de les característiques reproductives del mascle i de la femella del burro Català amb la finalitat d'aplicar tecnologies reproductives que potenciïn la seva cria i evitin l'extinció d'aquesta raça de burro.

Els objectius específics són els següents:

- Estudi del comportament sexual i anàlisi de les característiques del cicle estral i del zel, la seva durada i estacionalitat.
- Estudi de la dinàmica fol·licular, en especial durant el període preovulatori mitjançant l'ecografia transrectal.
- Anàlisi i selecció dels millors paràmetres per formular un model de predicció de l'ovulació en la burra Catalana, assegurant la màxima precisió en el moment d'aplicar les tecnologies reproductives.
- Determinar l'efecte del plasma seminal en el semen refrigerat (5ºC) de burro Català, observant si existeixen canvis en l'estructura subpoblacional espermàtica.
- Determinar quin mètode, la centrifugació o dilució del semen, és el més òptim pel manteniment de la qualitat seminal de dosis refrigerades (5ºC).
- Avaluar l'efecte de diferents temperatures (5, 15 i 20ºC) sobre la viabilitat i la motilitat del semen de ruc Català durant el transport al laboratori per la posterior centrifugació.
- Determinar l'efecte del procés de congelació/descongelació en l'estructura subpoblacional espermàtica en dues espècies de mamífers distants filogenèticament com el burro i el porc.
- Desenvolupar un model de valoració de la qualitat seminal del burro Català mitjançant l'interacció entre els espermatozoides de semen fresc i descongelat de ruc Català amb oòcits bovins lliures de zona pel·lúcida.

Capítols / Chapters

I

Oestrus cycle characteristics and prediction of ovulation in Catalonian jennies.

E. Taberner, A. Medrano, A. Peña, T. Rigau, J. Miró

Department of Animal Medicine and Surgery, School of Veterinary Medicine, Autonomous University of Barcelona,
Campus UAB, Edifici V, E-08193 Bellaterra, Spain

Received 7 April 2008; revised 10 June 2008; accepted 18 June 2008

Theriogenology 70 (2008) 1489-1497

Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 70 (2008) 1489–1497
Theriogenology
www.theriojournal.com

Oestrus cycle characteristics and prediction of ovulation in Catalonian jennies

E. Taberner *, A. Medrano, A. Peña, T. Rigau, J. Miró

Department of Animal Medicine and Surgery, School of Veterinary Medicine, Autonomous University of Barcelona, Campus UAB, Edifici V, E-08193 Bellaterra, Spain

Received 7 April 2008; received in revised form 10 June 2008; accepted 18 June 2008

Abstract

The Catalonian donkey breed is in danger of extinction, and much needs to be learned about the reproductive features of its females if breeding and conservation programmes are to be successful. This study reports the oestrous behaviour, oestrus cycle characteristics and dynamic ovarian events witnessed during 50 oestrous cycles (involving 106 ovulations) in 10 Catalonian jennies between March 2002 and January 2005. These jennies were teased, palpated transrectally and examined by ultrasound using a 5 MHz linear transducer—daily during oestrus and every other day during dioestrus. Predictors of ovulation were sought among the variables recorded.

The most evident signs of oestrus were mouth clapping (the frequent vertical opening and closing of the mouth with ears depressed against the extended neck) and occasional urinating and winking of the vulval lips (homotypical behaviour). Interactions between jennies in oestrus were also recorded, including mounting, herding/chasing, the Flehmen response, and vocalization (heterotypical behaviour).

Nine jennies ovulated regularly throughout the year; one had two anovulatory periods (54 and 35 days). The length of the oestrus cycle was 24.90 ± 0.26 days, with oestrus itself lasting 5.64 ± 0.20 days (mean ± S.E.M.) and dioestrus 19.83 ± 0.36 days. The incidence of single, double and triple ovulations was 55.66% ($n = 59$), 42.45% ($n = 45$) and 1.89% ($n = 2$), respectively. No significant difference was seen in the number of ovulations involving the left and right ovaries (52.63% [$n = 70$] compared to 47.37% [$n = 63$] respectively; $P > 0.05$). The mean interval between double ovulation was 1.44 ± 3.98 days. The mean diameter of the preovulatory follicle at day −1 was 44.9 ± 0.5 mm; the mean growth rate over the 5 days before ovulation was 3.7 mm/day.

Data on preovulatory changes in oestrous behaviour, follicle size, follicle texture, the echographic appearance of the follicle and uterus, and uterine tone were subjected to stepwise logistic regression analysis to detect predictors of ovulation. The logit function showed the best predictors to be follicle size, follicular texture and oestrous behaviour. Certain combinations of these three variables allow the prediction of ovulation within 24 h with a probability of >75%.

Keywords: Oestrus cycle; Prediction of ovulation time; Jennies

1. Introduction

The Catalonian donkey is an endangered local donkey breed that can be found in a number of Pyrenean and pre-Pyrenean areas of Catalonia (northeastern Spain) [1]. The Catalonian donkey has contributed to the development of other breeds, such as the American

* Corresponding author. Tel.: +34 935 811 959; fax: +34 935 812 006.
E-mail addresses: estertaberner@gmail.com (E. Taberner), jordi.miro@uab.cat (J. Miró).

0093-691X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2008.06.096

Mammoth Ass and the Italian Pantellaria, Ragusana and Martina Franca breeds.

Numerous publications describe the characteristics of the oestrus cycle and reproductive behaviour of mares (*Equus caballus*). However, studies on reproductive behaviour, the oestrus cycle and ovulation in donkeys are almost non-existent. Among the literature available, Nishikawa and Yamasaki [2] report the characteristics of the oestrus cycle in *Equus asinus*, Henry et al. [3] and Meira et al. [4] report the same in the Pega breed and in crossbreed jennies from Brazil, and Blanchard et al. [5] report the reproductive behaviour, the ovarian dynamics and the hormonal changes during the oestrous cycle of Mammoth Ass jennies in Texas. However, little is known about the differences between Catalonian and other jennies in these respects—a lack of basic knowledge that hinders the use of reproductive technologies [6].

The main aims of this study were to examine the oestrous cycle of the Catalonian donkey and to establish indicators that predict the exact moment of ovulation. Such knowledge should help maximize the production of live foals and help conserve the breed.

2. Materials and methods

2.1. Experimental animals and study location

This study was undertaken at the Experimental Farm and Countryside Service of the Veterinary Faculty of the Autonomous University of Barcelona (41°N) between March 2002 and January 2005. The animals involved were 10 clinically healthy, cycling Catalonian jennies aged 3–12 years. All were kept outdoors in a group and were fed grain forage, straw, hay, and water *ad libitum*. Body weight was monitored throughout the study; all the jennies showed a good body condition. Jacks were maintained in nearby paddocks and fed similar diets. Oestrous behaviour, oestrous cycle characteristics and the prediction of ovulation were studied during 50 oestrous cycles (106 ovulations).

2.2. Oestrus detection and ultrasonic examination

Jennies were teased, palpated transrectally and examined by ultrasound using a 5 MHz linear transducer (Aquila Vet, Esaote Pie Medical®, Pie Medical Equipment B.V., Maastricht, The Netherlands) daily during oestrus and every other day during dioestrus. The durations of 50 complete cycles were recorded, including the length of 36 oestrus periods and 23 dioestrus periods. The largest diameter of the preovulatory follicle and the changes in follicular shape were recorded. Ovulation was defined (day 0) as the day when the preovulatory follicle was irregular in outline or when the corpus rubrum was observed.

Six different variables were evaluated and categorized as below:

1. Oestrous behaviour

0 points	no oestrus, resistance
1 point	indifference
2 points	interested, mouth clapping
3 points	mouth clapping, urination, vulvar activity and immobilization response

2. Follicle size

0 points	<35 mm
1 point	35–39 mm
2 points	40–44 mm
3 points	>45 mm

3. Follicle texture

0 points	fibrous ovary
1 point	turgid
2 points	elastic
3 points	soft
4 points	oedematous and dolorous

4. Echographic appearance of the follicle

0 points	round
1 point	oval
2 points	triangular with ovulation conus
3 points	irregular

5. Echographic appearance of the uterus

0 points	grey and diffuse
1 point	irregular black zones
2 points	regular black zones

6. Uterine tone

0 points	poor
1 point	fair
2 points	good

2.3. Statistical analysis

All analyses were performed using the SAS® statistical package (Statistical Analysis System, SAS, Institute Inc., Release 8.2, Cary, NC, USA, 2001).

The durations of the oestrous cycle, oestrus and dioestrus were represented as means ± S.E.M. The PROC GLM procedure was used to compare the percentages of ovulation from the right and left ovaries. Differences between preovulatory follicle diameters in

simple and double ovulations were determined using the PROC GLM procedure.

Preovulatory changes in oestrous behaviour, follicle texture, follicle size, echographic follicle appearance, uterine tone and echographic uterine appearance were analysed taking into account the number of days to ovulation. Data for most jennies were recorded on two or more occasions and analysed using the PROC MIXED (SAS software) routine including the effect of the individual jenny as a random factor.

The independent variables that influence ovulation were selected by stepwise logistic regression. The relationship between these variables and time to ovulation (up to 5 days) was studied using stepwise logistic regression for ordinal responses, employing the logit function as the link function. The logit function is defined as

$$P(x_i) = \frac{1}{1 + e^{-x_i'\beta}}$$

where, $P(x_i)$ is the probability of ovulation (or non-ovulation) within a given time, and $x_i'\beta$ the product of the vectors of the observed variables (x_i') in each individual and the partial regression coefficients (β). This analysis was undertaken using the PROC LOGISTIC routine. Variables with P-values of <0.05 were selected as predictive of ovulation.

3. Results

3.1. Sexual behaviour

Sexual behaviour was observed when the jennies were teased. On some occasions, the simple arrival of the jennies at the place where they were teased evoked oestrous signs such as mouth clapping (frequent vertical opening and closing of the mouth with ears depressed against the extended neck). During pre-copulatory interaction the attending jack vocalized, mounted without erection, nibbled the head, neck and extremities of the jenny, sniffed the perineal area and showed the Flehmen response. The jennies showed mouth clapping, sialorrhea, immobility and no aggression towards the jack—except for one jenny that maintained a permanently aggressive attitude towards the males. This jenny nonetheless showed a preovulatory follicle with a diameter of >35 mm, although ovulation was delayed. Occasionally, the older jennies showed tail raising, urinating and contraction and relaxation of the labia of the vulva with eversion of the clitoris.

Within their group the jennies also expressed heterotypical sexual behaviour, included chasing one another, mounting, the Flehmen response and sniffing.

3.2. Oestrus cycle and ovulation

Over the 30 months of the study, 9 of the 10 jennies cycled throughout the entire period, with no anovulatory season. Only one jenny showed anovulatory oestrus periods at the end of 2002 and 2003 (54 and 35 days, respectively), with follicles <30 mm in diameter.

The mean length of the oestrus cycle, oestrus and dioestrus was 24.9 ± 0.26 (range 22–29), 5.64 ± 0.2 (range 4–8) and 19.83 ± 0.36 (range 17–23) days, respectively (mean ± S.E.M.). Table 1 shows the mean data for individual jennies. The duration of oestrus was longer in December to February than in other months (6.75 ± 0.25 compared to 5.52 ± 0.22 days [mean ± S.E.M]).

A slightly (but not significantly) greater frequency of ovulation was recorded for the left ovary (52.63% [$n = 70$] compared to 47.37 [$n = 73$]; $P > 0.05$)

Table 1
Mean duration of the oestrus cycle, length of oestrus, and length of dioestrus in 10 Catalonian jennies

Jenny	Oestrus cycle (days)	n	Oestrus (days)	n	Dioestrus (days)	n
1	25.25 ± 0.84	8	5.88 ± 0.48	8	20.17 ± 0.83	6
2	24.71 ± 0.66	7	4.75 ± 0.75	4	20 ± 1.00	3
3	27 ± 0.41	4	6.25 ± 0.75	4	20.67 ± 0.88	3
4	23 ± 0	2	5 ± 0	3	18 ± 0	2
5	24 ± 0.55	5	5.33 ± 0.88	3	18.5 ± 1.50	2
6	26.4 ± 1.03	5	8 ± 0	1	–	–
7	25.5 ± 1.50	2	–	–	–	–
8	24.6 ± 0.60	5	5.33 ± 0.33	3	21 ± 0	2
9	23.57 ± 0.43	7	6 ± 0.41	4	18.33 ± 0.33	3
10	25 ± 0.45	5	5.5 ± 0.43	6	21.5 ± 0.50	2
Mean	24.90 ± 0.26	50	5.64 ± 0.20	36	19.83 ± 0.36	23

Results are expressed as means ± S.E.M.

Table 2
Distribution of ovulation (left and right ovaries)

Jenny	Left ovary (*n*)	Right ovary (*n*)
1	4	6
2	5	4
3	10	8
4	8	9
5	5	4
6	8	5
7	6	7
8	7	6
9	10	8
10	7	6
Total	70	63
Percentage (%)	52.63	47.37

(Table 2). The incidence of single, double and triple ovulations was 55.66% ($n = 59$), 42.45% ($n = 45$) and 1.89% ($n = 2$), respectively (Table 3). Some 45% of multiple ovulations occurred in just three jennies (jenny 3 produced more than one follicle in 7 out of 14 cycles studied, jenny 9 did so in 11 out of 12, and jenny 10 did so in 6 out of 10). Fourteen (41.18%) double ovulations occurred on the same day, 7 (20.59%) occurred with an interval of 1 day between ovulations, 9 (26.47%) occurred with an interval of 2 days, and 1 (2.94%) occurred with an interval of 4, 5, 6 or 9 days between the ovulation events. The mean of the interval between multiple ovulations was 1.44 ± 3.98 days (range 0–9 days). These multiple ovulations involved the left ovary alone on 11 occasions (23.4%), the right ovary alone on 9 occasions (19.15%), and both ovaries on 27 occasions (57.45%).

3.3. Follicular dynamics

The mean diameter of the preovulatory follicles at 1 day before ovulation was 44.9 ± 0.5 mm (mean ± S.E.M.) (range 35–60 mm). In multiple ovulations the average size of the preovulatory follicles was 44.1 ± 0.6 mm (range 35–55.4 mm), and in single ovulations 46.3 ± 0.9 mm (range 37–60 mm) ($P > 0.05$) (Table 4). The preovulatory follicles involved in synchronic and asynchronic double ovulations had mean diameters of 44.1 ± 0.9 and 44.0 ± 1.0 mm, respectively ($P > 0.05$).

The growth rate of the preovulatory follicle was 3.7 mm per day over days −5 to −1, but this fell over the last 24 h before ovulation (Table 5).

Only in one jenny (in 2 of 14 oestrus cycles - in January and May) did the preovulatory follicles fail to reach the ovulation stage; these follicles were transformed into what seemed to be hemorrhagic follicles. The latter follicles reached 46 and 61 mm in diameter and showed an interior fibrin network; the follicular wall was also thicker than normal (4–7 mm). The next ovulation occurred after 23 and 46 days, respectively (Fig. 1).

Table 4
Diameters of preovulatory follicles at 1 day before ovulation in single and double ovulations

Mean diameter of preovulatory follicle (mm)				
Jenny	Single ovulation	*n*	Double ovulation	*n*
1	52.3 ± 2.9	4		0
2	39.5 ± 2.5	2	40.3 ± 1.8	7
3	48.6 ± 5.4	2	43.2 ± 1.2	11
4	46.7 ± 2.4	3	44.9 ± 1.3	10
5	43.6 ± 1.5	4		0
6	44.3 ± 3.4	3	46.1 ± 1.8	7
7	48.3 ± 2.5	6	48 ± 1.2	8
8	47.4 ± 2.4	7	48 ± 5.0	2
9	41	1	41.6 ± 1.2	11
10	43.1 ± 0.7	4	44.4 ± 3.7	4
Mean	46.3 ± 0.9	36	44.1 ± 0.6	60

Results are expressed as means ± S.E.M.

Table 3
Percentage of single, double and triple ovulations

Jennies	Single ovulations (%)	*n*	Double ovulations (%)	*n*	Triple ovulations (%)	*n*	Total ovulations
1	62.50	5	37.50	3			8
2	33.33	2	66.67	4			6
3	50.00	7	50.00	7			14
4	64.29	9	35.71	5			14
5	88.89	8	11.11	1			9
6	44.44	4	55.56	5			9
7	66.67	8	33.33	4			12
8	91.67	11	8.33	1			12
9	8.33	1	75.00	9	16.67	2	12
10	40.00	4	60.00	6			10
Total	55.66	59	42.45	45	1.89	2	106

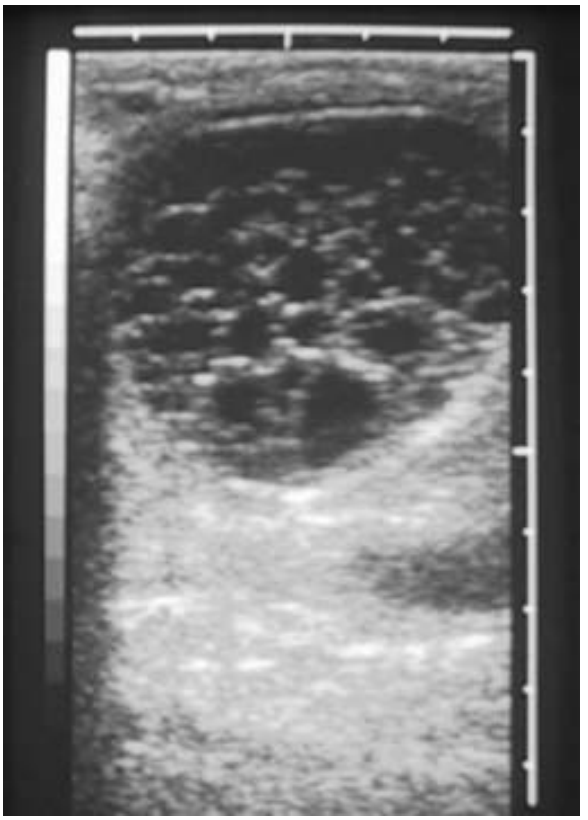

Fig. 1. Hemorrhagic follicle (diameter 46 mm).

Table 5
Growth rate of preovulatory follicles over days −5 to −1

Day before ovulation	Follicle growth rate (mm/day)	*n*
−5 to −4	4.3 ± 0.4	24
−4 to −3	3.8 ± 0.2	63
−3 to −2	3.9 ± 0.2	94
−2 to −1	3.3 ± 0.2	73
−5 to −2	3.9 ± 0.1	181
−5 to −1	3.7 ± 0.1	254

Results are expressed as means ± S.E.M.; day 0: ovulation.

A corpus luteum (CL) with a homogeneous echogenic texture was observed in the majority of jennies the first day after ovulation; the remainder showed a CL with a non-echogenic central area (Fig. 2).

3.4. Prediction of the ovulation

3.4.1. Changes in other variables over the days before ovulation

Table 6 shows the changes in the variables measured. Oestrous behaviour scores increased over oestrus as the jennies showed more interest in and greater receptivity towards jacks; significant differences were seen between day −5 and days −1, −2 and −3 ($P < 0.05$). Follicle size increased significantly on

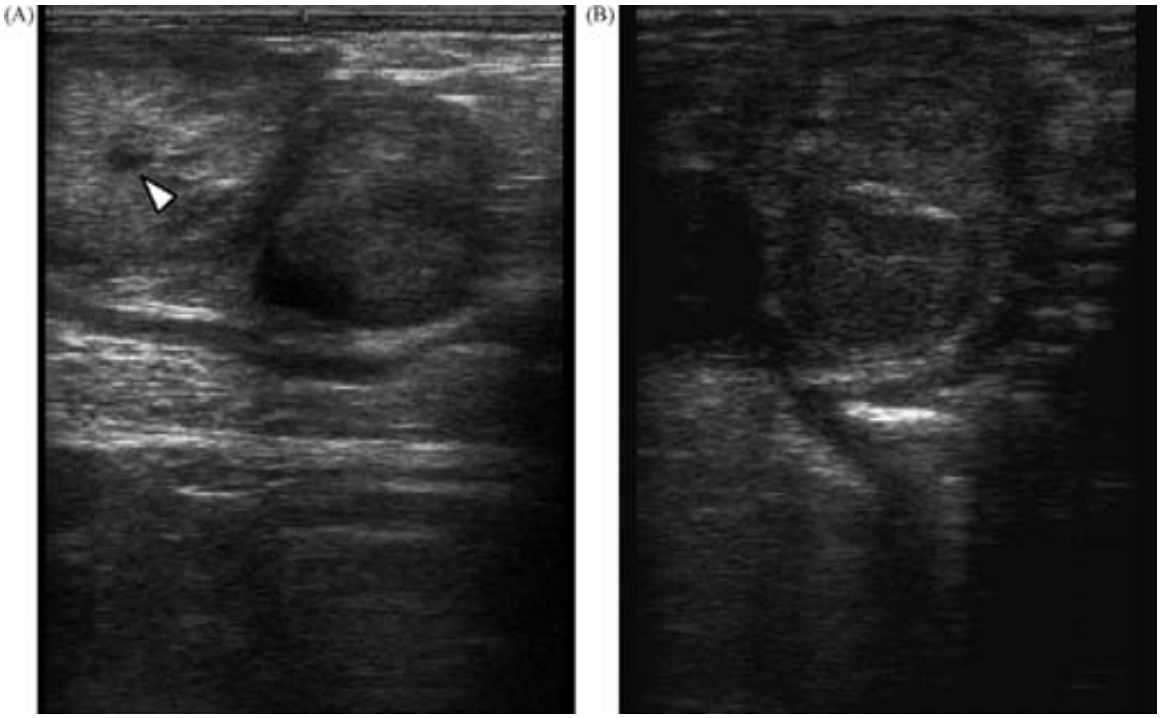

Fig. 2. Corpus luteum with non-echogenic central area (white arrow) (A), and another with a homogeneous echogenic texture (B).

Table 6
Values of the measured variables on different days before ovulation

Variables	Days before ovulation				
	5	4	3	2	1
Oestrous behaviour	1.24 ± 0.22^{c}	$1.66 \pm 0.20^{b,c}$	$1.89 \pm 0.15^{a,b}$	$2.03 \pm 0.14^{a,b}$	2.15 ± 0.11^{a}
Follicle size	0.36 ± 0.17^{c}	0.48 ± 0.10^{c}	1.23 ± 0.17^{b}	1.85 ± 0.13^{a}	2.36 ± 0.08^{a}
Follicle texture	1.69 ± 0.19^{c}	1.68 ± 0.11^{c}	$1.99 \pm 0.10^{b,c}$	2.23 ± 0.12^{b}	2.69 ± 0.08^{a}
Echographic follicular app.	0.14 ± 0.08^{b}	0.16 ± 0.06^{b}	0.47 ± 0.13^{b}	0.48 ± 0.10^{b}	1.05 ± 0.13^{a}
Uterine tone	0.81 ± 0.14^{b}	$0.66 \pm 0.14^{a,b}$	$0.59 \pm 0.11^{a,b}$	$0.55 \pm 0.12^{a,b}$	0.35 ± 0.08^{a}
Echographic uterine app.	0.95 ± 0.15^{b}	$1.03 \pm 0.10^{a,b}$	$1.14 \pm 0.11^{a,b}$	$1.30 \pm 0.09^{a,b}$	1.33 ± 0.09^{a}
N	22	31	39	41	66

Results are expressed as means ± S.E.M. Different superscript letters (a–c) within the same row indicate significant differences ($P < 0.05$). app. = appearance.

days −4, −3 and −2 ($P < 0.05$); on the day before ovulation further growth was slow. The consistency of the preovulatory follicle, measured by rectal palpation, decreased from the third day prior to ovulation. Echographic follicular appearance scores increased particularly on the day before ovulation ($P < 0.05$). The preovulatory follicles tended to elongate and took on a triangular shape over oestrus. Uterine tone, evaluated by rectal palpation, decreased between day −5 and −1 ($P < 0.05$), but day-to-day reductions were not significant. During oestrus the uterus appeared echographically as a series of black spaces arranged in a regular, radial fashion.

3.4.2. Probability of ovulation

Follicle size, oestrous behaviour and follicle palpation characteristics were revealed by stepwise logistic regression as the only variables to influence the probability of ovulation occurring in the following days.

Four intercept estimates corresponding to each to the cumulative distribution probabilities of the $n - 1$ response categories (1, 2, 3 and 4 days to ovulation) were determined: these were −5.39, −3.85, −2.36 and −0.67, respectively. The slopes for oestrous behaviour, follicle texture and follicle size were 0.40, 0.55 and 1.40, respectively. By introducing these values into the logit link function, the cumulated probabilities were

Table 7
Probability of ovulation within 24 h (>50% probability) determined from follicle size, follicle texture, and oestrous behaviour scores

Oestrous behaviour score	Follicle size score	Follicular texture score	Cumulative probability of ovulation (%)			
			−24 h	−48 h	−72 h	−96 h
3	3	4	90.21	97.72	99.48	99.90
3	3	3	84.16	96.11	99.09	99.83
3	3	2	75.39	93.43	98.44	99.71
3	3	1	63.85	89.14	97.33	99.50
3	3	0	50.46	82.55	95.46	99.14
3	2	4	69.47	91.36	97.91	99.61
3	2	3	56.75	85.90	96.44	99.33
2	3	4	86.01	96.62	99.22	99.86
2	3	3	78.00	94.27	98.65	99.75
2	3	2	67.15	90.47	97.68	99.57
2	3	1	54.10	84.55	96.05	99.25
2	2	4	60.29	87.58	96.91	99.42
1	3	4	80.40	95.01	98.83	99.78
1	3	3	70.28	91.66	97.99	99.63
1	3	2	57.69	86.36	96.57	99.35
1	2	4	50.32	82.47	95.43	99.13
0	3	4	73.24	92.71	98.26	99.68
0	3	3	61.21	87.99	97.02	99.44
0	3	2	47.64	80.86	94.94	99.03

determined for all possible combinations of values for the three variables selected. Table 7 shows the combinations predicting ovulation within 24 or 48 h, etc., with a probability of at least 50%.

4. Discussion

At the start of pre-copulatory interactions, the jacks vocalized and mounted without erection. They also nibbled the jennies head, neck and extremities to make sure they were submissive. These findings have been reported in other studies [7–10].

The main signs of oestrus detected were similar to those described by Trimeche and Tainturier [11] and Blanchard et al. [5] for domestic jennies. Mouth clapping was a clear sign of submission, as other authors have indicated for zebra, young mares and foals [11–13].

The present jennies showed heterotypical behaviour such as mounting, the Flehmen response and chasing one another, as described Henry et al. [14]. This type of behaviour (particularly mounting) is common among cows in oestrus, but not in mares [14].

Only one jenny showed a permanently aggressive attitude towards the jacks, although she did ovulate. Similar findings of altered oestrous behaviour have been reported for domestic jennies [3] and mares [12].

Nine of the studied jennies ovulated regularly throughout the year, only one jenny had two anovulatory periods (54 and 35 days) during the winter. It was generally observed, however, that the oestrous cycle was not affected by the season of the year. Ginther [12] indicates that the reproductive cyclicity of jennies is less affected by season than that of horses or ponies. In a study of different breeds in southern Wisconsin, Ginther et al. [15] reported that 64% of jennies ovulated in December and 82–100% during the other months. However, Asdell [16] found the breeding season of donkeys to be similar to that of horses.

The length of the oestrus cycle (24.9 ± 0.26 days) was similar to that indicated by Miró et al. [17], Vandeplassche et al. [18] and Trimeche and Tainturier [11], although Blanchard et al. [5] and Nishikawa and Yamazaki [2] have described shorter cycles (22.8 and 23.3 days, respectively). The mean length of oestrus in Catalonian donkeys is clearly longer than in mares (21 days) [19] but similar to that in ponies (25 days) [12].

The duration of oestrus in the present jennies was similar to that reported for other breeds of donkey [4,5], but shorter than the 7.9 ± 2.5 days reported by Henry et al. [3] for Pega and crossbreed jennies in Brazil, and the 7.5 ± 1.2 days reported by Trimeche and Tainturier [11] for Poitou jennies in France. Oestrus was longer during the winter months (December–February) (6.75 ± 0.25 days) compared to the rest of the year (5.52 ± 0.22 days). Ginther et al. [15] also described different durations of oestrus depending on the time of year (May–October: 5.7–6.9 days and November–April: 7.4–15.2 days).

The incidence of multiple ovulations in jennies has been reported to range from 5.3 to 61% [3,5,11,18]. In the present study the incidence of multiple ovulations was high (44.3%), with 45% occurring in just three jennies. Other authors have reported similar findings in individual mares and jennies [3,5,11,19]. In horses, breed is a determining factor in the occurrence of multiple ovulations. Large breeds have a higher incidence than small breeds and ponies (25% in thoroughbreds compared to 2% in ponies) [19,20]. The Catalonian and Mammoth breeds have the highest incidence of double ovulations (61 and 44.3%) recorded for donkeys. In fact, Catalonian donkeys were involved in the development of the Mammoth breed, the main characteristic contributed being their large size [1]. This suggests that double ovulations in jennies are affected by breed.

Henry et al. [3] indicate a high frequency of ovulation for the left ovary (61%). In the present study a slightly greater frequency of ovulation from the left ovary was seen, but the difference was not significant.

The mean interval between multiple ovulations in the present jennies was 1.44 ± 3.98 days (range 1–9 days). Ginther et al. [15] and Blanchard et al. [5] reported larger intervals of 3.1 and 2 days respectively in other jennies.

The mean diameter of the ovulating follicle in the present jennies was about 45 mm, a size similar to that described for mares by other authors [19,21,22]. Carluccio et al. [23] reported the mean diameter at ovulation to be 42.9 mm in Martina Franca jennies, similar to the mean preovulatory diameter seen in the present Catalonian jennies. However, in other donkey breeds the mean diameter of preovulatory follicles is reported to be smaller at around 36–41 mm [4,18,24]. These differences may be associated with the large size of Catalonian donkeys.

Pierson and Ginther [22] noted that the diameter of preovulatory follicles in double ovulations was smaller than in single ovulations. However, no significant differences were seen in the present work.

The growth rate of the preovulatory follicle was found to be 3.7 mm/day, although a reduction in this rate was seen on day −1 (in fact the follicle sometimes became smaller). Ginther [12] observed the same

growth rate in ponies, but a lower rate in mares (2.7 mm/day).

One jenny suffered a likely hemorrhagic follicle in two cycles—one in January and one in May. Carnevale [25] described ponies to more frequency suffer hemorrhagic follicles in autumn (21%) than in the April–August period (1%). In contrast, Ginther [12] concluded that season has no influence on the appearance of hemorrhagic follicles.

In the present work, most of the CLs observed showed a homogeneous echogenic texture; in contrast, 50–70% of mares have CLs with a non-echogenic central area [26].

Of the six variables (oestrous behaviour, follicle size, follicle texture, echographic follicle and uterine appearance) examined to determine which best predicted impending ovulation, stepwise multiple regression showed follicle size to be the most reliable. Koskinen et al. [27] concluded the same in mares. Signs of oestrous increased as ovulation approached, as recorded by other authors [13,28]. This variable was the second best predictor. As observed in the present study, many researchers have described the softening of the follicle prior to ovulation; in mares 80–90% of preovulatory follicles show a change in consistency from turgid to soft during oestrous [12,27,29]. However, some authors report that this does not always occur [19,30]. These differences may be related to the interval between examinations. In the present study the jennies showed a soft follicle on day −1 when the examination interval was 24 h. Thus, follicle texture also appeared as a predictor of ovulation, although it was the least reliable of those selected.

In mares, Miró et al. [31] reported only follicle size and texture to be selected by the same procedure; oestrous behaviour was not predictive of ovulation. This difference may be connected with the social organization of horses and donkeys. Wild horses breed in a harem group of one stallion to several mares, while donkeys are territorial with each male guarding a territory and breeding with jennies passing through or residing in that territory [32]. It may be that jennies undertake more obvious sexual behaviour than mares to attract the attention of males.

Neither the echographic appearance of the uterus and follicle, nor the uterine tone, were selected as predictors of ovulation. In mares, Pierson and Ginther [22] classified 85% of follicles as non-spherical from day −1 to ovulation. It is possible that the softening of follicles is related to their elongation into the ovulation fossa. In the present study the follicles showed a tendency to elongate, but this was not sufficiently marked to predict ovulation. When the follicle population was very large the smaller follicles took on a similar shape due to the pressure they exerted on one another, a consequence of the lack of free space. During oestrous, the uterus loses tonicity since the endometrial folds enlarge and become oedematous [33], but there are no important day-to-day changes during the oestrous period; uterine tone does not, therefore, help predict ovulation. Finally, echographic evaluation of the uterine appearance showed hypoechogenic regions linked to oedema, but without variation over the oestrus period [34]; it is therefore not able to predict ovulation.

In conclusion, the combination of oestrous behaviour, follicle size and follicle texture, measured daily, is able to predict the onset of ovulation. A jenny that shows mouth clapping, urination and immobility, that has a follicle larger than 45 mm, and that has very soft follicle texture, is likely to ovulate within 24 h with a probability of 90%. Different combinations of these three variables predict ovulation within 48 h with a probability of >70%.

Acknowledgements

The authors would like to thank Mr. José Manuel Fernandez Castro, owner of some of the jennies, for his help in this study. This work was funded by Grant RZ-03-021 (*Instituto Nacional de Investigaciones Agrarias*, Spain).

References

[1] Jordana J, Folch P. The endangered Catalonian donkey breed: the main ancestor of the American ass or mammoth. J Eq Vet Sci 1996;16:436–41.
[2] Nishikawa Y, Yamazaki Y. Studies on reproduction in asses. I. Breeding season, oestrous cycle and length of heat. Jpn J Zootech Sci 1949;19:119–24.
[3] Henry M, Figueiredo AEF, Palhares MS, Coryn M. Clinical and endocrine aspects of the oestrous cycle in donkeys (*Equus asinus*). J Reprod Fert Suppl 1987;35:297–303.
[4] Meira C, Ferreira JCP, Papa FO, Tornero MTT, Bicudo SD. Study of the estrous cycle in donkeys (*Equus asinus*) using ultrasonography and plasma progesterone concentrations. Biol Reprod Mono 1995;1:403–10.
[5] Blanchard TL, Taylor TS, Love CL. Estrous cycle characteristics and response to estrus synchronization in Mammoth Asses (*Equus asinus americanus*). Theriogenology 1999;52:827–34.
[6] Pukazhenthi BS, Wildt DE. Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reprod Fertil Dev 2004;16:33–46.
[7] Lobo VL. Comportamiento sexual y normalización de las características seminales del burro catalan. Universitat Autónoma de Barcelona. Facultat de Veterinària/Unitat de Reproduccció. Treball Master; 2003. p. 9–11.

[8] Henry M, Gastal EL, Meira C, Diaz AP. Caracteristicas do semen de jumentos da raça nordestina. In: Proceedings of VII Braz Cong Anim Reprod, vol. 72; 1987 (Abstr.).
[9] Gastal MO, Henry M, Beker AR, Gastal EL, Gonçalves A. Sexual behavior of donkey jacks: influence of ejaculatory frequency and season. Theriogenology 1996;46:593–603.
[10] Kreuchauf A. Reproductive physiology in the jackass. Anim Res Dev 1984;20:51–78.
[11] Trimeche A, Tainturier D. Étude échographique de la cinétique folliculaire des baudettes du Poitou pendant l'oestrus au cours du printemps et de l'été. Revue Méd Vét 1995;146:743–8.
[12] Ginther OJ. Reproductive biology of the mare: basic and applied aspects, 2nd ed., Wisconsin: Cross Plains, Equiservices Publishing; 1992. p. 75–232.
[13] Asa CS. Sexual behaviour of mares. Vet Clin N Am: Eq Pract 1986;2:519–34.
[14] Henry M, McDonnell SM, Lodi LD, Gastal EL. Pasture mating behaviour of donkeys (*Equus asinus*) at natural and induced oestrus. J Reprod Fert Suppl 1991;44:77–86.
[15] Ginther OJ, Scraba ST, Bergfelt DR. Reproductive seasonality of the jenney. Theriogenology 1987;27:587–92.
[16] Asdell SA. Patterns of mammalian reproduction. Ithaca, NY: Cornell University Press; 1964. p. 530.
[17] Miró J, Lobo V, Medrano A, Rigau T. Dinámica folicular en la burra catalana. In: Comunication, IV Congreso Ibérica de Reproducción Animal; 2003.
[18] Vandeplassche M, Wesson JA, Ginther OJ. Behavioral, follicular and gonadotropin changes during the estrous cycle in donkeys. Theriogenology 1981;16:239–49.
[19] Hughes JP, Stabenfeldt GH, Evans JW. Clinical and endocrine aspects of the oestrous cycle of the mare. Proc Annu Conv Am Assoc Eq Pract 1972;18:119–51.
[20] Ginther OJ, Gastal EL, Gastal MO, Bergfelt DR, Baerwald AR, Pierson RA. Comparative study of the dynamics of follicular waves in mares and women. Biol Reprod 2004;71: 1195–201.
[21] Lindeberg H, Kuntsi-Vaattovaara H, Katila T. Predicting ovulation in the mare. In: Communication, 12th International Congress on Animal Reproduction; 1992. p. 144–46.
[22] Pierson RA, Ginther OJ. Ultrasonic evaluation of the preovulatory follicle in the mare. Theriogenology 1985;24:359–68.
[23] Carluccio A, Panzani S, Tosi U, Faustini M, De Amicis I, Veronesi MC. Efficacy of hCG and GnRH for inducing ovulation in the jenny. Theriogenology 2007;68:914–9.
[24] Dadarwal D, Tandon SN, Purohit GN, Pareek PK. Ultrasonographic evaluation of uterine involution and postpartum follicular dynamics in French Jennies (*Equus asinus*). Theriogenology 2004;62:257–64.
[25] Carnevale EM. Folliculogenesis and ovulation. In: McKinnon AO, Rantanen NW, editors. Equine diagnostic ultrasonography. Williams & Wilkins; 1998. p. 201–11.
[26] Pierson RA, Ginther OJ. Ultrasonic evaluation of the corpus luteum of the mare. Theriogenology 1985;23:795–806.
[27] Koskinen E, Kuntsi H, Lindeberg H, Katila T. Predicting ovulation in the mare on the basis of follicular growth and serum oestrone sulphate and progesterone levels. J Vet Med A 1989;36:299–304.
[28] Squires EL. Estrous detection. In: McKinnon AO, Voss JL, editors. Equine reproduction. Philadelphia/London: Lea & Febiger; 1993. p. 186–95.
[29] Parker WG. Sequential changes of the ovulating follicle in the estrous mare as determined by rectal palpation. In: Proceedings of Annual Conference of Veterinarians, College of Veterinary Medicine and Biomedical Sciences. Fort Collins (CO): Colorado State University; 1971. p. 1–25.
[30] Witherspoon DM, Talbot RB. Nocturnal ovulation in the equine animal. Vet Rec 1970;87:302–4.
[31] Miró J, Muñoz F, Piedrafita J, Quintero-Moreno A. Prediction of the day of ovulation in mares through physiological parameters measured during estrous. Revista Científica FCV-LUZ 2004;XIV(1):54–8.
[32] McDonnell SM. Reproductive behavior of stallions and mares: comparison of free-running and domestic in-hand breeding. Anim Reprod Sci 2000;60/61:211–9.
[33] England G. Allen's fertility and obstetrics in the horse, 2nd ed., Oxford, London: Blackwell Science Ltd.; 1996. p. 9–12.
[34] Ginther OJ. Ultrasonic Imaging and Reproductive Events in the Mare. Cross Plains, WI: Equiservices Publishing; 1986.

II

Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen.

J. Miró, E. Taberner, M. Rivera, A. Peña, A. Medrano, T. Rigau, A. Peñalba.

Department of Animal Medicine and Surgery, School of Veterinary Medicine, Autonomous University of Barcelona,
Campus UAB, Edifici V, E-08193 Bellaterra, Spain

Received 23 September 2008; revised 17 June 2009; accepted 27 June 2009

Theriogenology, 72 (2009) 1017-1022

Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 72 (2009) 1017–1022
Theriogenology
www.theriojournal.com

Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen

J. Miró *, E. Taberner, M. Rivera, A. Peña, A. Medrano, T. Rigau, A. Peñalba

Department of Animal Medicine and Surgery, School of Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra, Spain

Received 23 September 2008; received in revised form 17 June 2009; accepted 27 June 2009

Abstract

The aim of this work was to study the effects of dilution and centrifugation (i.e., two methods of reducing the influence of the seminal plasma) on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey (*Equus asinus*) semen. Fifty ejaculates from nine Catalonian jackasses were collected. Gel-free semen was diluted 1:1, 1:5 or 1:10 with Kenney extender. Another sample of semen was diluted 1:5, centrifuged, and then resuspended with Kenney extender until a final dilution of 25×10^6 sperm/ml was achieved (C). After 24 h, 48 h or 72 h of refrigerated storage at 5 °C, aliquots of these semen samples were incubated at 37 °C for 5 min. The percentage of viable sperm was determined by staining with eosin-nigrosin. The motility characteristics of the spermatozoa were examined using the CASA system (Microptic, Barcelona, Spain). At 24 h, more surviving spermatozoa were seen in the more diluted and in the centrifuged semen samples (1:1 48.71%; 1:5 56.58%, 1:10 62.65%; C 72.40%). These differences were maintained at 48 h (1:1 34.31%, 1:5 40.56%, 1:10 48.52%, C 66.30%). After 72 h, only the C samples showed a survival rate of above 25%. The four known donkey motile sperm subpopulations were maintained by refrigeration. However, the percentage of motile sperms in each subpopulation changed with dilution. Only the centrifuged samples, and only at 24 h, showed exactly the same motile sperm subpopulation proportions as recorded for fresh sperm. However, the 1:10 dilutions at 24 and 48 h, and the centrifuged semen at 48 h, showed few variations compared to fresh sperm. These results show that the elimination of seminal plasma increases the survival of spermatozoa and the maintenance of motility patterns.

The initial sperm concentration had a significant ($P < 0.05$) influence on centrifugation efficacy, but did not influence the number of spermatozoa damaged by centrifugation. In contrast, the percentage of live spermatozoa in the fresh semen significantly influenced the number of spermatozoa damaged by centrifugation, but not centrifugation efficacy.

Keywords: Seminal plasma; Sperm subpopulations; Cooled semen; Donkey

1. Introduction

The Catalonian donkey (*Equus asinus*) is a large donkey found in a number of Pyrenean and pre-Pyrenean areas of Catalonia (northeastern Spain). It is the forerunner of several large donkey breeds around the world, including the Martina Franca, Pantelleria, or Ragusana breeds in Italy, the Pyrenean donkey of France, the Mammoth Jackass of the United States, the Mallorquin donkey of Spain, the Cyprus donkey, and others. The value of the Catalonian donkey in mule breeding has been known for centuries. Nonetheless, this breed is now in a danger of extinction [1]. Knowledge regarding the conservation of its semen is therefore not only important with respect to breeding

* Corresponding author. Tel.: +34 935811044; fax: +34 935812006.
E-mail address: jordi.miro@uab.cat (J. Miró).

0093-691X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2009.06.012

programs but also to the maintenance of its numbers and the prevention of its disappearance.

Making use of computer-assisted motility analysis (CASA), other studies have examined the sperm motility patterns of Catalonian donkey semen and report four specific motile sperm subpopulations [2]. Subpopulations of motile spermatozoa with specific motility characteristics have been reported in several species such as the common marmoset, gazelle, pig, dog, horse, and red deer [3–12]. The existence of such a structure in mammals of very different phylogenetic origin suggests the existence of a relationship between changes in the subpopulation structure of an ejaculate and its fertilizing ability [9,10].

The cooling, storing, and transport of semen for use in subsequent insemination is very important in the reproductive management of a number of species. Several authors report that sperm survival and the motility of refrigerated semen increases with the removal of the seminal plasma, and a number of seminal plasma proteins have been identified as a cause of reduced survival and motility loss in pig [13], cattle [14], goat [15], and horse [16] semen. These proteins make contact with the spermatozoa during ejaculation, interact with the spermatozoid membrane, and influence different characteristics, including motility [17]. The detrimental effects of seminal plasma on spermatozoa during cold storage may also be related to the action of several enzymes [16]. In horses, these undesirable effects can be reduced by semen dilution or the total or partial removal of the seminal plasma [18]. In the Zamorano-Leonés donkey, Serres et al. [19] observed that total motility, progressive motility, and HOST (Hipoosmotic Swelling Test) results were higher for centrifuged semen than for noncentrifuged semen.

The aims of the current study were to determine the effect of the seminal plasma on refrigerated Catalonian donkey semen, to investigate the maintenance of motile sperm subpopulation structure over time in different dilutions of semen and in centrifuged sperm (i.e., with the seminal plasma removed), and thus to determine the best protocol for refrigerating Catalonian donkey semen. The information obtained might help to increase more easily the population of Catalonian donkeys and avoid the extinction of this breed.

2. Materials and methods

2.1. Experimental animals

The study was performed at the experimental farm of the Veterinary Faculty, Autonomous University of Barcelona, between April 2006 and June 2007. Fifty ejaculates were collected from nine healthy, mature Catalonian jackasses of proven fertility aged 6 to 10 yr. Collections were made using an artificial vagina with an in-line gel filter. A jenny in natural or induced estrus was used to induce copulatory activity.

2.2. Sperm evaluation: Computer-assisted motility analysis

The sperm concentrations in aliquots of ejaculate were determined using a hemocytometer [20]; the pH was determined using a pH meter (micropH2000; Crison, Strumenti S.p.A., 41012 CARPI (MO), Italy). Gel-free semen was immediately diluted 1:1, 1:5, and 1:10 with dry skimmed milk extender [21] previously kept in a 37 °C water bath and stored in air-free 50-mL Corning tubes (Corning Incorporated, Corning, NY, 14831 USA). Another sample of gel-free semen was diluted 1:5 and centrifuged at $660 \times g$ for 15 min at 20 °C. The pellet, along with 5% to 20% of the supernatant, was resuspended with Kenney extender in air-free Corning tubes at a final dilution of 25×10^6 sperm/mL. All samples were then maintained at 5 °C for either 24, 48, or 72 h before analysis.

After storage, percentage viability was determined by eosin-nigrosin staining, examining 200 spermatozoa at ×1000 magnification, as described by Bamba [22]. Five-milliliter aliquots of semen samples were incubated for 5 min in a water bath at 37 °C. The pH was determined using a pH meter (micropH2000; Crison). Prior to sperm motility analysis, the above 1:1 and 1:5 samples were further diluted with Kenney extender until a 1:10 ratio was reached with respect to fresh semen. Sperm motility characteristics were determined using the Sperm Class Analyzer (Microptic; Barcelona, Spain) as described Miró et al. [2] for donkey semen. Three consecutive 5-μL samples of all of the studied ejaculates was observed using an optical phase-contrast microscope with a heatable plate (37 °C). Two fields per drop were examined; the number of spermatozoa examined in each field (including those not motile) was 50 to 100. The CASA system used is based on the analysis of 24 consecutive, digitalized photographic images obtained from a single field at ×200 magnification with dark-field illumination. These photographs were taken in a total time of 0.64 sec (image-capture rate, 1 photograph every 40 msec).

The CASA settings were as follows: cell size, 4 to 75 μm^2; connectivity, 12; progressive spermatozoa, >75% of the straightness coefficient (STR); and minimum number of images required to assess the

mean lateral head displacement (ALH), 10. The CASA system used takes into account 21 sperm motility descriptors. However, as described by other authors [2], only six of these variables are required to explain overall donkey sperm movement: mean velocity (VAP), mean lateral head displacement (ALH), the linear coefficient (LIN), the frequency of head displacement (BCF), the minor harmonic oscillation of the head (HLO), and the algebraic angular mean displacement (MAD). With these six variables, the FASTCLUS clustering procedure was used to separate the spermatozoa into their different motility subpopulations [9,10].

2.3. Statistical analysis

Data were processed using the SAS statistical package (version 8.2; Statistical Analysis System, SAS Institute Inc., Cary, NC, USA). Normality was assessed by the Shapiro-Wilks test (W) included in the UNIVARIATE procedure.

A general linear model (the PROC GLM routine) was used to test for significant differences in motility ($P < 0.05$) among the samples subjected to different treatments and refrigerated storage times. The LSMEANS procedure was used to identify significant differences. The chi-squared test was used to detect differences in viability and pH.

Finally, the centrifugation efficacy ([centrifuged spermatozoa mL^{-1}/fresh semen spermatozoa mL^{-1}] $\times$ 100) and the index of spermatozoa damaged by centrifugation ([fresh live spermatozoa/centrifuged live spermatozoa] $\times$ 100) were calculated. Correlations between the initial concentration and number of live spermatozoa mL^{-1} and the concentration and the number of live spermatozoa after centrifugation were determined by linear regression.

3. Results

Table 1 shows the sperm characteristics for the collected ejaculates. The composition of the semen and the values of the sperm motility variables varied between donkeys and even between ejaculates of the same animal ($P < 0.001$; data not shown).

The FASTCLUS procedure detected four subpopulations of motile sperm from the data for fresh semen. Table 2 shows the mean values for each motility variable in each subpopulation.

The spermatozoa of Subpopulation 1 showed the greatest progressiveness and were highly active (as inferred from the very high LIN and VAP values). The spermatozoa of Subpopulation 2 showed nonlinear trajectories, low progressiveness, and a very low VAP value. Subpopulation 3 contained spermatozoa with high VAP values, although their trajectories showed intermediate linear coefficients. Subpopulation 4 included spermatozoa with the least progressiveness and linearity.

Table 1
Mean values of the semen quality analysis.

Variable	Mean ± SEM
Filtered volume (mL)	64.1 ± 2.6
Sperm count ($\times 10^6$/mL)	338.2 ± 1.7
Total motility	81.3 ± 0.5
Progressive motility	68.4 ± 1.3
pH	7.6 ± 0.4
Sperm viability (%)	84.1 ± 1.5
Sperm immature tail (%)	10.3 ± 0.3
Sperm coiled-tail (%)	1.1 ± 0.2
Sperm head abnormality (%)	3.2 ± 0.4
Tailless spermatozoa (%)	2.5 ± 0.1
Immature sperm with proximal cytoplasmic droplets (%)	1.6 ± 0.0
Immature sperm with distal cytoplasmic droplets (%)	0.2 ± 0.1
Total abnormalities (%)	20.1 ± 0.2
Sperm curvilinear velocity (VCL; μm/sec)	82.7 ± 0.4
Sperm linear velocity (VSL; μm/sec)	90.9 ± 0.4
Mean velocity (VAP; μm/sec)	57.9 ± 0.1
Linear coefficient (LIN; %)	52.3 ± 0.5
Straightness coefficient (STR; %)	73.8 ± 0.6
Wobble coefficient (WOB; %)	79.2 ± 0.6
Mean lateral head displacement (ALHmed; μm)	2.5 ± 0.06
Frequency of head displacement (BCF; Hz)	12.3 ± 0.2
Minor harmonic oscillation of the head (HLO; μm)	0.4 ± 0.05

Table 3 shows the viability of the sperm after each treatment (different dilutions and centrifugation) and refrigerated storage time. Significant differences were seen between treatments for the same refrigeration time. Sperm cell survival increased with dilution, but the best results were recorded for the centrifuged semen. After 72 h of refrigerated storage, only the centrifuged samples showed greater than 25% survival.

The pH of the semen fell significantly between at 24 and 48 h when diluted by more than 1:1, with the lowest values seen for centrifuged semen. However, no significant differences were observed between the 1:5 and 1:10 dilutions at 24 and 48 h (Table 4).

The sperm subpopulation structure was perfectly maintained at 24 h only in the centrifuged semen (Table 5), although the 1:10 dilutions at 24 and 48 h and the centrifuged semen at 48 h showed few variations

Table 2
Fresh semen sperm subpopulations and motility descriptors.

Sperm subpopulation	Sperm motility descriptors					
	VAP (μm/sec)	LIN (%)	ALHmed (μm)	MADalg (°)	BCF (Hz)	HLO (μm)
1	154.4 ± 1.7	75.2 ± 2.2	4.89 ± 0.24	–1.72 ± 1.85	12.9 ± 0.6	1.11 ± 0.10
2	28.5 ± 0.9	44.3 ± 1.5	2.64 ± 0.12	4.85 ± 0.98	12.1 ± 0.3	0.32 ± 0.05
3	81.9 ± 0.8	59.8 ± 1.1	4.00 ± 0.12	0.57 ± 0.90	13.1 ± 0.3	0.67 ± 0.05
4	27.8 ± 1.2	26.4 ± 1.6	3.19 ± 0.17	–33.26 ± 1.30	15.0 ± 0.4	0.25 ± 0.07

Note: The motility descriptors are described in Section 2. Results are expressed as mean ± SEM for 50 semen samples from 9 Catalonian donkeys. The total number of spermatozoa analyzed was 5828.

Table 3
Sperm viability percentages at 24, 48, and 72 h for each treatment (dilutions 1:1, 1:5, 1:10, and centrifuged semen [C]).

Hours	Treatment			
	1:1	1:5	1:10	C
24	48.71 ± 23.42[a]	56.58 ± 21.42[b]	62.65 ± 19.87[c]	72.40 ± 14.50[d]
48	34.31 ± 22.46[e]	40.56 ± 19.96[e]	48.52 ± 23.28[f]	66.30 ± 17.97[d]
72	<25	<25	<25	42.10 ± 20.17[e]

[a–f]Different superscripts in the same row indicate significant differences. Results are expressed as mean ± SEM.

compared with that of fresh sperm. At 72 h, only the centrifuged semen maintained a population structure similar to that of fresh sperm, although with a predominance of Subpopulations 2 and 3.

Finally, the initial sperm concentration ($338.2 \times 10^6 \pm 1.7 \times 10^6$ spermatozoa/mL) had a significant influence on centrifugation efficacy (86.98 ± 1.93%) but no influence on the number of spermatozoa damaged by centrifugation (1.33 ± 1.06%), whereas the percentage of live spermatozoa in the fresh semen (84.1 ± 1.5%) significantly influenced the number of spermatozoa damaged by centrifugation but not the centrifugation efficacy (Table 6).

4. Discussion

In horses, the negative effects of refrigerating sperm can be diminished when a suitable extender is added to the semen; it can then be stored at 4 to 5 °C for 1 to 2 d [23–25]. In addition, the harmful effects of seminal plasma can be reduced by using high ratios (≥3:1) of extender to whole ejaculate semen [26,27] or totally or partially removing the seminal plasma [17]. Brinsko et al. [28] reported centrifugation and the partial removal of seminal plasma to be beneficial in the refrigerated storage of horse semen, especially for ejaculates with poor cooling and storage tolerance. In agreement, the results of the current study show that donkey sperm viability after storage is greater when more diluted or when the seminal plasma partially has been removed by centrifugation.

Table 4
Semen sample pH at 24, 48, and 72 h in each treatment (dilutions 1:1, 1:5, 1:10, and centrifuged semen [C]).

Hours	Treatment			
	1:1	1:5	1:10	C
24	7.14 ± 0.15[a]	7.05 ± 0.12[b]	7.02 ± 0.14[b]	6.92 ± 0.10[c]
48	7.15 ± 0.17[a]	7.06 ± 0.11[b]	7.04 ± 0.08[b]	6.91 ± 1.15[c]
72	—	—	—	7.05 ± 0.10[a]

[a–c]Different superscripts indicate significant differences. Results are expressed as mean ± SEM.

Serres et al. [19] reported that centrifugation and the removal of seminal plasma have a significant, positive effect on total motility, progressive motility, and HOST test results in Zamorano-Leonés donkey spermatozoa cooled in INRA (Institute National Recherche Agricole, France) 82. However, Rota et al. [29] indicated that the total removal of seminal plasma during in vitro preservation did not seem to offer any advantage over using semen diluted in INRA 82 + 2% egg yolk. Compared with the study by Rota et al. [29], the current conditions were different. The latter authors used only 12 ejaculates from four Amiata donkeys compared with the 50 ejaculates from nine Catalonian donkeys used here. A different extender was also used in the current work, and the different centrifugation conditions, initial dilution, temperature, and the almost complete removal of seminal plasma mark further variations in conditions.

Table 5
Sperm subpopulation proportions (SP) in donkey semen and their change with dilution or centrifugation and time (0, 24, 48, and 72 h).

SP	Fresh semen	24 h				48 h				72 h
		1:1	1:5	1:10	C	1:1	1:5	1:10	C	C
1	9.24[a]	3.37 [a]	1.66 [b]	1.31 [b]	3.71 [a]	5.26 [a]	2.56 [b]	2.11 [b]	1.60 [b]	0.70 [b]
2	33.01[a]	56.13[b]	40.85 [a]	39.02 [a]	35.43 [a]	45.61 [b]	48.08 [b]	42.11 [a]	40.52 [a]	51.75 [b]
3	39.07 [a]	7.36 [b]	24.77 [b]	33.77 [a]	39.57 [a]	21.05 [b]	14.10 [b]	34.42 [a]	30.34 [a]	27.97 [a]
4	18.68 [a]	33.13 [b]	32.72 [b]	25.89 [a]	21.29 [a]	28.07 [b]	35.26 [b]	17.37 [a]	27.54 [b]	19.58 [a]

C, centrifuged semen.
[a,b]Different superscripts indicate significant differences.

Table 6
Significance of correlations between sperm concentration or viability and centrifugation efficacy (initial concentration/postcentrifugation concentration × 100) and spermatozoa damaged by centrifugation (initial live spermatozoa/postcentrifugation live spermatozoa × 100).

	Centrifugation efficacy	Damaged spermatozoa
Fresh-semen spermatozoa/mL	0.023*	0.996
Percentage (%) fresh-semen live spermatozoa	0.910	0.000*

* $P < 0.05$.

Finally, Rota et al. analyzed their CASA results globally, which is nowadays thought to be inadequate.

Very different mammals have been found to have similar motile sperm subpopulation structures [3–12], the maintenance of which could be important in the general functioning of ejaculates. The four known donkey motile sperm subpopulations are maintained with freezing [30] and, according to the current results, with refrigerated storage. Nevertheless, the distribution of these subpopulations showed variations. Only the centrifuged samples, and only at 24 h, showed the same distribution of subpopulations as in fresh semen. However, the 1:10 diluted semen at 24 and 48 h, as well as the centrifuged semen at 48 h, showed relatively few variations compared with the fresh sperm. The centrifuged semen at 72 h showed a significant reduction in Subpopulation 1 (SP1) and an increase in Subpopulation 2 (SP2); overall sperm survival was good. In fresh semen, SP2 and Subpopulation 3 (SP3) showed the highest numbers (33.01% and 39.07% of the total number of sperm, respectively). The percentage of these subpopulations did not differ ($P < 0.05$) in the 1:10 diluted semen or the centrifuged semen at 24 or 48 h. At 72 h, however, whereas SP2 increased significantly, SP3 did not differ; nonetheless, both subpopulations remained the largest.

Brinsko et al. [28] observed a trend toward a reduction in the sperm curvilinear velocity (VCL) in diluted (1:3) and centrifuged horse semen after 24 or 48 h of refrigerated storage. The present diluted and centrifuged donkey semen showed an increase in the proportion of SP2 and Subpopulation 4 (SP4), but both showed low VAP values; the proportion of SP1 and SP3 were reduced, but their members showed higher VAPs.

Sperm survival and motile subpopulation structure in Catalonian donkey semen are therefore influenced by the presence of seminal plasma. High dilution ratios, but with an adequate number of normal motile spermatozoa ($>25 \times 10^6$ spermatozoa/mL), allow for optimum sperm cell survival and the maintenance of motile subpopulation structure in semen refrigerated for 24 or 48 h. If storage needs to be longer than 48 h, centrifugation should be performed to remove the seminal plasma. However, semen centrifugation can damage spermatozoa; the presence of semen extender and the concentration of spermatozoa are very important in reducing this damage [18,31]. The dilution rate normally used for horse semen centrifugation is 1:1 or 1:2, and centrifugation results in a recovery rate of about 75% of the spermatozoa in the pellet [25]. Matás et al. [32] (for pig semen) and Ecot et al. [33] (for horse semen) used a cushioned centrifugation method to minimize sperm loss. However, this is not easy to perform, and the cushion solution must be removed separately after centrifugation [25]. In the current study, the 1:5 dilution for centrifugation provided good sperm recovery results. However, the number of sperm damaged in centrifugation was not related to the precentrifugation dilution ratio. Rather, initial sperm viability correlated inversely with the numbers damaged; good initial semen results in good centrifuged semen.

Acknowledgments

The authors thank Mr. José Manuel Fernández, the owner of the jackasses used in our study, for his kind

collaboration. This work was funded by Grant RZ-03-021 from Instituto Nacional de Investigaciones Agrarias, Spain.

References

[1] Jordana J, Folch P, Aranguren JA. Microsatellite analysis of genetic diversity in the Catalonian donkey breed. J Anim Breed Genet 2001;118:57–63.
[2] 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.
[3] Renard P, Trimeche A, Le Pichon JP, Quero JC, Griveau JF, Chouteau P, et al. Sperm motility and flagellar motion: a comparison between boar and other mammalian species. Reprod Domest Anim 1996;31(1):249–50.
[4] Holt WV. Can we predict fertility rates? Making sense of sperm motility. Reprod Domest Anim 1996;31:17–24.
[5] Abaigar T, Holt W, Harrison R, 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.
[6] Abaigar T, Cano M, Pickard AR, Holt WV. Use of computerized-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.
[7] Rigau T, Farre M, Ballester J, Mogas T, Peña A, Rodríguez-Gil JE. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology 2001;56:801–15.
[8] Thurston LM, Watson P, Mileham A, Holt W. Morphologically distinct sperm subpopulation defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl 2001;22:382–94.
[9] Quintero-Moreno A, Miró J, Rigau T, Rodríguez-Gil JE. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 2003;58:1973–90.
[10] Quintero-Moreno A, Rigau T, Rodríguez-Gil JE. Regression analysis and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. Theriogenology 2004;61:673–90.
[11] Martinez-Pastor F, García-Macías V, Alvarez M, Herráez 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.
[12] Fernández-Santos M, Esteso MC, Soler AJ, Montoro V, Garde JJ. The effects of different cryoprotectants and the temperature of addition on the survival of red deer epididymal spermatozoa. CryoLetters 2005;26(1):25–32.
[13] Strzezek J, Kordan W, Kostyra H, Zaborniak A. Purification and partial characterization of 5700 Da sperm motility inhibiting factor for seminal plasma of boar. Anim Reprod Sci 1992;29:35–52.
[14] Wishwanath R, Shannon P, Curson B. Cationic extracts of egg yolk and their effects on motility, survival and fertilizing ability of bull sperm. Anim Reprod Sci 1992;29:185–94.
[15] Pellicer-Rubio M, Combarnous Y. Deterioration of goat spermatozoa in skimmed milk-based extenders as a result of oleic acid released by the bulbourethral lipase BUSgp60. J Reprod Fertil 1998;112:95–105.
[16] Carver DA, Ball BA. Lipase activity in stallion seminal plasma and effect of lipase on stallion spermatozoa during storage at 5 °C. Theriogenology 2002;58:1587–95.
[17] Braun J, Torres-Boggino F, Hochi S, Oguri N. Effect of seminal plasma on motion characteristics of epididymal and ejaculated stallion spermatozoa during storage at 5 °C. Dtsch Tieärtzl Wschr 1994;101:319–22.
[18] Pickett BW, Sullivan JJ, Byers WW, Pace MM, Remmenga EE. Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. Fertil Steril 1975;26:167–74.
[19] Serres C, Rodríguez A, Alvarez AL, Santiago I, Gabriel J, Gómez-Cuétara C, Mateos E. Effect of centrifugation and temperature on the motility and plasma membrane integrity of Zamorano-Leonés donkey semen. Theriogenology 2002;58:329–32.
[20] Pickett BW, Back DG. Procedures for preparation, collection, evaluation and inseminating of stallion semen. Bull Colo St Univ Agric Exp STN Anim Reprod Lab Gen Ser 1973;935:3–52.
[21] Kenney RM, Bergman RV, Cooper WL, Morse GW. Minimal contamination techniques for breeding mares: technique and preliminary finding. Proc Am Assoc Equine Pract 1975;327–36.
[22] Bamba K. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. Theriogenology 1988;29:1245–51.
[23] Douglas-Hamilton DH, Burns PJ, Driccoll DD, Viale KM. Fertile characteristics of slow-cooled equine semen. J Reprod Fertil 1987;35(Suppl):649–50.
[24] Douglas-Hamilton DH, Burns PJ, Driscoll DD, Viale KM. A field study of the fertility of transported equine semen. Theriogenology 1984;22:291–303.
[25] Aurich C. Recent advances in cooled-semen technology. Anim Reprod Sci 2008;107:268–75.
[26] Jasko DJ, Moran DM, Farlin ME, Squires EL. Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. Theriogenology 1991;35:1059–68.
[27] Varner DD, Blanchard TL, Love CL, García MC, Kenney RM. Effects of semen fractionation and dilution ratio on equine spermatozoal motility parameters. Theriogenology 1987;28:709–23.
[28] Brinsko SP, Crockett EC, Squires EL. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. Theriogenology 2000;54:129–36.
[29] Rota A, Magelli C, Panzani D, Camillo F. Effect of extender, centrifugation and removal of seminal plasma on cooled-preserved Amiata donkey spermatozoa. Theriogenology 2008;69:176–85.
[30] Flores E, Taberner E, Rivera M, Peña A, Rigau T, Miro J, Rodríguez-Gil JE. Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates. Theriogenology 2008;70:936–45.
[31] Cochran JD, Amann RP, Froman DP, Pickett BW. Effects of centrifugation, glycerol level, cooling to 5 °C, freezing rate and thawing rate on the post-thaw motility of equine sperm. Theriogenology 1984;22:25–38.
[32] Matás C, Decuadro G, Martínez-Miró S, Gadea J. Evaluation of a cushioned method for centrifugation and processing for centrifugation and processing for freezing boar semen. Theriogenology 2007;67:1087–91.
[33] Ecot P, Decuadro-Hansen G, Delhomme G, Vidament M. Evaluation of a cushioned technique for processing equine semen for freezing. Anim Reprod Sci 2005;89(1–4):245–8.

III

Effect of pre-centrifugation short-term conservation temperature on the survival and motility of Catalonian donkey spermatozoa

E. Taberner, A. Peñalba, A. Peña, J. Miró

Department of Animal Medicine and Surgery, School of Veterinary Medicine, Autonomous University of Barcelona,
Campus UAB, Edifici V, E-08193 Bellaterra, Spain

Submitted for publication

Effect of pre-centrifugation short-term conservation temperature on the survival and motility of Catalonian donkey spermatozoa

Abstract

The aim of this study was to assess the effect of three storage temperatures on the survival and motility of Catalonian donkey sperm during transport to the laboratory for centrifugation. The response to refrigeration and centrifugation of the different sperm cell subpopulations in donkey ejaculates was also investigated. Semen was collected from seven Catalonian jackasses and diluted with a skimmed milk-based (Kenney) extender at 37ºC. Sperm survival and motility were examined in aliquots that underwent different treatments: freshly collected semen (FRESH), fresh semen immediately centrifuged (660 *g* for 15 min) to remove the seminal plasma before resuspension in Kenney extender (FRESH+CENTRIFUGATION), fresh semen stored at 5, 15 or 20ºC for 2 h (STORAGE 5/15/20ºC), and STORAGE 5/15/20ºC semen then centrifuged as above (STORAGE 5/15/20ºC+CENTRIFUGATION). All centrifugations were performed at the corresponding storage temperature. Survival was examined using eosin-nigrosin stained smears. Motion characteristics were assessed by means of a computer-assisted sperm analyzer (CASA). The sperm cells of the STORAGE 5ºC and 20ºC treatments showed an overall motility similar to that seen in FRESH samples. However, the STORAGE 15ºC treatment led to an important reduction in mean motility parameters. No differences were seen between the FRESH and STORAGE 5/15/20ºC+CENTRIFUGATION treatments with respect to progressive motility. However, the STORAGE 5/15/20ºC+CENTRIFUGATION treatments all reduced total motility, and the STORAGE 15ºC+CENTRIFUGATION treatment led to reduced survival. The 4-subpopulation

structure of donkey semen was maintained after the STORAGE 5/15/20ºC+CENTRIFUGATION treatments, although the STORAGE 15ºC +CENTRIFUGATION treatment led to important changes in sperm motion characteristics, especially in subpopulations 2 and 4. The STORAGE 5/20ºC+CENTRIFUGATION treatments, in contrast, only induced slight changes in mean motion indices in each subpopulation. The STORAGE 20ºC+CENTRIFUGATION treatment was associated with no change in the percentage of sperm cells belonging to each subpopulation compared to FRESH sperm, and only slight changes were induced by the STORAGE 5/15ºC+CENTRIFUGATION treatments. Subpopulation 4 showed significant differences in terms of motility indices after the STORAGE 5ºC/15ºC+CENTRIFUGATION treatments. In conclusion, the present results indicate that 2 h of storage at 20ºC followed by centrifugation is suitable for the short-term storage of donkey semen. However, 5ºC would also appear to be an adequate storage temperature before centrifugation if, for some reason, such conditions were necessary.

1. Introduction

The Catalonian donkey breed has suffered a substantial reduction in its numbers, a consequence of the intense mechanization of agriculture [1]. This could lead to high levels of inbreeding, which would only increase the risk of the breed's extinction. The use of reproductive technologies and the setting up of gene banks can, however, contribute to the preservation of endangered species, and might help the Catalonian donkey. In this respect, the optimization of the cooling of collected semen for transport would facilitate the involvement of animals far from facilities where artificial insemination or semen cryopreservation are performed.

Cooling semen reduces the metabolic activity of the spermatozoa it contains, reduces microbial growth, and helps to extend the time over which the viability of sperm is maintained. However, cooling between 18 and 8ºC is a critical step that can lead to "cold shock", a problem associated with damage to sperm cell plasma membranes [2]. Indeed, semen processing involves a number of factors that can cause such damage, including the addition of semen extender, centrifugation and storage [3]. Damage to the plasma membrane results in the irreversible loss of its functions. A loss of motility and fertilizing capacity can be caused, at least in part, by the peroxidation of lipids in the membrane [4]. Injuries can be reduced if the cooling rate is slow (<0.3ºC/min), but there is no real consensus regarding the optimal final storage temperature for the liquid preservation of equine spermatozoa. Temperatures as low as 4-6°C have been reported to provide better environments for the maintenance of motility [5,6] and fertility [7], but some authors [8] indicate temperatures of 15 or even 20ºC to better maintain motility and fertility (if storage is no longer than 12 h).

The centrifugation of equine semen is necessary to limit the harmful effects of seminal plasma on sperm motility during storage. It is also necessary for the addition of cryoprotectants and the adjustment of sperm concentrations before freezing [9]. Centrifugation has to be performed with great care since damage can result from the mechanical forces induced by the close packing of the spermatozoa. This can be manifested as structural damage of the sperm acrosome and an important loss of motility and enzymatic activity [10]. Some studies have assessed the use of different extenders, storage temperatures and the elimination of seminal plasma by centrifugation as means of better preserving donkey semen [11-15].

The aim of the present study was to determine the effect of three storage temperatures (5, 15 and 20ºC for 2 h) with and without centrifugation on donkey sperm survival and motility, and to study the changes induced by these treatments in the different sperm subpopulations.

2. Materials and Methods

2.1. Animals and semen collection

Donkey semen was obtained from seven healthy, mature Catalonian donkeys aged 4-6 years, all of which were previously reported as fertile. Animals were housed at the Experimental Farm and Countryside Service of the School of Veterinary Medicine of the Autonomous University of Barcelona (Bellaterra, Spain). Semen was collected using an artificial vagina (Hannover model) with an in-line gel filter to allow the collection of gel-free semen. Ejaculates were obtained at 2-3 day intervals in the presence of an ovariectomized female donkey brought into estrus with estrogens. Immediately after

collection, the gel-free semen was diluted (proportion 1:5, v/v) with dry skimmed milk extender (24 mg/mL dry skimmed milk and 49 mg/mL glucose) kept at 37ºC in a water bath. The diluted semen was immediately cooled in a water bath to 20ºC before all other treatments.

2.2. Storage conditions and centrifugation

One aliquot of freshly collected semen was immediately analyzed (rewarmed to 37ºC) for sperm survival and motility (FRESH). A further aliquot was immediately centrifuged at 660 x g for 15 min at 20ºC to remove the seminal plasma. The pellet obtained was resuspended with skimmed milk-based (Kenney) extender at a final dilution of 25 x 10^6 sperm/mL, incubated at 37ºC for 5 min and reanalyzed for sperm survival and motility (FRESH+CENTRIFUGATION). Other aliquots of each sample were maintained at 5ºC, 15ºC or 20ºC for 2 h. They were then re-evaluated at 37ºC for viability and motility (STORAGE 5/15/20ºC) before being centrifuged at 660 *g* for 15 min at the corresponding storage temperature in a programmable refrigerated centrifuge (Medifriger BL-S; JP Selecta; Barcelona, Spain). The supernatant was eliminated and the sperm re-suspended in Kenney extender. These centrifuged samples were then incubated in a water bath at 37ºC for 15 min and viability and motility re-evaluated once more (STORAGE 5/15/20ºC+CENTRIFUGATION).

2.3 Analysis of semen quality variables

Fresh semen was subjected to standard analysis to determine the sperm concentration, total sperm number, sperm survival, morphological abnormalities and motility. After the

different treatments each sample was analyzed to determine sperm cell survival and motility. Sperm concentration and total sperm number were determined using a Neubauer hemocytometer. Sperm survival and total morphological abnormalities were determined by eosin-nigrosin staining as described by Bamba [16]. Viable spermatozoa show uniform white staining over the entire cell; the presence of a partial or totally pinkish stain is indicative of non-viable sperm cells. This determination was made after examining a minimum of 200 spermatozoa/sample by light microscopy (magnification: 1000x).

The motion characteristics of the samples were determined using a computer-assisted sperm analyzer (CASA, ISAS v1.0; Proiser SL, Valencia, Spain). An aliquot of each sample of treated semen was incubated for 5 min in a water bath at 37ºC. Five μL drops of each sample were observed using a phase contrast microscope with a heatable stage (37ºC). Three fields per drop were analyzed. The CASA system is based on the analysis of 50 consecutive, digital images of a single field at a magnification of x200 (dark ground). The settings for the system were: 50 images acquired over 1 s (1 every 20 ms), minimum contrast 80, minimum cell size 4 pixels. Total motility was defined as the percentage of spermatozoa with an average path velocity (VAP) of >10 μm/s. Progressive motility was defined as the percentage of spermatozoa with a VAP of >90 μm/s plus a straightness coefficient (STR) of >75%.

The sperm motility descriptors obtained by the CASA system are:

Curvilinear Velocity (VCL)	μm/s	Measures the sequential progression along the true trajectory
Linear Velocity (VSL)	μm/s	Measures the straight trajectory of the spermatozoa per unit time
Average Path Velocity (VAP)	μm/s	Measures the mean trajectory of the

		spermatozoa per unit time
Linearity Coefficient (LIN)	%	VSL/VCL x 100
Straightness Coefficient (STR)	%	VSL/VAP x 100
Wobble Coefficient (WOB)	%	VAP/VCL x 100
Mean lateral head displacement (ALH)	μm	Measures the mean head displacement along the curvilinear trajectory
Frequency of head displacement (BCF)	Hz	Frequency with which the sperm trajectory crosses the average path trajectory

2.4. Statistical analysis

The results were analyzed using the SAS statistical package [17]. Normality was assessed by the Shapiro-Wilks test (W) included in the UNIVARIATE procedure. The FASTCLUS clustering procedure (which performs a disjointed cluster analysis based on Euclidean distances calculated from one or more quantitative variables - in this case the sperm motility variables measured by the CASA system) was then used to separate the spermatozoa into subpopulations. Sperm cells were divided into clusters such that every observation belonged to a single cluster. Sperm cells that shared similar motility characteristics were assigned to the same cluster. The PROC GLM procedure was used to detect differences between the values for the sperm motility descriptors of these subpopulations. The LSMEANS procedure was used to determine the degree of significance of these differences. A Chi-squared test was used to examine the percentage of sperm cells belonging to each cell subpopulation after each treatment. New PROC GLM

and LSMEANS procedures were used to determine and list, respectively, any differences in the number of sperm cells belonging to the different subpopulations after the different treatments. The total number of spermatozoa analyzed following this protocol was 14,860.

3. Results

Quality of refrigerated and centrifuged donkey semen

Table 1 shows the characteristics of the semen samples processed under the different conservation and centrifugation conditions. No differences were seen between the FRESH and FRESH+CENTRIFUGATION sperm in terms of total or progressive motility or survival. However, the LIN and STR values of the FRESH+CENTRIFUGATION samples were significantly higher, and the VCL value significantly smaller than those of the FRESH samples ($P<0.05$).

No important differences were seen in sperm survival or progressive or total motility between the STORED 5/15/20ºC and FRESH treatments. The CASA system showed the overall motility associated with the FRESH and STORAGE 5ºC treatments to be very similar; in fact, only the VSL showed a significant reduction in the latter treatment (from 72.1 μm/s in FRESH semen to 68.8 μm/s). The STORED 15ºC treatment was associated with significant reductions in the VCL and mean ALH values (VCL falling from 114.2 μm/s in FRESH semen to 109.2 μm/s, and mean ALH falling from 3.50 μm to 3.20 μm). The same treatment was also associated with an overall motility more linear than that seen at other temperatures, as the increases in the VSL, LIN, STR and WOB values show. The STORAGE 20ºC treatment was associated with a significant increase in VCL, VAP and mean ALH.

After 2 h of storage at the different temperatures, centrifugation had different effects on viability and mean motility. No differences were seen in terms of viability between the STORED 5ºC+CENTRIFUGATION and FRESH treatments, nor between the STORED 20ºC+CENTRIFUGATION and FRESH treatments. However, the STORED 15ºC+CENTRIFUGATION treatment was associated with a significant reduction in survival ($P<0.05$). In addition, all the STORED 5/15/20ºC+CENTRIFUGATION treatments were associated with a significant reduction in total motility compared to the FRESH treatment, although no differences were seen with respect to progressive motility. No differences were seen between the STORED 5ºC+CENTRIFUGATION and FRESH treatments in terms of sperm motion characteristics, except for BCF, which was significantly increased. In contrast, the STORED 15ºC+CENTRIFUGATION treatment was associated with important changes in velocity characteristics. The values of all the velocity variables were significantly lower than in FRESH sperm. Finally, the STORED 20ºC+CENTRIFUGATION treatment was associated with significant reductions in the VSL, LIN and STR values compared to FRESH samples, while a significant increase was observed in mean ALH.

Table 1. Semen quality analysis before and after centrifugation at different storage temperatures.

	FRESH	FRESH+CTR	STORAGE 5ºC	STORAGE 5ºC+CTR	STORAGE 15ºC	STORAGE 15ºC+CTR	STORAGE 20ºC	STORAGE 20ºC+CTR
Viability (%)	71.0 ± 3.8^{a}	69.7 ± 4.0^{ab}	71.9 ± 4.1^{a}	65.2 ± 4.7^{ab}	68.0 ± 3.7^{ab}	63.1 ± 4.0^{b}	73.3 ± 3.7^{a}	69.0 ± 4.1^{ab}
Total Motility (%)	94.8 ± 1.4^{a}	88.6 ± 2.7^{ab}	86.4 ± 3.5^{ab}	78.0 ± 4.5^{b}	88.0 ± 1.9^{ab}	82.1 ± 3.1^{bc}	92.6 ± 2.4^{ac}	82.6 ± 3.2^{bc}
Progressive Motility (%)	50.4±2.6	44.0±3.0	41.1±3.1	38.6±3.7	41.8±2.6	41.3±3.2	48.8±3.2	38.5±2.8
VCL (μm/sec)	114.2 ± 0.5^{ae}	112.2 ± 0.5^{b}	112.8 ± 0.7^{ab}	114.1 ± 0.7^{abe}	109.2 ± 0.5^{c}	107.4 ± 0.6^{c}	117.8 ± 0.6^{d}	115.4 ± 0.6^{e}
VSL(μm/sec)	72.1 ± 0.4^{a}	$72.3\pm0.5^{a,c}$	68.8 ± 0.6^{b}	$70.7\pm0.6^{a,b}$	74.1 ± 0.5^{c}	65.6 ± 0.6^{d}	$72.0\pm0.5^{a,e}$	$70.2\pm0.5^{b,e}$
VAP (μm/sec)	91.2 ± 0.4^{a}	89.6 ± 0.5^{a}	89.5 ± 0.6^{a}	$91.0\pm0.6^{a,c}$	89.9 ± 0.5^{a}	84.6 ± 0.5^{b}	93.0 ± 0.5^{c}	91.1 ± 0.5^{a}
LIN (%)	$58.3\pm0.4^{a,c}$	60.1 ± 0.5^{b}	$56.6\pm0.6^{a,d}$	$59.3\pm0.6^{b,c,e}$	61.0 ± 0.5^{b}	$58.0\pm0.5^{a,d,e}$	$57.0\pm0.5^{a,d}$	56.5 ± 0.5^{d}
STR (%)	$74.6\pm0.4^{a,c}$	76.8 ± 0.5^{b}	$72.8\pm0.6^{c,d}$	$75.6\pm0.6^{a,b}$	76.7 ± 0.5^{b}	$74.8\pm0.5^{a,c,d}$	$73.6\pm0.5^{c,d}$	73.4 ± 0.5^{d}
WOB (%)	$75.7\pm0.3^{a,c}$	$76.2\pm0.3^{a,b}$	$75.2\pm0.4^{a,c}$	$76.5\pm0.4^{a,b}$	77.2 ± 0.3^{b}	$75.3\pm0.4^{a,c}$	$75.3\pm0.4^{a,c}$	74.9 ± 0.4^{c}
mean ALH (μm)	3.50 ± 0.02^{a}	3.44 ± 0.03^{a}	3.48 ± 0.03^{a}	$3.50\pm0.04^{a,d}$	3.20 ± 0.03^{b}	3.40 ± 0.03^{a}	3.66 ± 0.03^{c}	$3.61\pm0.03^{c,d}$
BCF (Hz)	$6.72\pm0.07^{a,c,d}$	$7.01\pm0.08^{a,b}$	$6.96\pm0.10^{a,b,c}$	7.15 ± 0.10^{b}	6.48 ± 0.08^{d}	$6.65\pm0.10^{c,d}$	$6.67\pm0.09^{c,d}$	$6.66\pm0.09^{c,d}$

Results are expressed as means ± SEM of 34 different experiments with a total analyzed sperm number of 14,860. VCL: curvilinear velocity; VSL: linear velocity; VAP: mean velocity; LIN: linearity coefficient; STR: straightness coefficient; WOB: wobble coefficient; mean ALH: mean lateral head displacement, BCF: frequency of head displacement; CTR, centrifugation. Different superscripts between rows indicate significant differences ($P<0.05$).

Sperm subpopulation structure

The CASA system revealed the FRESH samples to show the typical four-subpopulation structure.

Subpopulation 1: This subpopulation showed the highest VCL value (170.51±0.84 μm/s) and the highest velocity and linearity characteristics, as indicated by the VCL, VSL, VAP, LIN and STR values. The oscillatory movement of the spermatozoa was also very notable, as indicated by the high WOB, mean ALH and BCF values. This subpopulation made up 28.0±2.6% of all sperm cells (Table 2).

Subpopulation 2: This subpopulation had a high VCL value (160.52±0.95 μm/s). Its cells showed high velocity and low linearity, as indicated by the low LIN and STR values. They also showed quite notable oscillatory movement, as indicated by the WOB, mean ALH and BCF values. This subpopulation made up 20.3±2.1% of all sperm cells (Table 2).

Subpopulation 3: This subpopulation had medium VCL value (93.76±0.87 μm/s). The cells showed a medium velocity (as indicated by the VCL, VSL and VAP values), medium linearity, and notable oscillatory movement (as indicated by their WOB, mean ALH and BCF values). This subpopulation made up 23.6±1.5% of all sperm cells (Table 3).

Subpopulation 4: This subpopulation showed the lowest VCL value (31.89±0.78 μm/s), as well as low VSL and VAP values, reduced oscillatory movement (as indicated by the WOB, mean ALH and BCF values) and low linearity (as indicated by the values for LIN and STR). This subpopulation made up 28.1±3.4% of all sperm cells (Table 3).

Refrigeration and later centrifugation induced different changes in the mean motion characteristics of each subpopulation.

Subpopulation 1: This subpopulation showed modifications only in terms of velocity variables, mainly after the STORAGE 15ºC+CENTRIFUGATION treatment. The STORAGE 15ºC treatment actually led to increases in the VCL, VSL and VAP values compared to the FRESH treatment, but later centrifugation led to their reduction. The STORAGE 5ºC treatment led to reduced VSL and VAP values, while the STORAGE 5ºC+CENTRIFUGATION treatment led to no significant differences in the values of velocity variables compared to the FRESH treatment. No difference in any motility variable was seen between the STORAGE 20ºC and FRESH treatments. Finally, the STORAGE 20ºC+CENTRIFUGATION treatment had no effect on the velocity variables except for VAP, which was significantly reduced.

Subpopulation 2: This subpopulation suffered the most important changes in terms of motility variables. The STORAGE 5ºC treatment led to reductions in the VSL and STR values compared to the FRESH treatment. The STORAGE 5ºC+CENTRIFUGATION treatment was associated with the same changes plus a reduction in the LIN value. The STORAGE 15ºC treatment was associated with important changes in motility. The VCL and mean ALH values were reduced while the VSL, LIN, STR and WOB values increased. The STORAGE 15ºC+CENTRIFUGATION treatment led to a reduction in the values of VCL, VSL, VAP, LIN and STR. The STORAGE 20ºC treatment led to no changes in any variable compared to the FRESH treatment, while the STORAGE 20ºC+CENTRIFUGATION treatment was associated with reduced VSL and STR values.

Subpopulation 3: No differences were seen in any motility variable between the STORAGE 5ºC and FRESH treatments. The STORAGE 5ºC+CENTRIFUGATION treatment, however, led to increases in the LIN, STR and WOB values. The STORAGE

15ºC treatment led to a reduction in VCL, while the STORAGE 15ºC+CENTRIFUGATION treatment induced an increase in sperm linearity, as indicated by the LIN and STR values. The STORAGE 20ºC treatment induced an increase in VCL values. The STORAGE 20ºC+CENTRIFUGATION treatment led to no changes in motility variables.

Subpopulation 4: Changes in motility variables were induced in this subpopulation, especially by storage at 5ºC and 15ºC. The STORAGE 15ºC treatment led to important reductions in all velocity variables (VCL, VSL and VAP) and in the mean ALH and BCF values compared to the FRESH treatment. However, in the STORAGE 15ºC+CENTRIFUGATION treatment only the STR values increased significantly. After the STORAGE 5ºC treatment the motility characteristics of the sperm were similar to those of FRESH sperm. However, STORAGE 5ºC+CENTRIFUGATION treatment led to a reduction of all velocity variables and the BCF value, along with an increase in the LIN and STR values. The STORAGE 20ºC treatment led to increased VCL and mean ALH values; the STORAGE 20ºC+CENTRIFUGATION treatment also led to increased mean ALH values.

Table 2. Effects of refrigeration and centrifugation at different temperatures on motility parameters of Subpopulations 1 and 2 from donkey semen samples.

Subpopulation 1	FRESH	FRESH+CTR	STORAGE 5ºC	STORAGE 5ºC+CTR	STORAGE 15ºC	STORAGE 15ºC+CTR	STORAGE 20ºC	STORAGE 20ºC+CTR
VCL (μm/s)	170.5±0.8^{a}	167.9±1.0^{a}	167.1±1.16^{ab}	167.1±1.3^{ab}	177.0±1.2^{c}	161.7±1.1^{b}	172.4±1.0^{ac}	167.8±1.1^{a}
VSL(μm/s)	139.7±0.8^{a}	138.8±0.9^{ab}	135.0±1.05^{bc}	136.2±1.2^{ac}	146.6±1.1^{d}	130.7±1.0^{c}	140.3±0.9^{a}	135.8±1.0^{ab}
VAP (μm/s)	153.1±0.7^{ab}	150.8±0.8^{abc}	148.6±1.0^{cd}	148.3±1.1^{acd}	160.3±1.0^{e}	143.7±0.9^{d}	153.9±0.8^{b}	148.8±0.9^{c}
LIN (%)	82.6±0.7	83.4±0.8	81.7±1.0	82.0±1.1	83.6±1.0	82.0±0.9	81.9±0.8	81.5±0.9
STR (%)	91.3±0.7	92.3±0.8	90.7±1.0	92.0±1.1	91.5±1.0	91.2±0.9	91.1±0.8	91.3±0.9
WOB (%)	90.2±0.5	90.3±0.6	89.1±0.7	89.0±0.8	91.0±0.7	89.6±0.7	89.6±0.	89.0±0.7
mean ALH (μm)	3.9±0.0	3.8±0.1	3.8±0.1	3.9±0.1	3.8±0.1	3.8±0.1	3.9±0.1	4.0±0.06
BCF (Hz)	8.3±0.1	8.4±0.2	8.9±0.2	8.9±0.2	8.3±0.2	8.0±0.2	8.4±0.2	8.4±0.2
Subpopulation 2	FRESH	FRESH+CTR	STORAGE 5ºC	STORAGE 5ºC+CTR	STORAGE 15ºC	STORAGE 15ºC+CTR	STORAGE 20ºC	STORAGE 20ºC+CTR
VCL (μm/s)	160.5±1.0^{a}	153.9±1.2^{bc}	157.3±1.6^{ac}	160.0±1.9^{ac}	148.0±1.0^{d}	147.2±1.6^{bd}	163.0±1.2^{a}	164.6±1.3^{a}
VSL(μm/s)	77.4±0.9^{a}	75.0±1.1^{ab}	68.4±1.4^{bc}	65.3±1.7^{ce}	87.1±0.9^{d}	59.8±1.4^{e}	72.1±1.1^{abc}	71.8±1.2^{bc}
VAP (μm/s)	119.9±0.8^{a}	113.4±1.1^{b}	117.2±1.3^{ab}	117.3±1.6^{ab}	117.5±0.9^{ab}	105.9±1.3^{c}	120.2±1.1^{a}	122.2±1.1^{a}
LIN (%)	49.2±0.8^{a}	49.4±1.1^{a}	44.2±1.4^{ab}	41.2±1.6^{b}	61.0±0.9^{c}	41.3±1.3^{b}	45.1±1.1^{ab}	44.0±1.1^{ab}
STR (%)	65.5±0.8^{ad}	66.9±1.1^{a}	59.1±1.4^{b}	56.5±1.6^{b}	75.4±0.9^{c}	57.9±1.4^{b}	61.2±1.1^{bd}	59.5±1.2^{b}
WOB (%)	75.1±0.6^{a}	74.3±0.8^{a}	74.7±0.1^{a}	73.7±1.2^{a}	80.3±0.6^{b}	72.1±0.1^{a}	74.1±0.8^{a}	74.6±0.8^{a}
mean ALH (μm)	5.3±0.1^{a}	5.3±0.1^{a}	5.2±0.1^{a}	5.4±0.1^{a}	4.6±0.1^{b}	5.2±0.1^{a}	5.5±0.1^{a}	5.5±0.1^{a}
BCF (Hz)	6.6±0.2	7.0±0.2	6.8±0.3	6.7±0.3	7.0±0.2	6.2±0.2	6.3±0.2	6.3±0.2

Results are expressed as means ± SEM of 34 different experiments with a total number of analyzed spermatozoa of 14,860. Different superscripts between rows indicates significant (P<0.05) differences. CTR = centrifugation.

Table 3. Effects of refrigeration and centrifugation at different temperatures on motility parameters of Subpopulations 3 and 4 from donkey semen samples.

Subpopulation 3	FRESH	FRESH+CTR	STORAGE 5ºC	STORAGE 5ºC+CTR	STORAGE 15ºC	STORAGE 15ºC+CTR	STORAGE 20ºC	STORAGE 20ºC+CTR
VCL (μm/s)	93.8±0.9^{a}	95.2±0.9^{a}	96.5±1.1^{ab}	100.6±1.0^{ab}	85.1±0.9^{b}	90.1±0.9^{a}	99.5±1.0^{c}	97.6±1.0^{a}
VSL(μm/s)	60.4±0.8^{ab}	63.3±0.8^{a}	61.8±1.0^{ab}	70.0±0.9^{ab}	53.6±0.9^{b}	60.7±0.9^{ab}	62.8±0.9^{a}	62.9±0.9^{ab}
VAP (μm/s)	73.5±0.7^{ab}	75.1±0.8^{a}	74.7±0.9^{ab}	80.6±0.9^{ab}	66.2±0.8^{b}	70.6±0.8^{ab}	76.7±0.8^{a}	75.5±0.8^{ab}
LIN (%)	65.5±0.8^{a}	67.3±0.8^{b}	65.2±0.9^{a}	70.4±0.9^{c}	64.3±0.8^{a}	68.7±0.8^{b}	63.8±0.8^{ab}	65.9±0.8^{a}
STR (%)	82.1±0.8^{a}	84.0±0.8^{bc}	82.6±0.9^{a}	86.7±0.9^{c}	81.2±0.8^{a}	86.3±0.8^{b}	81.5±0.8^{ab}	83.4±0.8^{a}
WOB (%)	79.1±0.6^{ad}	79.5±0.6^{bc}	78.4±0.7^{ab}	80.7±0.6^{c}	78.5±0.6^{ab}	79.1±0.6^{bcd}	77.7±0.6^{ab}	78.4±0.6^{a}
mean ALH (μm)	3.3±0.1^{ab}	3.2±0.1^{ac}	3.4±0.1^{ac}	3.2±0.1^{a}	3.1±0.1^{a}	3.1±0.1^{ac}	3.5±0.1^{b}	3.4±0.1^{bc}
BCF (Hz)	6.6±0.1^{ab}	7.2±0.1^{a}	7.0±0.2^{ab}	7.8±0.2^{ab}	5.9±0.2^{b}	7.3±0.2^{ab}	6.4±0.2^{a}	7.0±0.2^{ab}
Subpopulation 4	FRESH	FRESH+CTR	STORAGE 5ºC	STORAGE 5ºC+CTR	STORAGE 15ºC	STORAGE 15ºC+CTR	STORAGE 20ºC	STORAGE 20ºC+CTR
VCL (μm/s)	31.9±0.8^{ad}	31.8±0.7^{ae}	30.3±0.9^{b}	28.6±0.8^{b}	26.7±0.7^{c}	30.7±0.7^{d}	36.3±0.8^{be}	31.7±0.7^{ab}
VSL(μm/s)	10.7±0.7^{a}	12.3±0.6^{a}	9.9±0.8^{a}	11.5±0.7^{b}	9.0±0.7^{c}	11.1±0.6^{a}	12.7±0.8^{a}	10.3±0.6^{a}
VAP (μm/s)	18.4±0.7^{ad}	19.3±0.6^{a}	17.3±0.7^{ad}	17.6±0.7^{b}	15.5±0.6^{c}	18.1±0.6^{d}	21.3±0.7^{ab}	18.1±0.6^{a}
LIN (%)	35.9±0.7^{ac}	40.1±0.6^{ab}	35.3±0.7^{ac}	43.4±0.7^{b}	35.2±0.6^{a}	40.0±0.6^{bc}	37.3±0.7^{a}	34.7±0.6^{ac}
STR (%)	59.6±0.7^{a}	64.0±0.6^{ab}	59.0±0.8^{b}	67.0±0.7^{b}	58.7±0.6^{a}	63.7±0.6^{b}	60.7±0.7^{a}	59.3±0.6^{ab}
WOB (%)	58.5±0.5	61.0±0.4	58.6±0.5	62.7±0.5	58.8±0.5	60.6±0.4	59.7±0.5	57.6±0.5
mean ALH (μm)	1.6±0.0^{ab}	1.5±0.0^{abd}	1.5±0.1^{c}	1.4±0.0^{abc}	1.4±0.0^{b}	1.5±0.0^{ab}	1.7±0.0^{cd}	1.6±0.0^{d}
BCF (Hz)	5.4±0.1^{ad}	5.4±0.1^{abd}	5.1±0.1^{abd}	5.3±0.1^{b}	4.9±0.1^{c}	5.0±0.1^{ab}	5.6±0.1^{cd}	5.0±0.1^{ad}

Results are expressed as means ± SEM of 34 different experiments with a total number of analyzed spermatozoa of 14,860. Different superscripts between rows indicates significant (P<0.05) differences. CTR = centrifugation.

The percentage of sperm cells belonging to each subpopulation experienced slight changes after the different treatments, with only subpopulation 4 experiencing significant differences. Figure 1 shows that the STORAGE 5ºC+CENTRIFUGATION and STORAGE 15ºC+CENTRIFUGATION treatments significantly ($P<0.05$) increased the percentage of sperm cells in subpopulation 4 (from 28.1±3.4% in FRESH samples to 47.6±4.7% and 47.4±3.5%, respectively).

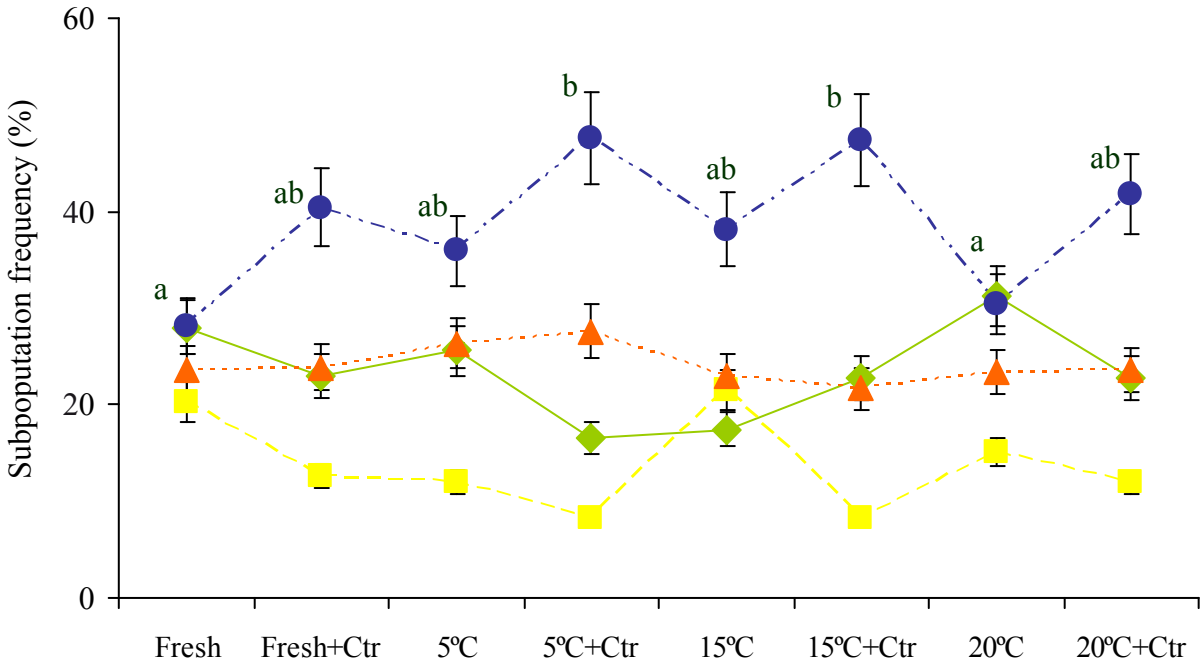

Figure 1. Number of cells belonging to each subpopulation before and after refrigeration and centrifugation. Results are means ± SEM for 34 different experiments. Different letters (a-b) indicate significant differences ($P<0.05$) between FRESH samples and after refrigeration/centrifugation treatment. ◆: Subpopulation 1. ■: Subpopulation 2. ▲: Subpopulation 3. ●: Subpopulation 4

4. Discussion

Equine semen is commonly cooled to reduce the metabolic activity of the spermatozoa, to reduce microbial growth, and to maintain the viability of the sperm for extended periods of time. However, semen processing is known to damage the plasma membrane, contributing significantly to a loss of motility and fertilizing ability. Sometimes only a short storage period is required before artificial insemination or semen freezing, but no data are available on the best short-term storage conditions for donkey semen. Success in the use of cooled/stored semen depends on damage being avoided as far as possible since mature spermatozoa can no longer call upon repair mechanisms [18]. The decision was therefore made to evaluate sperm motility and survival after short-term storage at different temperatures followed by centrifugation (or not) at the same temperatures.

The optimum storage temperature for maintaining the motility and fertility of horse semen has previously been reported as 4-6ºC [5,6]. However, other authors have reported 15 or 20ºC to be better than 5ºC for maintaining motility and fertility – although the duration of storage was between 4 and 12 h [8,19]. Cottorello et al. [12] concluded that a 5ºC maintenance temperature and the use of modified Baken extender (10% egg yolk) was more appropriate than a 10ºC or 0ºC storage temperature for the preservation of donkey sperm motility *in vitro*, while Serres et al. [13] reported it to be better preserved at 4ºC or 15ºC in the presence of INRA82 extender than at 20ºC. The latter authors also observed that the storage of donkey semen diluted with INRA 82 under aerobic conditions at 15ºC best maintained the integrity of the plasma membrane. The results of the present study indicate that, after 2 h of cool storage, survival, total motility and progressive motility were maintained at all the temperatures investigated, although 5ºC or 20ºC seem to be the best for maintaining the mean motion characteristics as determined by the CASA system. The

discrepancies between the present results and those of other authors might be due to the use of different storage times or diluents, or differences in the accuracy of sperm motility assessments.

Stallion semen is routinely centrifuged to reduce the harmful effect of seminal plasma and to adjust the sperm cell concentration before freezing. Certainly, an improvement in the maintenance of sperm motility in cooled stallion and indeed donkey semen has been observed after reducing the concentration of seminal plasma [13, 15, 20, 21]. However, Rota et al. [14] reported higher total motility values and percentages of rapid spermatozoa in non-centrifuged donkey samples. These authors also observed that the removal of seminal plasma increased the number of spermatozoa showing high progressive motility, a consequence of the greater straightness of their tracks after 48 h of storage. In the present study, the FRESH+CENTRIFUGATION treatment had a significant, positive effect on sperm linearity and reduced the curvilinear velocity, in agreement with other authors [14].

Several authors [9, 22, 23] have reported better post-thaw motility and fertility for stallion semen centrifuged at 22-25ºC and then cooled to 5ºC before freezing than for semen centrifuged at 5ºC. Backman et al. [24] compared the post-thaw motility of ejaculates first cooled to 5ºC for 18 h and then centrifuged to that of ejaculates centrifuged at room temperature and then cooled to 5ºC for 18 h, and reported no differences with respect to post-thaw total and progressive motility. The present results show that after all STORAGE+CENTRIFUGATION treatments, total motility was significantly reduced, while progressive motility was not affected. Moreover, sperm viability decreased in the STORAGE 15ºC+CENTRIFUGATION treatment. The sperm in the STORAGE 5ºC+CENTRIFUGATION treatment showed mean motion values more similar to those of FRESH samples. These results are not comparable with those of other authors since, in the present study, post-thaw motility was not evaluated [24]. Further studies are needed to

determine the effect of storing and centrifuging donkey sperm at different temperatures on post-thaw motility and fertility.

Motile sperm subpopulations have been described in a large number of mammals, including the donkey [25-30]. Previous studies have established that motility changes induced by centrifugation, cooling or freezing/thawing procedures are linked to changes in specific motion variables and the percentage of sperm cells belonging to each subpopulation [15, 30]. In the present study, the 4-subpopulation structure of donkey semen was maintained after cooling and centrifugation at all the temperatures investigated. The STORAGE 15ºC+CENTRIFUGATION treatment was associated with important changes in the sperm motion characteristics, especially in subpopulations 2 and 4. However, the STORAGE 5/20ºC+CENTRIFUGATION treatments induced only slight changes on the mean motion characteristics of each subpopulation. No differences were seen with respect to FRESH sperm in terms of the percentage of sperm cells belonging to each subpopulation when the semen was subjected to the STORAGE 20ºC+CENTRIFUGATION treatment; significant changes were only seen in the percentage of spermatozoa belonging to subpopulation 4 after the STORAGE 5/15ºC+CENTRIFUGATION treatments. In conclusion, fresh donkey semen can be maintained in adequate condition for around 2 h if kept at 20ºC and then centrifuged. However, 5ºC would also appear to be an adequate storage temperature before centrifugation if, for some reason, such conditions were necessary.

Acknowledgments

This work has been supported by Grant RZ2007-00017-00-00 (Instituto Nacional de Investigaciones Agrarias, Spain). The authors are grateful to Mr. José Manuel Fernández Castro for providing us some of the jackasses.

References

[1] Aranguren-Méndez JA, Jordana J, Gomez M. Genetic diversity in Spanish donkey breeds using microsatellite DNA markers. Genet Sel Evol 2001;33:433-442.

[2] Amann RP, Pickett BW. Principle of cryopreservation and a review of cryopreservation of stallion spermatozoa. J Equine Vet Sci 1987;7:145-173.

[3] Aurich C. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. Anim Repro Sci 2005;89:65-75.

[4] Storey BT. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Mol Hum Reprod 1997;3:203-213.

[5] Varner DD, Blanchard TL, Meyers PJ and Meyers SA. Fertilizing capacity of equine spermatozoa stored for 24 hours at 5 and 20ºC. Theriogenology 1989;32(4):515-525.

[6] Moran DM, Jasko DJ, Squires EL and Amann RP. Determination of temperature and cooling rate which induce cold shock in stallion spermatozoa. Theriogenology 1992; 38:999-1012.

[7] Squires E, Amann RP, McKinnon AO, Pickett BW. Fertility of equine spermatozoa cooled to 5 or 20ºC. In: Proceedings of the 11th International Congress on Animal Reproduction, Dublin, 1988; 3: 297-299.

[8] Province CA, Squires EL, Pickett BW, Amann RP. Cooling rates, storage temperatures and fertility of extended equine spermatozoa. Theriogenology 1985; 23: 925-934.

[9] Vidament M, Ecot P, Noue P, Bourgeois C, Magistrini M, Palmer E. Centrifugation and addition of glycerol at 22ºC instead of 4ºC improve post-thaw motility and fertility of stallion spermatozoa. Theriogenology 2000; 54:907-919.

[10] Mátas C, Decuadro G, Martínez-Miró S, Gadea J. Evaluation of a cushioned method for centrifugation and processing for freezing boar semen. Theriogenology 2007; 67:1087-1091.

[11] Mello SLV, Henry M, Souza MC, Oliveira SMP. Effect of split ejaculation and seminal extenders on longevity of donkey semen preserved at 5ºC. Arq Bras Med Vet Zootec 2000; 52:372-378.

[12] Cottorello ACP, Amancio RC, Henry M, Borges I. Effect of storage temperature and extenders on “in vitro” activity of donkey spermatozoa. Theriogenology 2002; 58:325-328.

[13] Serres C, Rodriguez A, Alvarez AL, Santiago I, Gabriel J, Gómez-Cuétara C, Mateos E. Effect of centrifugation and temperature on the motility and plasma membrane integrity of Zamorano-Leonés donkey semen. Theriogenology 2002; 58:329-332.

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

[15] Miró J, Taberner E, Rivera M, Peña A, Medrano A, Rigau T, Peñalba A. 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; 72:1017-1022.

[16] Bamba K. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. Theriogenology 1988; 29:1245-1251.

[17] SAS. SAS/STAC Software; Cary, NC,USA. SAS Inst. Inc.; 2000.

[18] Eddy EM, O’Brien DA. The spermatozoon. In: Knobil E, Neill JD (Eds.), The Physiology of Reproduction. Raven Press, New York. 1994; pp 29-77.

[19] Francl AT, Amann RP, Squires EL, Pickett BW. Motility and fertility of equine spermatozoa in a milk extender after 12 or 24 hours at 20ºC. Theriogenology 1987; 27:517-525.

[20] Varner DD, Blanchard TL, Love CL, Garcia MC, Kenney RM. Effects of semen fractionation and dilution on equine spermatozoal motility parameters. Theriogenology 1987; 28: 709-723.

[21] Pruitt JA, Arns MJ, Pool KC. Seminal plasma influences recovery of equine spermatozoa following in vitro culture (37ºC) and cold-storage (5ºC). Theriogenology 1993; 39:291.

[22] Cochran JD, Amann RP, Froman DP, Pickett BW. Effects of centrifugation, glycerol level, cooling to 5°C, freezing rate and thawing rate on the post-thaw motility of equine sperm. Theriogenology 1984;22:25–35.

[23] Crockett EC, Graham JK, Bruemmer JE, Squires EL. Effect of cooling equine spermatozoa before freezing on post-thaw motility : preliminary results. Theriogenology 2001; 55:793-803.

[24] Backman T, Bruemmer JE, Graham JK, Squires EL. Pregnancy rates of mares inseminated with semen cooled for 18 h and then frozen. J Anim Sci 2004; 82:690-694.

[25] Abaigar T, HoltW, Harrison R, Del Barrio G. Sperm subpopulation 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.

[26] Rigau T, Farré M, Ballester J, Mogas T, Peña A, Rodríguez-Gil JE. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology 2001;56:801–15.

[27] Quintero-Moreno A, Miró J, Rigau T, Rodríguez-Gil JE. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 2003;58:1973–90.

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

[29] 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.

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

IV

Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates

E. Flores, E. Taberner, M.M. Rivera, A. Peña, T. Rigau, J. Miró, J.E. Rodríguez-Gil

Department of Animal Medicine and Surgery, School of Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra, Spain

Received 19 November 2007; revised 23 May 2008; accepted 26 May 2008

Theriogenology, 70 (2008) 936-945

Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 70 (2008) 936–945
Theriogenology
www.theriojournal.com

Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates

E. Flores [1], E. Taberner [1], M.M. Rivera, A. Peña, T. Rigau, J. Miró [2,*], J.E. Rodríguez-Gil [2,*]

Department of Animal Medicine and Surgery, School of Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra, Spain

Received 19 November 2007; received in revised form 23 May 2008; accepted 26 May 2008

Abstract

The main aim of this study is to assess the influence of freeze/thawing on motile sperm subpopulations in ejaculates from two phylogenetically different mammalian species, boar and donkey. Our results indicate that, whereas boar and donkey sperm respond very differently in their mean motion characteristics to freezing/thawing, this process did not change the existence of a 4-subpopulations structure in the ejaculates in either species when these subpopulations were defined by taking values of curvilinear velocity (VCL) as reference. Moreover, the freezing/thawing-linked changes in mean sperm-motion characteristics in both boar and donkey semen were especially due to changes in the proportion among each concrete subpopulation. In this way, the freezing/thawing-induced mean increase in motion characteristics observed in boar sperm was a result of the decrease in the percentage of sperm in Subpopulation 1 (from 53.9% ± 4.7% to 31.2% ± 3.9% after thawing) and a concomitant increase of sperm from Subpopulations 3 (from 13.3% ± 2.5% to 32.6% ± 3.9% after thawing) and 4 (from 3.4% ± 0.9% to 8.0% ± 1.1% after thawing). On the contrary, changes in mean motility of frozen/thawed donkey sperm were linked to an increase in the percentage of sperm in Subpopulation 1 (from 31.5% ± 4.3% to 58.8% ± 4.9% after thawing) and a concomitant decrease of sperm from Subpopulations 3 (from 32.4% ± 3.2% to 6.6% ± 1.8% after thawing) and 4 (from 12.2% ± 2.5% to 7.3% ± 1.9% after thawing). In conclusion, our results seem to indicate that motility changes induced by the freezing/thawing protocol are linked to concomitant changes in both the specific parameters and, more importantly, to the specific percentage of each of the motile sperm subpopulations. These changes did not affect the overall proportion of motile sperm present in both boar and donkey, which is conserved despite the detrimental effect caused by freezing/thawing in both species. Finally, the presence of some kind of motile sperm subpopulations structure has been described in mammalian species with a very great phylogenetic distance, thus suggesting that this structure could play some role in the maintenance of the overall function of mammalian ejaculates.

Keywords: Freezing/thawing; Sperm subpopulations; Boar; Donkey

1. Introduction

It has been well established that the freezing/thawing-induced decrease in the fertilising ability of mammalian sperm is associated with important changes in both the percentage of motile sperm and their motion quality. In this way, the mean characteristics of motility in frozen/thawed spermatozoa have been described as

* Corresponding author. Tel.: +34 935811044/45; fax: +34 935812006.
E-mail addresses: jordi.miro@uab.cat (J. Miró), juanenrique.rodriguez@uab.cat (J.E. Rodríguez-Gil).
[1] Both authors should be considered as first authors.
[2] Both authors should be considered as last authors.

0093-691X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2008.05.056

being similar to those observed in capacitated cells. This phenomenon, called ''cryocapacitation'', has been described in several species [1–4], although there is not a general consensus about it. For instance, in boar sperm, whereas several authors have shown that freezing/thawing induced a significant increase in the mean velocity of sperm, concomitantly with a decrease in the mean linearity of the sperm progression [5], others, on the contrary, have shown a general decrease of all of the studied parameters related to sperm velocity [6]. These discrepancies can be due to a myriad of causes, from functional differences among ejaculates to the precise media in which boar semen is diluted prior to its freezing. In any case, and as a general rule in all of the mammalian species studied, it is well known that freezing/thawing modifies sperm function in a way in which sperm motility is greatly altered, although the specific mechanisms by which these modifications are produced (i.e., changes in mitochondrial activity, increase in intracellular, reactive oxygen species levels, etc.; see [2,3]) need to be more precisely defined.

In recent years, it has been reported that ejaculates for an increasing number of mammals are comprised of well-defined subpopulations, which are characterised by precise values of the motion parameters obtained after a computer-assisted motility analysis (CASA; 7–13). Notwithstanding, although the presence of such an organisation seems to be practically universal in mammals, there is no consensus about the physiological role for these motile sperm subpopulations in the ejaculate. In this way, the presence of specific motile sperm subpopulations has been related to semen characteristics, such as cryosurvival and semen quality characteristics in species like boar and horse [10,14,15]. Moreover, the specific sperm selection and competition that is observed after sperm colonisation of oviducts in pigs is modulated by the existence of a specific, motile sperm subpopulation structure pattern, based on the sperm's response to bicarbonate, in boar semen [16]. Hence, the existence of a specific, motile sperm subpopulations structure in an ejaculate would be strongly related, at least in boar, to the semen quality characteristics of this ejaculate. However, the physiological role of sperm subpopulations in other aspects like the sperm's survival ability to freezing/thawing is not well documented, thus leaving many unanswered questions in this area.

The main aim of this study is to determine how a freezing/thawing protocol affects the specific, motile sperm subpopulations structure in a mammalian ejaculate. For this purpose, semen from two mammalian species with great phylogenetic differences and in which semen motility in fresh samples is very different was studied. One species is boar, whose sperm from fresh ejaculates shows low velocity combined with medium-to-low linear movement characteristics [4,15]. The other species is donkey, in which sperm from fresh ejaculates shows very high velocity combined with high linear movement characteristics [12]. Moreover, both species had a 3-to-4 motile sperm subpopulations structure [4,8,12,15], which allows for a direct comparison between them. The freezing/thawing protocol applied to boar is standard, thus allowing for a comparison of our results with those previously published. The results obtained in both species indicate that the freezing/thawing-linked changes in mean motility parameters of both boar and donkey semen samples are strongly related to changes in the specific percentage of sperm included in each subpopulation.

2. Material and methods

2.1. Animals and samples collection

Eleven healthy boars of about 2–3 years of age from a commercial farm were used in this study. The boars were from 3 separate lines (4 Landrace, 3 Large White and 4 Pietrain). All boars had proven fertility after AI using extended, liquid semen. The sperm-rich fraction of each ejaculate utilised in this study was manually collected twice weekly using the gloved-hand method and analysed to ensure the quality and the homogeneity of the ejaculates. Immediately after collection, the ejaculated spermatozoa were suspended (1:2; v/v) in a commercial extender (MR-A, Kubus SA, Majadahonda, Spain). The extended semen samples were cooled and maintained at 17 °C for shipment to the laboratory of the Autonomous University of Barcelona within 24-h post-collection, for further processing and analyses.

Donkey semen was obtained from 7 healthy, mature Catalonian donkeys aged 4–8 years, all of which were previously reported as being fertile. Animals were housed at the Experimental Farm and Countryside Service of the School of Veterinary Medicine of the Autonomous University of Barcelona (Bellaterra, Spain). Semen collection was performed at 2–3-day intervals using an artificial vagina (Hannover model) and an ovariectomised female donkey brought into oestrus with estrogens. The artificial vagina was equipped with an in-line gel filter to permit collection of gel-free semen. Immediately after collection, ejaculates underwent standard analysis for volume, pH, sperm concentration and total sperm number, viability, morphological abnormalities and motility.

Gel-free semen was immediately diluted (proportion 1:5, v/v) with dry skimmed-milk extender (24 mg/mL dry skimmed-milk and 49 mg/mL glucose) kept at 37 °C in a water bath. Diluted semen was immediately sent to the laboratory, where the samples were placed at 20 °C in a water bath until beginning the freezing process.

2.2. Semen cryopreservation

Regarding boar sperm, immediately after receiving the shipped semen samples, an aliquot was taken to perform the appropriate semen quality parameters (fresh semen sample). Only those samples displaying a minimum of 70% progressive motile, and 80% morphologically normal spermatozoa were further processed by adapting a proven protocol [17]. The extended semen was centrifuged in a programmable, refrigerated centrifuge (Medifriger BL-S, JP Selecta, Barcelona, Spain) set at 17 °C, at 600 × *g* for 10 min. After centrifugation, the supernatant was discarded. The remaining pellets were re-extended with a lactose-egg yolk (LEY) extender (80 mL [80%, v/v 310 mM] of β-lactose + 20 mL egg yolk), at a ratio that led to a final concentration of 1.5×10^9 spermatozoa/mL. The sperm concentration was manually assessed in a Thoma or Neubauer haemocytometer. At this point, and after thorough mixing, the semen was further cooled to 5 °C for 2 h in the refrigerator. Afterwards, the semen was slowly mixed with a third extender consisting of 89.5 mL LEY extender, 9 mL glycerol and 1.5 mL of Equex STM (Nova Chemicals Sales Inc., Scituate, MA, USA), which is equivalent to Orvus Es Paste [18], at a ratio of two parts of semen to one part of extender, yielding a final concentration of 3% (v/v) glycerol and a concentration of 1×10^9 spermatozoa/mL at 5 °C, which was verified by counting in a Thoma or Neubauer haemocytometer. Spermatozoa were packaged at 5 °C in a cool cabinet (IMV, L'Aigle, France) in 0.5-mL polyvinyl chloride (PVC) plastic straws (Minitüb, Tiefenbach, Germany), which were sealed with PVC powder and placed on racks for freezing [19]. The racks were transferred to the chamber of a programmable freezer (Ice-Cube 14S, Minitüb) set at 5 °C. The cooling/freezing rate used was: 6 °C/min from 5 to −5 °C, 30 s for crystallisation, and thereafter 50 °C/min from −5 to −140 °C. The samples were then plunged into liquid N_2 (−196 °C) for storage. Frozen samples were stored in liquid N_2 for at least 21 days. After this, samples were thawed by the plunging of samples into a water bath at 37 °C for 20 s. Immediately afterwards, straws were carefully wiped and opened, and samples were immediately analysed to determine the appropriate semen quality parameters (frozen/thawed semen sample).

The donkey-semen freezing protocol was based on [20], including the following modifications. The protocol started with a first centrifugation of the previously diluted samples at 660 × *g* for 15 min at 20 °C. Supernatants were eliminated and sperm was re-suspended with 2 mL of the commercial Gent A® extender containing egg yolk (Minitub Ibérica, S.A., Riudoms, Spain). After this dilution, percentages of viability, sperm concentration, total sperm number and motility were re-evaluated. Only semen samples displaying a minimum of 70% progressive motility, and 80% morphologically normal spermatozoa were further processed. Afterwards, commercial Gent B® extender containing egg yolk and glycerol (Minitub Ibérica, S.A.) was added to obtain a final concentration of 2×10^8 viable sperm/mL. Diluted semen samples were then packaged into 0.5-mL straws and immediately placed in an Ice-Cube 14S programmable freezer. Immediately thereafter, samples were subjected to a three-step cooling/freezing programme. The first step was a cooling phase from 20 to 5 °C at a cooling rate of −0.26 °C/min. The second step was to lower the temperature of the samples from 5 to −90 °C at a freezing rate of −4.75 °C/min. The third and final step was to further freeze samples from −90 to −120 °C at a freezing rate of −11.11 °C/min. Afterwards, frozen samples were stored in liquid N_2 for at least 21 days. Thawing was performed through immersion of the straws in a water bath at 37 °C for 30 s. Thawed semen was immediately evaluated to determine post-thaw sperm motility, viability and concentration (frozen/thawed semen samples).

2.3. Analysis of semen quality parameters

Percentages of viability, altered acrosomes and morphological abnormalities in boar samples were determined by using the Eosin–Nigrosin stain [21]. Similarly, percentages of sperm viability and total morphological abnormalities in donkey samples were determined in samples stained following the same Eosin–Nigrosin technique. In both species, this technique shows viable spermatozoa as being those with a uniform, white stain in all of the cells, whereas the presence of a partial or a totally pinkish stain was indicative of non-viable sperm cells. Moreover, acrosome integrity of boar spermatozoa was evaluated by observing the presence of a regular and intact acrosomal ridge after the Eosin–Nigrosin stain. Any part of the

acrosomal ridge that did not have a regular and intact aspect was considered as being altered acrosomes. In both species, the determinations of the above-mentioned percentages were performed after analysing a minimum of 200 spermatozoa/sample through optical microscopy (magnification: 1000×). Sperm concentration and total sperm number was determined after counting in a haemocytometer chamber. On the other hand, the Osmotic Resistance Test (ORT Test) in boar semen was carried out as described in [22].

In both boar and donkey semen, the computer-assisted analysis of sperm motility (CASA) was performed by using a commercial system (Integrated Sperm Analysis System V1.0, Proiser SL, Valencia, Spain). In this system, samples were previously warmed at 37 °C for 5 min in a water bath and 5-μL aliquots of these samples were then placed on a warmed (37 °C) slide and covered with a 22-mm^2 coverslip. Our CASA system was based upon the analysis of 25 consecutive, digitalized photographic images obtained from a single field at a magnification of 200× on a dark field. These 25 consecutive photographs were taken in a time lapse of 1 s, which implied a velocity of image-capturing of one photograph every 40 ms. Two to three separate fields were taken for each sample. The obtained sperm motility descriptors are described following [10]. Motility descriptors obtained after CASA analysis are:

Curvilinear velocity (VCL): The mean path velocity of the sperm head along its actual trajectory (units: μm/s).
Linear velocity (VSL): The mean path velocity of the sperm head along a straight line from its first to its last position (units: μm/s).
Mean velocity (VAP): The mean velocity of the sperm head along its average trajectory (units: μm/s).
Linearity coefficient (LIN): (VSL/VCL) × 100 (units: %).
Straightness coefficient (STR): (VSL/VAP) × 100 (units: %).
Wobble coefficient (WOB): (VAP/VCL) × 100 (units: %).
Mean amplitude of lateral head displacement (ALH): The mean value of the extreme side-to-side movement of the sperm head in each beat cycle (units: μm).
Frequency of head displacement (BCF): the frequency with which the actual sperm trajectory crosses the average path trajectory (units: Hz).

Finally, total motility was defined as the percentage of spermatozoa which showed a VAP above 10 μm/s.

2.4. Statistical analysis

Data were processed by using the SAS statistical package [23]. Normality of data distributions was assessed by the Shapiro–Wilks Test, which is included in the UNIVARIATE procedure. Afterwards, the FASTCLUS clustering procedure included in the SAS package was utilised to separate motile spermatozoa into specific subpopulations. The FASTCLUS procedure performs a disjointed cluster analysis based on Euclidean distances computed from one or more quantitative parameters. In this case, these variables are the different sperm motility parameters measured by the CASA system. Spermatozoa were divided into clusters such that every observation belonged to a single cluster. Sperm cells that shared similar motility characteristics were assigned to the same cluster, whereas spermatozoa that differed in motility characteristics were assigned to different clusters. A PROC GLM procedure was applied to evaluate significant differences ($P < 0.05$) and the LSMEANS procedure was applied to list these differences. Finally, a Chi-square procedure was applied to determine the subpopulational distribution percentage in every single experiment. Once the distribution percentage per experiment was determined, new PROC GLM and LSMEANS procedures were applied to determine and list, respectively, the differences among the different treatments. The total number of spermatozoa analysed following this protocol was 3744 in boar samples and 1673 in donkey analyses.

3. Results

3.1. Comparison between mean quality parameters of fresh boar and donkey semen and frozen/thawed

As expected, freezing/thawing induced great changes in the values of the mean quality parameters of both boar and donkey sperm. As shown in Table 1, frozen/thawed samples from both species showed a significant ($P < 0.05$) decrease in the percentages of viability and total motility, which was accompanied by a concomitant increase in the percentage of altered acrosomes. Furthermore, boar sperm underwent a significant ($P < 0.05$) decrease in the response to the ORT Test, which fell from 80.6% ± 2.9% in fresh samples to 34.8% ± 2.9% after thawing (Table 1). The response of the mean motility parameters of both boar and donkey spermatozoa to freezing/thawing was not similar, most probably due to the specific motility characteristics that both species showed. Thus, boar sperm from fresh, diluted ejaculates had an overall

Table 1
Mean values of the semen quality analysis of both boar and donkey sperm before and after freezing/thawing

	Boar		Donkey	
	Before freezing	After freezing	Before freezing	After freezing
Viability (%)	85.1 ± 1.2^{a}	51.9 ± 1.2^{b}	86.7 ± 1.0^{a}	49.3 ± 0.9^{b}
Altered acrosomes (%)	13.7 ± 0.4^{a}	47.2 ± 0.9^{b}	14.2 ± 0.4^{a}	51.8 ± 1.1^{b}
ORT Test (%)	80.6 ± 2.9^{a}	34.8 ± 2.9^{b}	N.D.	N.D.
Total motility (%)	75.9 ± 1.2^{a}	46.9 ± 0.9^{b}	83.6 ± 1.3^{a}	48.4 ± 1.0^{b}
VCL (μm/s)	38.8 ± 0.7^{a}	85.2 ± 2.8^{b}	93.9 ± 1.0^{a}	91.7 ± 1.7^{a}
VSL(μm/s)	21.9 ± 0.5^{a}	49.6 ± 2.2^{b}	40.3 ± 0.6^{a}	35.2 ± 1.1^{b}
VAP (μm/s)	28.7 ± 0.7^{a}	62.6 ± 2.7^{b}	58.5 ± 0.7^{a}	50.2 ± 1.7^{b}
LIN (%)	52.6 ± 0.5^{a}	53.0 ± 2.2^{a}	47.4 ± 0.6^{a}	46.2 ± 1.1^{a}
STR (%)	70.8 ± 0.5^{a}	73.8 ± 1.9^{a}	68.1 ± 0.7^{a}	68.7 ± 1.1^{a}
WOB (%)	70.8 ± 0.4^{a}	68.6 ± 1.8^{a}	64.4 ± 0.6^{a}	60.3 ± 0.9^{a}
Mean ALH(μm)	1.65 ± 0.02^{a}	2.98 ± 0.08^{b}	3.65 ± 0.06^{a}	3.72 ± 0.10^{a}
BCF (Hz)	5.8 ± 0.1^{a}	7.0 ± 0.3^{b}	12.2 ± 0.2^{a}	14.4 ± 0.4^{b}

The parameters shown here have been determined as explained in Section 2. Results are expressed as means ± S.E.M. of 15 (boar) and 8 (donkey) different experiments with a total number of analysed spermatozoa of 1981 (boar) and 770 (donkey). N.D. = not determined. Different superscript letters between rows in the same species indicates significant ($P < 0.05$) differences.

motility that was much less rapid, but more linear, than that from donkey samples (VAP: 28.7 μm/s ± 0.7 μm/s in boar vs. 58.5 μm/s ± 0.7 μm/s in donkey; LIN: 52.6% ± 0.5% in boar vs. 47.4% ± 0.6% in donkey; $P < 0.05$). Moreover, boar motile spermatozoa also showed a less intense sperm-head movement than donkey cells, as inferred when the mean values of mean ALH (1.65 ± 0.02 μm in boar vs. 3.65 ± 0.06 μm in donkey) and BCF (5.8 ± 0.1 Hz in boar vs. 12.2 ± 0.2 Hz in donkey cells, see Table 1) were compared. Freezing/thawing induced a great, significant ($P < 0.05$) increase in boar sperm in the mean values of VCL, VSL and VAP, whereas LIN, STR and WOB did not undergo significant changes. In contrast, donkey sperm underwent a slight, but significant ($P < 0.05$) decrease in VSL and VAP, which was accompanied by no modifications in VCL, LIN, STR and WOB values (Table 1). Additionally, both boar and donkey samples increased BCF values after freezing/thawing (boar BCF: from 5.8 ± 0.1 to 7.0 ± 0.3 Hz; donkey BCF: from 12.2 ± 0.2 to 14.4 ± 0.4 Hz, Table 1). Mean ALH was significantly increased in boar semen (from 1.65 ± 0.02 to 2.98 ± 0.08 μm in frozen/thawed samples), while the increase of this parameter after thawing on donkey samples was not significant (Table 1).

3.2. Motile sperm subpopulations structure in boar and donkey samples before freezing

Both boar and donkey samples had a similar, four-subpopulations structure when sperm motility was analysed through CASA. These results were similar to those published before [12,24,25]. In our experimental design, subpopulations were classified by taking VCL as the marker point in both species. Under this point of view, boar motile-sperm subpopulations are characterised as follows (see Table 2):

Subpopulation 1: This subpopulation showed the lowest values of VCL (25.5 ± 2.6 μm/s). Subpopulation 1 was defined by overall low values of velocity, based on the results of VCL, VSL and VAP, low values of linearity, as indicated by values of LIN and STR, and low values of oscillatory movement, as indicated by WOB, mean ALH and BCF values (Table 2). Subpopulation 1 was made up of the highest percentage of cells, since it included 53.9% ± 4.7% of all of motile sperm (Fig. 1A).

Subpopulation 2: This subpopulation was characterised by the second lowest values of VCL (43.9 ± 2.1 μm/s). Sperm included in Subpopulation 2 showed middle-to-high velocity, as indicated by VCL, VSL and VAP, high linearity, as indicated by LIN and STR, and high values of oscillatory movement, as indicated by WOB, mean ALH and BCF. The percentage of spermatozoa included in this subpopulation was 29.4% ± 3.5% of all motile sperm (Table 2, Fig. 1A).

Subpopulation 3: This subpopulation had high values of VCL (69.2 ± 2.6 μm/s). Subpopulation 3 was made up of sperm with high velocity and relatively high linearity, as indicated by VCL, VSL, VAP, LIN and STR. Moreover, sperm included in this subpopulation also had a relatively high oscillatory movement, as indicated by values of WOB, mean ALH and BCF. The cells included in this subpopula-

Table 2
Effects of freezing/thawing on motility parameters of the motile-sperm subpopulations determined in boar samples

	Mean values		Subpopulation 1		Subpopulation 2	
	Before freezing	After freezing	Before freezing	After freezing	Before freezing	After freezing
Total motility (%)	75.9 ± 1.2^{a}	46.9 ± 0.9^{b}				
VCL (μm/s)	38.8 ± 0.7^{a}	85.2 ± 2.8^{b}	25.5 ± 2.6^{a}	34.4 ± 5.3^{b}	43.9 ± 2.1^{a}	68.6 ± 7.3^{b}
VSL(μm/s)	21.9 ± 0.5^{a}	49.6 ± 2.2^{b}	9.8 ± 1.8^{a}	10.1 ± 3.3^{a}	28.0 ± 3.1^{a}	42.1 ± 6.8^{b}
VAP (μm/s)	28.7 ± 0.7^{a}	62.6 ± 2.7^{b}	15.2 ± 2.0^{a}	15.3 ± 3.4^{a}	35.1 ± 2.8^{a}	57.0 ± 4.9^{b}
LIN (%)	52.6 ± 0.5^{a}	53.0 ± 2.2^{a}	38.2 ± 5.7^{a}	27.6 ± 5.3^{b}	67.5 ± 5.3^{a}	65.4 ± 5.0^{a}
STR (%)	70.8 ± 0.5^{a}	73.8 ± 1.9^{a}	63.9 ± 5.2^{a}	62.8 ± 5.3^{a}	82.0 ± 4.4^{a}	76.9 ± 7.0^{a}
WOB (%)	70.8 ± 0.4^{a}	68.6 ± 1.8^{a}	61.4 ± 5.3^{a}	44.2 ± 4.3^{b}	83.9 ± 3.9^{a}	87.6 ± 6.4^{a}
Mean ALH(μm)	1.65 ± 0.02^{a}	2.98 ± 0.08^{b}	1.40 ± 0.03^{a}	1.62 ± 0.15^{a}	1.79 ± 0.06^{a}	2.44 ± 0.15^{b}
BCF (Hz)	5.8 ± 0.1^{a}	7.0 ± 0.3^{b}	4.5 ± 1.5^{a}	3.4 ± 1.3^{a}	7.0 ± 0.6^{a}	7.9 ± 0.9^{a}

	Subpopulation 3		Subpopulation 4	
	Before freezing	After freezing	Before freezing	After freezing
VCL (μm/s)	69.2 ± 2.6^{a}	80.2 ± 4.3^{b}	102.3 ± 6.6^{a}	122.6 ± 4.3^{b}
VSL(μm/s)	47.2 ± 2.3^{a}	51.1 ± 4.5^{a}	78.0 ± 4.9^{a}	104.5 ± 8.6^{b}
VAP (μm/s)	58.6 ± 3.2^{a}	70.2 ± 4.2^{b}	92.4 ± 4.0^{a}	115.8 ± 4.8^{b}
LIN (%)	73.0 ± 6.1^{a}	66.5 ± 3.8^{a}	79.3 ± 4.2^{a}	88.4 ± 4.8^{a}
STR (%)	84.3 ± 5.7^{a}	79.5 ± 6.1^{a}	87.6 ± 4.5^{a}	93.6 ± 3.5^{a}
WOB (%)	88.1 ± 3.0^{a}	91.1 ± 4.2^{a}	91.7 ± 4.3^{a}	95.9 ± 3.7^{a}
Mean ALH(μm)	2.31 ± 0.08^{a}	3.08 ± 0.14^{b}	2.71 ± 0.09^{a}	3.14 ± 0.16^{a}
BCF (Hz)	7.7 ± 1.6^{a}	8.7 ± 2.3^{a}	8.2 ± 1.6^{a}	8.6 ± 2.2^{a}

The motility parameters shown have been determined as explained in Section 2. Results are expressed as means ± S.E.M. of 15 different experiments, which implies the total number of analysed sperm for motility characteristics were of 1981 (before freezing) and 1763 (after freezing). Different superscript letters between rows in the same subpopulation or between the mean values indicates significant ($P < 0.05$) differences.

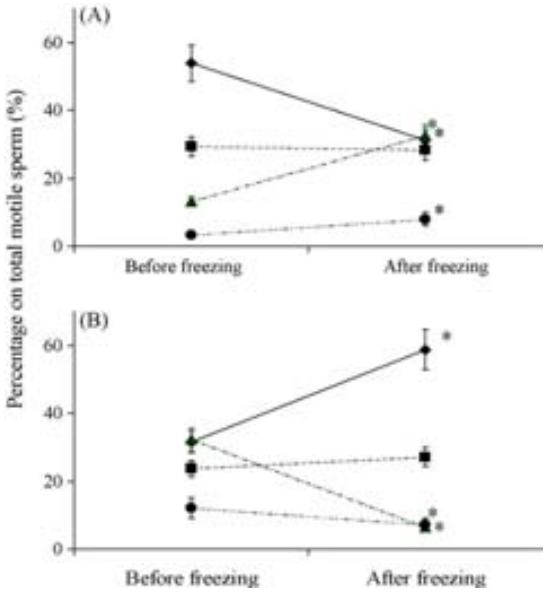

Fig. 1. Changes in the proportion of each motile sperm subpopulation in boar and donkey samples before and after freezing. The proportion of sperm in each subpopulation has been determined as described in Section 2. Results are means ± S.E.M. for 15 (boar) and 8 (donkey) different experiments. Asterisks indicate significant ($P < 0.05$) differences between samples before and after freezing. (A) Boar spermatozoa. (B) Donkey spermatozoa. Rhombuses: Subpopulation 1. Squares: Subpopulation 2. Triangles: Subpopulation 3. Circles: Subpopulation 4.

tion accounted for 13.3% ± 2.5% of all motile sperm (Table 2, Fig. 1A).

Subpopulation 4: Finally, this subpopulation included that sperm with the highest VCL (102.3 ± 6.6 μm/s). Cells included in Subpopulation 4 showed the highest velocity and linearity characteristics, as indicated by values of VCL, VSL, VAP, LIN and STR. Furthermore, the overall oscillatory movement of these spermatozoa was also very high, as indicated by WOB, mean ALH and BCF values. The percentage of motile sperm included in this subpopulation was the lowest, including only 3.4% ± 0.9% of the total motile-sperm subpopulation (Table 2, Fig. 1A).

When the motile sperm subpopulations structure of donkey samples was analysed, they also showed a structure that was composed of four separate subpopulations, with very specific motility characteristics (Table 3, Fig. 1B), namely:

Subpopulation 1: This subpopulation was composed of motile sperm with overall, relatively low velocity characteristics, high linear movement and both low BCF and mean ALH values. These spermatozoa comprised 31.5% ± 4.3% of the total motile-sperm subpopulation.

Table 3
Effects of freezing/thawing on motility parameters of the motile-sperm subpopulations determined in donkey samples

	Mean values		Subpopulation 1		Subpopulation 2	
	Before freezing	After freezing	Before freezing	After freezing	Before freezing	After freezing
Total motility (%)	83.6 ± 1.3^{a}	48.4 ± 1.0^{b}				
VCL (μm/s)	93.9 ± 1.0^{a}	91.7 ± 1.7^{a}	31.7 ± 1.7^{a}	27.4 ± 1.5^{a}	67.5 ± 2.0^{a}	62.3 ± 2.2^{a}
VSL(μm/s)	40.3 ± 0.6^{a}	35.2 ± 1.1^{b}	19.9 ± 1.1^{a}	16.6 ± 0.9^{a}	15.3 ± 1.2^{a}	12.2 ± 1.4^{a}
VAP (μm/s)	58.5 ± 0.7^{a}	50.2 ± 1.7^{b}	22.4 ± 1.2^{a}	18.6 ± 1.0^{a}	33.9 ± 1.3^{a}	25.8 ± 1.5^{b}
LIN (%)	47.4 ± 0.6^{a}	46.2 ± 1.1^{a}	63.2 ± 1.1^{a}	61.6 ± 1.0^{a}	22.8 ± 1.2^{a}	19.5 ± 1.4^{a}
STR (%)	68.1 ± 0.7^{a}	68.7 ± 1.1^{a}	87.7 ± 1.1^{a}	87.5 ± 1.0^{a}	46.2 ± 1.3^{a}	45.5 ± 1.4^{a}
WOB (%)	64.4 ± 0.6^{a}	60.3 ± 0.9^{a}	71.2 ± 0.9^{a}	69.4 ± 0.8^{a}	50.0 ± 1.1^{a}	45.5 ± 1.4^{b}
Mean ALH(μm)	3.65 ± 0.06^{a}	3.72 ± 0.10^{a}	1.37 ± 0.10^{a}	1.17 ± 0.09^{a}	3.59 ± 0.12^{a}	3.11 ± 0.14^{b}
BCF (Hz)	12.2 ± 0.2^{a}	14.4 ± 0.4^{b}	11.3 ± 0.4^{a}	11.9 ± 0.3^{a}	11.2 ± 0.4^{a}	12.0 ± 0.5^{a}

	Subpopulation 3		Subpopulation 4	
	Before freezing	After freezing	Before freezing	After freezing
VCL (μm/s)	111.8 ± 1.7^{a}	92.3 ± 4.5^{b}	164.6 ± 2.7^{a}	184.7 ± 4.3^{b}
VSL(μm/s)	90.9 ± 1.0^{a}	87.3 ± 3.1^{a}	34.9 ± 1.7^{a}	36.7 ± 2.7^{a}
VAP (μm/s)	95.4 ± 2.0^{a}	87.3 ± 3.1^{b}	82.4 ± 1.9^{a}	78.9 ± 3.0^{a}
LIN (%)	81.8 ± 1.0^{a}	94.4 ± 3.1^{b}	21.6 ± 1.7^{a}	20.0 ± 2.7^{a}
STR (%)	85.8 ± 0.9^{a}	85.4 ± 2.5^{a}	43.5 ± 1.8^{a}	44.2 ± 2.8^{a}
WOB (%)	85.8 ± 0.9^{a}	96.4 ± 2.5^{b}	50.8 ± 1.5^{a}	44.2 ± 2.4^{a}
mean ALH(μm)	2.14 ± 0.10^{a}	1.94 ± 0.28^{a}	7.43 ± 0.17^{a}	8.67 ± 0.27^{b}
BCF (Hz)	13.1 ± 0.6^{a}	17.0 ± 1.0^{b}	13.4 ± 0.4^{a}	12.7 ± 1.0^{a}

The motility parameters shown have been determined as explained in Section 2. Results are expressed as means ± S.E.M. of 8 different experiments, which implies the total number of analysed sperm for motility characteristics were of 770 (before freezing) and 903 (after freezing). Different superscript letters between rows in the same subpopulation or between the mean values indicates significant ($P < 0.05$) differences.

Subpopulation 2: This subpopulation was composed of motile sperm with overall, relatively low velocity characteristics, but with much lower linear movement characteristics than that showed by Subpopulation 1.Furthermore, BCF was similar to Subpopulation 1, whereas mean ALH was relatively high. These spermatozoa comprised 23.8% ± 2.9% of the total motile sperm subpopulation.

Subpopulation 3: Motile sperm with high velocity characteristics combined with a very high linearity. This was combined with high values of BCF and mild mean ALH characteristics. This subpopulation contained 32.4% ± 3.2% of the total motile-sperm subpopulation.

Subpopulation 4: Spermatozoa with high velocity and low linearity. Moreover, both BCF and specially mean ALH were very high. Spermatozoa included in this subpopulation were 12.2% ± 2.5% of the total motile-sperm subpopulation.

3.3. Effects of freezing/thawing on the mean motile sperm subpopulations structure of boar and donkey semen samples

Freezing/thawing of boar sperm from fresh ejaculates induced important changes in the mean motion parameters of motile sperm included in each specific subpopulation. However, it is worth noting that the effects of freezing/thawing were different on each subpopulation. Furthermore, the combined, observed changes in each subpopulation by themselves did not entirely explain the observed changes in the mean overall values of motility parameters after thawing. Thus, whereas VCL significantly ($P < 0.05$) increased in all subpopulations after thawing, only VSL from Subpopulation 2 (from 28.0 ± 3.1 to 42.1 ± 6.8 μm/s after thawing) and Subpopulation 4 (from 78.0 ± 4.9 to 104.5 ± 8.6 μm/s after thawing, see Table 2) experienced a similar increase. Thawing induced an increase in VAP parameters in only Subpopulation 2 (from 35.1 ± 2.8 to 57.0 ± 4.9 μm/s after thawing), Subpopulation 3 (from 58.6 ± 3.2 to 70.2 ± 4.2 μm/s after thawing) and Subpopulation 4 (from 92.4 ± 4.0 to 115.8 ± 4.8 μm/s after thawing, see Table 2). A similar increase was also observed in mean ALH after thawing in Subpopulation 2 (from 1.79 ± 0.06 to 2.44 ± 0.15 μm after thawing) and Subpopulation 3 (from 2.31 ± 0.08 to 3.08 ± 0.14 μm after thawing). On the other hand, freezing/thawing induced a significant ($P < 0.05$) decrease in the LIN and WOB of Subpopulation 1 (from 61.4% ± 5.3% to 44.2% ± 4.3% after thawing, see Table 2).

As in boar, the effects of freezing/thawing on motility characteristics of each motile-sperm subpopulation in fresh donkey samples was different and specific for each subpopulation and, on the whole, these changes did not entirely explain the observed changes in the mean overall values of motility parameters after thawing. However, the effects of freezing/thawing of motile descriptors of donkey-sperm motile subpopulations were different from those observed in boar samples. Thus, as shown in Table 3, freezing/thawing did not significantly change any motility parameter of Subpopulation 1, whereas Subpopulation 2 underwent a significant ($P < 0.05$) decrease of VAP, WOB and mean ALH, with Subpopulation 3 having a significant ($P < 0.05$) decrease of VCL and VAP that was concomitant to a significant ($P < 0.05$) increase of LIN, WOB and BCF. Finally, Subpopulation 4 had a significant ($P < 0.05$) increase of VCL (from 164.6 ± 2.7 to 184.7 ± 4.3 μm/s after thawing) and mean ALH (from 7.43 ± 0.17 to 8.67 ± 0.27 μm after thawing, see Table 3).

On the other hand, the percentage of motile sperm included in each subpopulation experienced important changes after freezing/thawing in both boar and donkey samples. Thus, as shown in Fig. 1A, freezing/thawing induced a significant ($P < 0.05$) decrease in the percentage of motile sperm included in Subpopulation 1 in boar samples (from $53.9\% \pm 4.7\%$ to $31.2\% \pm 3.9\%$ after thawing). This decrease was compensated for by concomitant, significant ($P < 0.05$) increases in the percentages of sperm included in Subpopulations 3 (from $13.3\% \pm 2.5\%$ to $32.6\% \pm 3.9\%$ after thawing) and 4 (from $3.4\% \pm 0.9\%$ to $8.0\% \pm 1.1\%$ after thawing). Strikingly, an inverse effect was observed in donkey semen. In these samples, freezing/thawing induced a significant ($P < 0.05$) increase in sperm included in Subpopulation 1 (from $31.5\% \pm 4.3\%$ to $58.8\% \pm 4.9\%$ after thawing) that was concomitant with significant ($P < 0.05$) decreases in the percentages of both Subpopulation 3 (from $32.4\% \pm 3.2\%$ to $6.6\% \pm 1.8\%$ after thawing) and Subpopulation 4 (from $12.2\% \pm 2.5\%$ to $7.3\% \pm 1.9\%$ after thawing, see Fig. 1B).

4. Discussion

Our results clearly establish that motility changes induced by the freezing/thawing protocol are linked to concomitant changes in both the specific parameters and the specific percentage of each of the motile sperm subpopulations present in both boar and donkey ejaculates. Furthermore, it is noteworthy that these changes did not affect the general motile-sperm structure present in both boar and donkey. This indicates that in both species the maintenance of an overall subpopulation structure could be important in order to maintain the general function of the ejaculate, regardless of the specific functional status. These results pointed to a specific, important role for the maintenance of a specific subpopulational structure in mammalian ejaculates, regardless of the species in which the studies were carried out. In this way, the existence of an specific subpopulational structure has been described in very different mammals like boar [8,15,24,25], horse, [10], donkey [12], dog [9], red deer [11], gazelle [8], golden hamster [7] and rabbit [13], indicating that it is a common feature of all mammalian ejaculates. More importantly, recent studies from our laboratory indicate that motility changes associated with processes like ''in vitro'' capacitation in boar semen [26] do not modify the overall subpopulational structure of these samples, the mean motility changes linked to these processes being induced mainly to concomitant changes in the percentage of motile sperm included in each specific subpopulation. Thus, the maintenance of this specific subpopulational structure would be important in the maintenance of the overall semen function in mammals.

It is noteworthy that, whereas freezing/thawing causes a significant increase in the motility characteristics of boar sperm, the same process did not exert a similar increase on donkey cells. In fact, this effect has already been described in both species [5,20], and it could be linked to two attendant causes. The first, the great differences observed in the physiological and metabolic characteristics of boar and donkey spermatozoa. Thus, boar sperm from fresh ejaculates shows very low mitochondrial activity together with a very high glycolytic rate [27]. These metabolic characteristics are linked to a mean, low motility pattern when compared with other species, such as dog and equine [9,10]. On the other hand, donkey sperm from fresh samples shows a glycolytic rate that was lower than that observed in boar sperm [12]. This would be linked to high mitochondrial activity in these cells that, in turn, could be at the basis of the very high velocity characteristics observed in fresh donkey semen [12,20]. These differences in the physiological and metabolic characteristics of boar and donkey sperm could lead to different responses in each species when faced with a very stressing procedure such as freezing/thawing. In this way, whereas boar sperm would respond to freezing/thawing by inducing the already-described cryocapacitation phenomenon [1–4,6], donkey cells would respond to a relative impairment of their

motion characteristics related to a different sensitivity in response to the stress linked to freezing/thawing.

Nevertheless, although the specific characteristics are important in explaining the different behaviours of boar and donkey sperm in front of freezing/thawing, there are surely other factors implied in these differences. One of these factors would be the initial extender utilised in the first stages of the freezing/thawing process. Thus, in our procedure, whereas donkey semen was initially utilised without any previous storage step, boar samples were previously diluted in a commercial extender for storage at 16–17 °C for a time lapse of never less than 4 h. This can be important, since practically all commercial extenders for boar semen have, as a constitutive element, some substances intended for decreasing sperm motility, such as ethylenediaminotetraacetic acid (EDTA) salts. EDTA is a strong calcium chelant, thus interfering with the biochemical processes linked to the maintenance of sperm flagellar movement [28]. In this way, boar sperm diluted in these media has motion characteristics that are lower than those observed in undiluted samples. However, in our working conditions, the utilisation of undiluted boar-semen samples was impossible without producing considerable stress on cells, thus compelling the utilisation of previously diluted ejaculates. All of these points explain why the motility of boar samples prior to freezing/thawing was low. The first steps of the freezing/thawing protocol include a centrifugation/washing phase, in which boar sperm is separated from its diluting medium. During this phase, EDTA is eliminated, in this way allowing boar sperm to recover a fully functional flagellar movement. Thus, the separation of boar sperm from the initial commercial extender could have an activating effect on sperm movement, which could be one of the reasons for the increase in overall motility in frozen/thawed boar sperm.

The maintenance of the overall, four-subpopulations structure in both boar and donkey ejaculates after freezing/thawing, despite the very different response to the process in both species, was linked to symmetrical changes in the specific percentage of each subpopulation. Hence, the overall increase in mean motility of frozen/thawed boar sperm was linked to a decrease in the percentage of sperm in Subpopulation 1 and a concomitant increase of sperm from Subpopulations 3 and 4. On the contrary, changes in mean motility of frozen/thawed donkey sperm were linked to an increase in the percentage of sperm in Subpopulation 1 and a concomitant decrease of sperm from Subpopulations 3 and 4. This suggests that the specific response to freezing/thawing of an ejaculate could depend on the concrete relationship among the percentages of sperm included in Subpopulations 1, 3 and 4. However, we have to stress that subpopulations with the same numerical identification would not necessarily have the same functional and/or physiological meaning in boar and in donkey semen (i.e., Subpopulation 1 of boar semen is not necessarily equivalent to Subpopulation 1 in donkey samples). Concomitantly, a subpopulation designed with the same number does not necessarily to be equivalent between fresh and frozen/thawed samples from a concrete species (i.e., Subpopulation 1 of boar fresh semen does not necessarily coincide with Subpopulation 1 in frozen/thawed samples). Taking into account all of these precautions, at this moment we cannot specify what the precise mechanism/s is/are related with this assert. In this way, further experiments regarding ejaculates with different sensitivities to freezing/thawing are in progress in order to evaluate this assumption.

In conclusion, our results seem to establish that the motility changes induced by the freezing/thawing protocol are linked to concomitant changes in both the specific parameters and the specific percentage of each of the motile sperm subpopulations present in mammalian species with very different sperm motility characteristics, such as boar and donkey. Moreover, these changes did not affect the general motile-sperm structure present in both boar and donkey, which is conserved despite the detrimental effect caused by freezing/thawing in both species. All of these results suggest, then, that a specific, motile sperm subpopulations structure is a common feature in mammalian ejaculates, which seems to play some role in the maintenance of the overall function of the whole mammalian ejaculates.

Acknowledgments

We would like to thank Mr. Chuck Simmons for his accurate revision of the English grammar of this manuscript. This work has been supported by Grant AGL2004-04756-C02/02/GAN (Dirección General de Investigación, Ministerio de Educación y Ciencia, Spain) and Grant RZ03-021 (Instituto Nacional de Investigaciones Agrarias, Spain).

References

[1] Maxwell WMC, Johnson LA. Membrane status of boar spermatozoa after cooling or cryopreservation. Theriogenology 1997;48:209–19.

[2] Curry MR. Cryopreservation of semen from domestic livestock. Rev Reprod 2000;5:46–52.

[3] Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000;60–61:481–92.
[4] Cremades T, Roca J, Rodríguez-Martínez H, Abaigar T, Vázquez JM, Martínez EA. Kinematic changes during the cryopreservation of boar spermatozoa. J Androl 2005;26:610–8.
[5] Maldjian A, Pizzi F, Gliozzi T, Cerolini S, Penny P, Noble R. Changes in sperm quality and lipid composition during cryopreservation of boar sperm. Theriogenology 2005;63:411–21.
[6] Guthrie HD, Welch GR. Impact of storage prior to cryopreservation on plasma membrane function and fertility of boar sperm. Theriogenology 2005;63:396–410.
[7] Holt WV. Can we predict fertility rates? Making sense of sperm motility. Reprod Domest Anim 1996;31:17–24.
[8] Abaigar T, Holt WV, Harrison R, 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.
[9] Rigau T, Farré M, Ballester J, Mogas T, Peña A, Rodríguez-Gil JE. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology 2001;56: 801–15.
[10] Quintero-Moreno A, Miró J, Rigau T, Rodríguez-Gil JE. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 2003;58:1973–90.
[11] 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.
[12] 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.
[13] 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.
[14] Hirai M, Boersma A, Hoeflich A, Wolf E, Foll J, Aumuller R, et al. Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): relation to fertility and seminal plasma growth factors. J Androl 2001;22:104–10.
[15] 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.
[16] Satake N, Elliott RMA, Watson PF, Holt WV. Sperm selection and competition in pigs may be mediated by the differential motility activation and suppression of sperm subpopulations within the oviduct. J Exp Biol 2006;209:1560–72.
[17] Sancho S, Casas I, Ekwall H, Saravia F, Rodríguez-Martínez H, Rodríguez-Gil JE, et al. Effects of cryopreservation on semen quality and the expression of sperm membrane hexose transporters in the spermatozoa of Iberian pigs. Reproduction 2007;134:111–21.
[18] Graham EF, Schmehl MLO, Brown KI, Crabo BG, Ertel G. Some chemical studies of the female reproductive tract and seminal plasma of the male turkey and their relationship to fertility. Poult Sci 1971;50:1170–81.
[19] Saravia F, Wallgren M, Nagy S, Johannisson A, Rodriguez-Martines H. Deep freezing of concentrated boar semen for intrauterine insemination: effects on sperm viability. Theriogenology 2005;63:1320–33.
[20] Vidament M, Ecot P, Noue P, Bourgeois C, Magistrini M, Palmer E. Centrifugation and addition of glycerol at 22 °C instead of 4 °C improve post-thaw motility and fertility of stallion spermatozoa. Theriogenology 2000;54:907–19.
[21] Bamba K. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscope using an eosin-nigrosin stain. Theriogenology 1988;29:1245–51.
[22] Rodríguez-Gil JE, Rigau T. Effects of slight agitation on the quality of refrigerated boar sperm. Anim Reprod Sci 1995;39: 141–6.
[23] SAS. SAS/STAC Software. Cary, NC, USA: SAS Inst. Inc.; 2000.
[24] Rivera MM, Quintero-Moreno A, Barrera X, Palomo MJ, Rigau T, Rodríguez-Gil JE. Natural Mediterranean photoperiod does not affect the main parameters of boar-semen quality analysis. Theriogenology 2005;64:934–46.
[25] 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.
[26] Ramió L, Rivera MM, Ramírez A, Concha II, Peña A, Rigau T, et al. Dynamics of motile sperm subpopulations structure in boar ejaculates subjected to "in vitro" capacitation and further "in vitro" acrosome reaction. Theriogenology 2008;69:501–12.
[27] Marín S, Chiang K, Bassilian S, Lee WN, Boros LG, Fernández-Novell JM, et al. Metabolic strategy of boar spermatozoa revealed by a metabolomic characterization. FEBS Lett 2003; 554:342–6.
[28] Rikmenspoel R, Orris SE, Isles CA. Effect of vanadate Mg^{2+} and electric current injection on the stiffness of impaled bull spermatozoa. J Cell Sci 1981;51:53–61.

V

Ability of Catalonian donkey sperm to penetrate zona pellucida-free bovine oocytes matured *in vitro*

E. Taberner, R. Morató, T. Mogas, J. Miró

Department of Animal Medicine and Surgery, School of Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra, Spain

Received 26 May 2009; revised form 31 July 2009 ; accepted 17 August 2009

Animal Reproduction Science, 118 (2010) 354-361

Animal Reproduction Science 118 (2010) 354–361

Contents lists available at ScienceDirect

Animal Reproduction Science

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

Ability of Catalonian donkey sperm to penetrate zona pellucida-free bovine oocytes matured *in vitro*

E. Taberner, R. Morató, T. Mogas, J. Miró*

Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

ARTICLE INFO

Article history:
Received 26 May 2009
Received in revised form 31 July 2009
Accepted 17 August 2009
Available online 21 August 2009

Keywords:
Penetrability
Pronucleus
Sperm–oocyte interaction
Sperm functionality assay
Motility

ABSTRACT

An experiment was designed to study the interaction between fresh/frozen-thawed donkey spermatozoa and zona pellucida (ZP)-free bovine oocytes in an attempt to develop a model for assessing cryopreserved Catalonian donkey sperm function. Semen from five donkeys was collected using an artificial vagina. Sperm motility and viability were immediately assessed and the semen sample cryopreserved. Sperm viability and motility were then reassessed immediately after thawing. The motion characteristics of the fresh and frozen-thawed spermatozoa were determined using a computer-assisted sperm analysis system. *In vitro*-matured cow oocytes were inseminated with different percent live donkey sperm (high (>60%) or low (<40%) viability donkey sperm). After 18 h of co-incubation, the oocytes were fixed, stained with 4′,6-diamidino-2-phenylindole (DAPI) and examined for sperm penetration, the number of penetrated spermatozoa per oocyte, and male pronucleus formation. Frozen-thawed spermatozoa from high viability semen showed significantly lower VCL, VAP and mean ALH values than did high viability fresh spermatozoa. In contrast, frozen-thawed spermatozoa of low viability had significantly higher velocity values than fresh spermatozoa of low viability. A significant positive correlation ($P < 0.01$) was detected between percentage fertilization and viability ($r = 0.84$), and between percentage fertilization and certain CASA parameters (VAP, $r = 0.56$; VCL, $r = 0.61$ and mean ALH, $r = 0.68$). Fresh or frozen-thawed high viability spermatozoa penetrated 90.1% and 85.4% of bovine oocytes respectively. Lower rates of penetration were observed for fresh and frozen-thawed low viability spermatozoa (34% and 22.5% respectively). The donkey spermatozoa were able to fuse with the oolema and even to decondense and form the male pronucleus (85–94%). Larger numbers of penetrated spermatozoa per oocyte were recorded when high viability sperm samples were used, whether fresh (3.02 vs. 1.12 for low viability sperm) or frozen-thawed (3.41 vs. 1.47). Consequently, low viability sperm samples showed higher percentages of monospermic penetration (91.17% and 61.97% for fresh and frozen-thawed sperm samples respectively). These findings suggest that bovine oocytes provide a useful model for assessing the penetration potential of frozen-thawed donkey sperm.

1. Introduction

The laboratory assessment of sperm quality is essential if assisted reproduction in domestic species is to be efficient. However, it is becoming ever more apparent that the standard variables of sperm motility, morphology and concentration are insufficient for predicting fertility or even for identifying subfertile individuals (Gadea et al., 2004). The search for more appropriate variables that can be measured quickly by sensitive and repeatable methods is therefore the focus of increasing interest.

In vitro fertilization (IVF) tests might afford an adequate means of assessing fertility. This type of procedure allows the evaluation of sperm–oocyte interactions that

* Corresponding author. Tel.: +34 935811044; fax: +34 935812006.
E-mail address: jordi.miro@uab.cat (J. Miró).

0378-4320/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.anireprosci.2009.08.005

occur during *in vivo* fertilization, and permits different endpoints in the early stages of embryo development to be monitored. Some authors have used homologous IVF assays to predict male fertility using zona pellucida (ZP)-intact oocytes (Papadopoulos et al., 2005; Schneider et al., 1999; Zhang et al., 1997). However, it is often difficult to obtain oocytes of the same species as the sperm donor, especially if this species is wild or endangered. In such animals, heterologous IVF would appear to be an attractive method for evaluating the fertilizing capacity of fresh or frozen-thawed sperm. Further, compared to artificial insemination, heterologous IVF requires fewer sperm cells, allowing sperm function to be thoroughly investigated while sparing valuable male gametes. Nonetheless, the potential of heterologous IVF remains largely unexplored.

Mammalian oocytes have been used as a model for assessing human sperm functionality (Canovas et al., 2007; Liu and Baker, 1992; Terada et al., 2004; Yanagimachi et al., 1976), and cross-species fertilization of the oocytes of domestic farm and laboratory species by the cryopreserved sperm of endangered felids (Baudi et al., 2008; Swanson and Wildt, 1997), non-domestic bovines (McHugh and Rutledge, 1998), oryx (Kouba et al., 2001) and equines (Brackett et al., 1982; Campos-Chillón et al., 2007; Choi et al., 2003) has been reported. However, the literature contains no references on the interaction between donkey spermatozoa and bovine oocytes.

The aim of the present work was to determine whether the functionality of Catalonian donkey sperm (fresh and frozen-thawed) could be assessed by studying its interaction with bovine oocytes at the oolema and ooplasm levels. The objectives were: (i) to evaluate the ability of donkey spermatozoa to fuse with *in vitro*-matured bovine ZP-free oocytes (sperm–oolema interaction), (ii) to assess the ability of donkey spermatozoa to decondense and transform into a male pronucleus following the *in vitro* fertilization of bovine oocytes (sperm–ooplasm interaction), and (iii) to study the correlation between donkey spermatozoa motility and viability and its ability to penetrate bovine oocytes.

2. Materials and methods

2.1. Reagents and laboratory supplies

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Plastic dishes, four-well plates and tubes were obtained from Nunc (Roskilde, Denmark).

2.2. Bovine oocyte collection and in vitro maturation

Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory in phosphate buffered saline (PBS) at 36–38 °C. Cumulus oocyte complexes (COCs) were obtained by aspirating 2–10 mm follicles. Only COCs with three or more layers of cumulus cells and showing homogeneous cytoplasm were selected for maturing *in vitro*. Groups of up to 50 COCs were placed in 500 μL of maturation medium in four-well dishes and cultured for 24 h at 38.5 °C in a 5% CO_2 humidified air atmosphere. The maturation medium was comprised of TCM-199 supplemented with 10% (v/v) foetal calf serum (FCS), 10 ng/mL epidermal growth factor and 50 μg/mL gentamicin.

2.3. Animals, semen collection and cryopreservation

The donkeys used in this study were five Catalonian jackasses between four and eight years of age. All were in good condition and were known to be fertile. Semen was collected using a Hanover model artificial vagina with an in-line gel filter. Collections were performed using an ovariectomized female donkey brought into oestrus with estrogens. After collection, gel-free semen from each donkey was immediately diluted 1:5 (v/v) with prewarmed (37 °C) dry skimmed milk extender. Semen samples were evaluated upon collection for volume, viability, morphology and motility. Aliquots were then taken as needed for analysis. The sperm concentration of the ejaculate was determined using a Neubauer haemocytometer. Sperm viability was classified and determined using a standard percent live spermatozoon assay. Samples were stained with eosin–nigrosin as described by Bamba (1988). Following this stain, viable spermatozoa were defined as those that showed an uniform, white colour under observation, whereas non-viable sperm were defined as those which showed any sign of both partial and total pinkish-purple staining.

Sperm processing and cryopreservation were conducted as previously described (Flores et al., 2008). Briefly, the diluted semen samples were centrifuged at $660 \times g$ for 15 min at 20 °C in a programmable refrigerated centrifuge to remove the seminal plasma. The supernatant was eliminated and the spermatozoa re-suspended in Gent A diluent® (Minitüb, Tiefenbach, Germany). The sperm cell concentration was then re-determined and Gent B diluent® (Minitüb, Tiefenbach, Germany) added to obtain a final concentration of 200×10^6 viable sperms/mL (50% final volume). Diluted semen was packaged into 0.5 mL polyvinyl chloride plastic straws (Minitüb, Tiefenbach, Germany). These were positioned horizontally on a metal rack and cooled in a programmable liquid nitrogen freezer (Ice-Cube 14S; Minitüb, Tiefenbach, Germany) at a rate of 0.25 °C/min from 20 °C to 5 °C, and 5.50 °C/min from 5 °C to −120 °C. The straws were then plunged into the liquid nitrogen (−196 °C) for storage. Frozen semen was thawed by immersing the straws in a water bath at 37 °C for 30 s. Sperm motility and viability was then determined.

2.4. Sperm motility

The motion characteristics of fresh and frozen-thawed sperm samples were determined using a computer-assisted sperm analysis system (CASA system) (Integrated Sperm Analysis System V1.0; Proiser SL, Valencia, Spain). Samples were incubated for 5 min in a water bath at 37 °C. Five microlitres aliquots of these samples were then observed using a phase contrast microscope equipped with a heat stage (37 °C). Three fields per drop were analysed. The CASA system is based on the analysis of 50 consecutive, digital images of a single field at a magnification of 200×

(dark ground). These 50 consecutive images were obtained over 1 s – an image capture rate of one photograph every 20 ms. The sperm motility descriptors obtained by CASA are described below:

Curvilinear velocity (VCL)	μm/s	Measures the sequential progression along the true trajectory
Linear velocity (VSL)	μm/s	Measures the straight trajectory of the spermatozoa per unit time
Mean velocity (VAP)	μm/s	Measures the mean trajectory of the spermatozoa per unit time
Linearity coefficient (LIN)	%	VSL/VCL × 100
Straightness coefficient (STR)	%	VSL/VAP × 100
Wobble coefficient (WOB)	%	VAP/VCL × 100
Mean lateral head displacement (mean ALH)	μm	Measures the mean head displacement along the curvilinear trajectory
Frequency of head displacement (BCF)	Hz	The frequency with which the sperm trajectory crosses the average path trajectory

Total motility was defined as the percentage of spermatozoa with a VAP of >10 μm/s, and progressive motility as the percentage of spermatozoa with a VAP of >90 μm/s plus an STR of >75%.

2.5. Sperm preparation and ZP-free fertilization

For *in vitro* fertilization, cow oocytes were collected and matured as described above. After maturation, they were washed twice in PBS and then denuded (removal of the cumulus cells) by gentle pipetting. Oocytes with polar bodies were then selected and incubated in 50 μL droplets of prewarmed 0.3% pronase (w/v) in fertilization medium (Tyrode's medium supplemented with 25 mM sodium bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA and 10 mg/mL heparin sodium salt [Calbiochem, Darmstadt, Germany]) for 2–3 min to remove the ZP. Its digestion was observed continuously using a stereomicroscope. When the ZP was no longer visible, the oocytes were immediately removed from the pronase solution, washed, and transferred in groups of up to 20–25 to four-well plates containing 250 μL of fertilization medium per well. The oocytes were then allowed to recover for 30 min prior to insemination. ZP-free oocytes were randomly assigned to two groups; those of one were inseminated with fresh spermatozoa, while those of the second were inseminated with frozen-thawed spermatozoa. Presumptive zygotes were fixed 18–20 h after insemination and examined for sperm cell penetration. In order to exclude parthenogenetic activation of bovine oocytes, a sample of oocytes was cultured in presence of ionomycin without sperm cells (data not shown).

Motile spermatozoa were obtained by centrifuging fresh or frozen-thawed sperm in Hepes-buffered Tyrode's medium for 5 min at 700 × *g* at room temperature. The supernatant was removed and the pellet resuspended in 8 mL of Hepes-buffered Tyrode's and centrifuged again at 700 × *g* for 5 min. Spermatozoa were counted in a haemocytometer and diluted in an appropriate volume of fertilization medium to give a final concentration of 2×10^6 spermatozoa/mL. In order to induce *in vitro* capacitation, the spermatozoa were treated with 0.1 μM ionomycin for 15 min (adding 1 μL of 0.1 mM ionomycin to 1 mL of the final diluted sperm). A 250 μL aliquot of this suspension was then added to each fertilization well to obtain a final concentration of 1×10^6 spermatozoa/mL. Plates were incubated for 18–20 h at 38.5 °C in a 5% CO_2 humidified air atmosphere. In order to exclude parthenogenetic activation of bovine oocytes, a sample of oocytes ($n = 51$) was cultured with a 250 μL aliquot of 0.1 μM ionomycin without sperm cells.

2.6. Evaluation of penetration

At 18–20 h post-insemination, the oocytes were washed three times, fixed for 30 min in 2% (v/v) paraformaldehyde in PBS, stained with DAPI (4′,6-diamidino-2-phenylindole; Vysis Inc., Downer's Grove, IL, USA) (125 ng/mL) and mounted on glass slides. Nuclear stage, sperm penetration and the formation of the male pronucleus were assessed at 400× under an epifluorescence microscope (see Fig. 1).

2.7. Experimental design

Fifteen ejaculates – three from each of the five donkeys – were collected and sperm viability and motility immediately assessed. All collected semen was cryopreserved as described above. After thawing, viability and motility were checked again. The motion characteristics of the fresh and thawed spermatozoa were determined using a CASA system. The ZP of *in vitro*-matured cow oocytes was removed and the oocytes fertilized with fresh or frozen-thawed spermatozoa from the five donkeys. Four experimental groups were established: (1) fertilization attempts involving fresh, high viability (HV; >60%) semen, (2) fertilization attempts involving low viability (LV; <40%) semen, (3) fertilization attempts involving frozen-thawed HV (>60%) semen and (4) and fertilization attempts involving frozen-thawed LV (<40%) semen. Criteria for HV (>60%) and LV (<40%) were established according to Flores et al. (2008). After an 18 h co-incubation period, the oocytes were fixed and examined for sperm penetration, the number of penetrated spermatozoa per oocyte, and male pronucleus formation.

2.8. Statistical analysis

All calculations were performed using the Statistical Analysis Systems Package (SAS, 2000). The normality of the distribution of the sperm motility results was assessed using the Shapiro–Wilks test (W) included in the UNIVARIATE procedure. The PROC GLM procedure was used to determine the mean values of the motility variables and to analyse differences between the results for the sperm motility descriptors. The total number of spermatozoa analysed following this protocol was 1322. Total *in vitro* penetration and the mean number of penetrated sperm per oocyte were analysed by ANOVA. All experiments were replicated three times. Percentage data were subjected to arcsine transformation before analysis. When

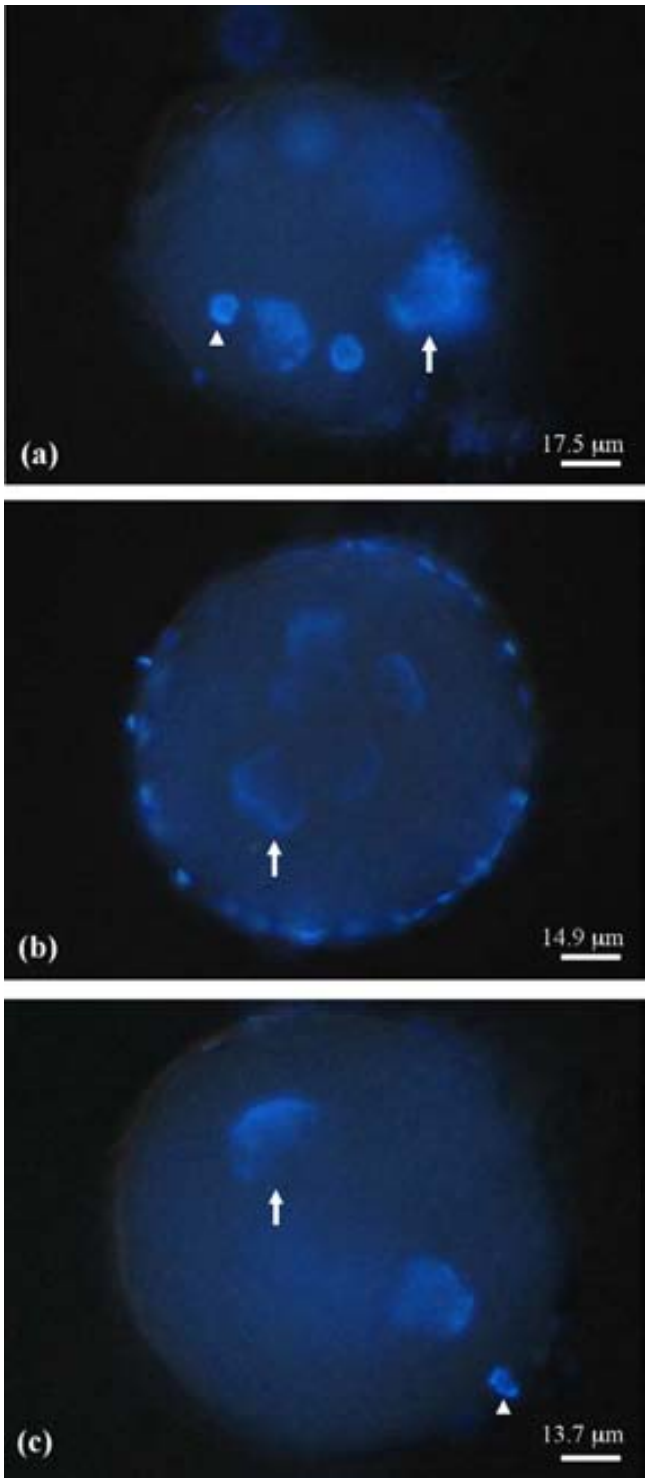

Fig. 1. Epifluorescence microscope images of *in vitro*-matured bovine oocytes after 18 h of co-incubation with donkey spermatozoa. Oocytes were immunocytochemically stained using DAPI to visualize the nuclear stage, sperm heads and the male pronucleus (blue). (a) Oocyte with five pronuclei (PN) (arrow) and one enlarged sperm head (arrowhead); (b) Oocyte with 5 PNs; (c) Oocyte showing 2 PN and 1 enlarged sperm head.

Table 1
Quality analysis of high and low viability fresh and frozen-thawed donkey sperm.

	Fresh HV	Fresh LV	F-T HV	F-T LV
Viability (%)	69.30 ± 3.81[a]	22.00 ± 4.66[b]	71.26 ± 2.95[a]	33.87 ± 3.30[b]
Total motility (%)	71.66 ± 15.81	62.00 ± 19.36	82.40 ± 12.24	43.75 ± 13.69
Progressive motility (%)	31.33 ± 10.59	37.5 ± 12.97	34.20 ± 8.20	23.25 ± 9.17
VCL (μm/s)	121.02 ± 0.84[a]	73.89 ± 1.15[b]	93.15 ± 0.83[c]	78.79 ± 0.93[d]
VSL(μm/s)	64.30 ± 0.82[a]	52.36 ± 1.12[b]	62.21 ± 0.82[ac]	59.11 ± 0.91[c]
VAP (μm/s)	88.76 ± 0.68[a]	58.99 ± 0.93[b]	76.41 ± 0.68[c]	66.85 ± 0.76[d]
LIN (%)	48.30 ± 0.99[a]	62.48 ± 1.35[bc]	60.39 ± 0.98[b]	66.73 ± 1.10[c]
STR (%)	67.82 ± 1.01[a]	80.31 ± 1.38[bc]	76.54 ± 1.00[b]	82.45 ± 1.12[c]
WOB (%)	68.41 ± 0.69[a]	75.42 ± 0.94[b]	76.41 ± 0.68[bc]	78.63 ± 0.76[c]
Mean ALH (μm)	3.90 ± 0.05[a]	2.44 ± 0.06[b]	2.75 ± 0.05[c]	2.19 ± 0.05[d]
BCF (Hz)	8.95 ± 0.16[a]	8.13 ± 0.22[b]	8.49 ± 0.16[ab]	8.85 ± 0.18[ab]

Results are expressed as means ± SEM of four different semen preparations involving a total of 1322 spermatozoa. Fresh HV and fresh LV: fresh semen of high and low viability respectively; F-T HV and F-T LV: frozen-thawed semen of high and low viability respectively; VCL: curvilinear velocity; VSL: linear velocity; VAP: mean velocity; LIN: linearity coefficient; STR: straightness coefficient; WOB: wobble coefficient; mean ALH: mean lateral head displacement, BCF: frequency of head displacement. Different superscripts between rows indicate significant differences ($P < 0.05$).

ANOVA revealed a significant effect, the results for the different treatments were compared using Fisher's protected LSD test. Significance was set at $P < 0.05$.

3. Results

3.1. Quality of fresh and frozen-thawed donkey sperm

Table 1 shows the characteristics of the fresh and frozen-thawed sperm from the five Catalonian donkeys used in this study. Significant differences were seen in the viability of HV and LV sperm, although their total and progressive motility values were not significantly different. The overall motility of the frozen-thawed HV samples was lower but more linear than that of the fresh HV samples ($P < 0.05$). Further, the frozen-thawed HV samples presented lower mean ALH and VCL values than the fresh HV samples ($P < 0.05$). Moreover, the STR and WOB values were significantly higher in the frozen-thawed HV samples. In contrast, frozen-thawed LV samples showed significant higher velocity variables (VAP, VCL and VSL) and linearity coefficients than the fresh LV samples. The WOB was significantly higher in the LV samples, while the mean ALH was significantly lower in the LV samples. The BCF values of the different types of sample were not significantly different.

3.2. Penetration of ZP-free bovine oocytes by donkey sperm

Results are presented in Table 2. When ZP-free bovine oocytes were inseminated with HV sperm, significantly higher penetration rates were obtained – for both fresh (90.12%) and frozen-thawed (85.40%) samples – than with LV sperm (34% and 22.47% for fresh and frozen-thawed samples respectively). High viability sperm were associated with a larger number of penetrated spermatozoa per oocyte ($P > 0.05$) than LV sperm – both for fresh and frozen-thawed samples. Consequently, LV sperm samples showed higher percentages of monospermic. The donkey spermatozoa were seen to be able to fuse with the oolema, decondense and form male pronuclei (85–94%). Parthenogenetic activation of bovine oocytes was 7% (4/51) and only 1 PN was observed on activated oocytes.

3.3. Correlation between sperm characteristics and penetration rate

Table 3 shows the correlations detected between sperm motion characteristics, viability and the penetration rate. Progressive motility was strongly correlated with the CASA variables (VCL, VSL, VAP, WOB, mean ALH and BCF). Penetration frequency was found to be strongly and positively correlated ($P < 0.01$) with percentage viability ($r = 0.84$). Only the VCL and mean ALH values correlated strongly with percentage fertilization ($r = 0.61$ and 0.68, respectively; $P < 0.01$). The VAP and total motility correlated with the penetration rate ($P < 0.05$). The remaining CASA variables were not correlated with penetration percentage. Finally, percentage viability correlated only with total motility and mean ALH ($P < 0.05$).

4. Discussion

The Catalonian donkey is an endangered local donkey breed that can be found in a number of Pyrenean and pre-Pyrenean areas in Catalonia (northeastern Spain).

Table 2
Results of *in vitro* heterologous oocyte penetration testing involving fresh and frozen-thawed donkey sperm of different viability.

Sperm type	Examined, *n*	Penetration, *n* (%)	Monospermic, *n* (%)	N sperm/oocyte
Fresh HV	81	73 (90.12)[a]	21 (28.77)[a]	3.02 ± 0.52[ab]
Fresh LV	100	34 (34)[b]	31 (91.17)[b]	1.12 ± 0.06[ac]
F-T HV	226	193 (85.40)[a]	61 (31.61)[a]	3.41 ± 0.97[b]
F-T LV	316	71 (22.47)[b]	44 (61.97)[b]	1.47 ± 0.09[c]

Data are means (SEM of three replicates). Letters a–c denote significant differences within columns ($P < 0.05$). Fresh HV and fresh LV: fresh semen of high and low viability respectively; F-T HV and F-T LV: frozen-thawed semen of high and low viability respectively.

Table 3
Pearson's correlation coefficient between motion characteristics, viability and penetration rate.

	%Penetration	%Viability	PM	TM	VCL	VSL	VAP	LIN	STR	WOB	ALH
%Viability	0.84**										
PM	0.41	0.32									
TM	0.57*	0.60*	0.91**								
VCL	0.61**	0.48	0.73**	0.66**							
VSL	0.42	0.30	0.71**	0.52*	0.91**						
VAP	0.56*	0.43	0.75**	0.63**	0.98**	0.97**					
LIN	−0.07	−0.18	0.43	0.14	0.40	0.73**	0.55*				
STR	−0.23	−0.39	0.23	−0.07	0.22	0.59*	0.38	0.97**			
WOB	0.13	0.07	0.61**	0.36	0.57*	0.84**	0.70**	0.96**	0.88**		
ALH	0.68**	0.56*	0.65**	0.66**	0.94**	0.74**	0.87**	0.12	−0.05	0.30	
BCF	0.38	0.34	0.81**	0.69**	0.88**	0.89**	0.90**	0.60*	0.46	0.71**	0.78**

PM: progressive motility; TM: total motility; VCL: curvilinear velocity; VSL: linear velocity; VAP: mean velocity; LIN: linearity coefficient; STR: straightness coefficient; WOB: wobble coefficient; mean ALH: mean lateral head displacement, BCF: frequency of head displacement.
* $P < 0.05$.
** $P < 0.01$.

Knowledge regarding the cryopreservation of its semen is important for breeding programs, the maintenance of the breed's numbers, and the prevention of its disappearance. Sperm cryopreservation can, however, damage sperm cells, reducing their fertilization capacity (Bailey et al., 2000). The damage caused to membranes during cooling to 5 °C or freezing is often subtle and difficult to detect in laboratory assays.

Sperm penetration assays can assess a number of sperm functions simultaneously (e.g., motility, ability to undergo an acrosome reaction, oocyte penetration and DNA decondensation) and may better assess sperm quality than assays that evaluate a single sperm characteristic (Bousquet and Brackett, 1981, 1982; Bousquet et al., 1983; Brahmkshtri et al., 1999). The ZP-free oocyte penetration assay has been used to assess the spermatozoa of many species, including horses (Blue et al., 1989; Graham et al., 1987; Okolski et al., 1987; Samper et al., 1989; Zhang et al., 1990) and cattle (Bousquet and Brackett, 1981, 1982; Bousquet et al., 1983; Eaglesome and Miller, 1989; Graham and Foote, 1987a,b). Wilhelm et al. (1996) reported that frozen-thawed horse spermatozoa could penetrate ZP-free hamster oocytes after treatment with dilauroylphosphatidylcholine (PC12) to induce the acrosome reaction, and indicated the percentage of penetrated hamster oocytes to be highly correlated to stallion fertility. When bovine ZP-free oocytes were used, the penetration rates for fresh HV stallion sperm (capacitated with 8-bromoadenosine cyclic monophosphate (8BrcAMP) + 0.1 μM of ionomycin in PVA-containing media) varied from 18% to 47% depending on the concentration of equine preovulatory follicular fluid added to the bovine oocyte maturation medium (Choi et al., 2003). These authors concluded that ZP-free bovine oocytes might be useful for assaying the *in vitro* capacitation and fertilization potential of stallion sperm. However, as far as we know, the literature contains no studies on heterologous bovine fertilization involving donkey sperm. The present work, shows that donkey sperm can penetrate bovine oocytes, fuse with the oolema, decondense, and form male pronuclei.

The penetration rates of fresh (90.12%) and frozen-thawed (85.40%) HV donkey sperm were higher than those obtained by Choi et al. (2003) and Landim-Alvarenga et al. (2001) for equine spermatozoa. It is known that spermatozoa from different species can have different penetration rates depending on the ZP-free oocyte used. For example, Graham et al. (1986) and Landim-Alvarenga et al. (2001) observed that bull spermatozoa penetrated a higher percentage of ZP-free hamster oocytes than did stallion spermatozoa. One reason for the higher penetration capacity of the donkey spermatozoa observed in the present work compared to that of stallion spermatozoa may be the differences in the lipid composition of the sperm cell membranes. Differences in the membrane lipid composition of mammalian spermatozoa have been studied in an attempt to understand the susceptibility of spermatozoa to cold shock. Parks and Lynch (1992) observed that cholesterol was the major sterol present in spermatozoa membranes of boar, bull, stallion and rooster spermatozoa, and that the molar ratio of cholesterol to phospholipid was higher in bull spermatozoa than in stallion spermatozoa. Cholesterol may help regulate the fluidity and permeability of the lipid bilayers of the membrane. During capacitation, cholesterol efflux from the spermatozoa increases the fluidity and permeability of their membranes, eventually leading to membrane fusion and the acrosome reaction. Davis (1981) first suggested that differences in the capacitation rates of human spermatozoa from different individuals might be related to differences in the cholesterol:phospholipid ratio of the membranes.

Stallion spermatozoa can be capacitated and the acrosome reaction induced by treatment with 8BrcAMP, heparin, PC-12, lysophosphatidyl-serine, ionophore calcium and ionomycin (Choi et al., 2003; Landim-Alvarenga et al., 2001; Wilhelm et al., 1996). In the present work, spermatozoa were incubated with ionomycin (0.1 μM) for a short time (15 min) to induce capacitation and thus allow the oocyte penetration capacity to be assessed (Choi et al., 2003). Spermatozoa that did not undergo ionomycin treatment were incapable of penetrating the present ZP-free oocytes (data not shown), while those that were thus treated penetrated them at rates of 22.5–90.1%. This study was not, however, designed to test which compound is most effective at inducing the acrosome reaction. In the present work, use was made of a concentration of ionomycin previously shown to induce the acrosome reaction in stallion and bull spermatozoa.

Motility is essential for the transport of sperm cells through the female reproductive tract and for oocyte penetration. The mean sperm motility values obtained in this study are comparable to those indicated in previous reports (Flores et al., 2008; Miro et al., 2005). However, the BCF recorded in the present work was higher in all experimental groups. The values of the velocity variables for the fresh HV sperm were higher than those recorded in other works, perhaps due to variability between the animals used. The frozen-thawed sperm motion characteristics recorded in the present study are dissimilar to those reported in previous reports (Flores et al., 2008). The velocity and linearity variables had higher values in the present work, whereas the mean ALH and BCF values were lower. This discrepancy might also be due to variability between the animals used. However, the changes in VCL and VAP before and after freezing were similar. Whereas freezing/thawing is reported to cause a significant increase in the values of boar sperm motility characteristics, the same was not seen for the present donkey cells. This discrepancy might be related to the notable differences observed in the physiological and metabolic characteristics of boar and donkey spermatozoa.

Relationships between motility characteristics in human and bovine spermatozoa, measured by CASA in both *in vitro* and *in vivo* fertility studies, have been reported by different authors. Fetterolf and Rogers (1990), working with human spermatozoa, showed that the total motility and VCL were highly correlated with the homologous IVF rate. Similarly, in bovines (*in vivo*), Farrell et al. (1998) reported a strong correlation between several motility characteristics (BCF, LIN, VAP, VSL and VCL) and fertility ($r^2 = 0.97$), where fertility was defined as 59 days of non-return service in cows inseminated with frozen-thawed semen. More recently, Kathiravan et al. (2008) reported that the progressive motility, VAP and VSL of bull spermatozoa were highly correlated with penetration in ZP-free hamster oocytes. Wilhelm et al. (1996) also reported that the percentage viability was correlated with stallion fertility ($r = 0.68$). In agreement with the above reports, the present penetration rates were correlated with viability, total motility, VAP and VCL. In contrast, no correlation was reported between penetration rate and sperm motility in the pig (Martinez et al., 1993; Suzuki et al., 1996). It has been suggested that the lack of correlation between conventional semen quality tests and sperm penetration assays is due to the fact that they measure different aspects of sperm fertilizing capacity (Gadea, 2005). Certainly, the influence of sperm characteristics on the success of *in vitro* fertilization is incompletely understood (Ivanova and Mollova, 1993; Martinez et al., 1993; Vazquez et al., 1993) and this lack of correlation may be due to differences in protocol, the number of penetrated sperm per oocyte, and differences among ejaculates selected.

In conclusion, this work shows that fresh and frozen-thawed donkey spermatozoa are able to fuse with *in vitro*-matured bovine ZP-free oocystes, and to decondense and form male pronuclei. Some of the CASA motility characteristics (VAP, VCL, mean ALH and total motility) and sperm viability are highly correlated with the *in vitro* fertilization rate. Heterologous IVF would seem to be a good way to evaluate the quality of frozen-thawed donkey semen and to verify the storage quality of banked sperm samples. Further studies are needed to correlate sperm variables and heterologous penetration with *in vivo* fertility.

Acknowledgments

This work has been supported by Grant RZ2007-00017-00-00 (Instituto Nacional de Investigaciones Agrarias, Spain). The authors are grateful to Mr. José Manuel Fernández Castro for providing us some of the jackasses.

References

Bailey, J.L., Bilodeau, J.F., Cormier, N., 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J. Androl. 21, 1–7.

Bamba, K., 1988. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin–nigrosin stain. Theriogenology 29, 1245–1251.

Baudi, D.L., Jewgenow, K., Pukazhenthi, B.S., Spercoski, K.M., Santos, A.S., Reghelin, A.L., Candido, M.V., Javorouski, M.L., Muller, G., Morais, R.N., 2008. Influence of cooling rate on the ability of frozen-thawed sperm to bind to heterologous zona pellucida, as assessed by competitive in vitro binding assays in the ocelot (*Leopardus pardalis*) and tigrina (*Leopardus tigrinus*). Theriogenology 69, 204–211.

Blue, B.J., McKinnon, A.O., Squires, E.L., Seidel Jr., G.E., Muscari, K.T., 1989. Capacitation of stallion spermatozoa and fertilisation of equine oocytes in vitro. Equine Vet. J. 8, 111–116.

Bousquet, D., Brackett, B.G., 1981. Penetration of zona-free hamster ova by bull sperm after frozen storage. Theriogenology 15, 117.

Bousquet, D., Brackett, B.G., 1982. Penetration of zona-free hamster ova as a test to assess fertilizing ability of bull sperm after frozen storage. Theriogenology 17, 199–213.

Bousquet, D., Brackett, B.G., Dressel, M.A., Allen, C.H., 1983. Efforts to correlate laboratory with field observations on bull sperm fertility. Theriogenology 20, 601–613.

Brackett, B.G., Cofone, M.A., Boice, M.L., Bousquet, D., 1982. Use of zona-free hamster ova to assess fertilizing ability of bull and stallion. Gamete Res. 5, 217–227.

Brahmkshtri, B.P., Edwin, M.J., John, M.C., Nainar, A.M., Krishnan, A.R., 1999. Relative efficacy of conventional sperm parameters and sperm penetration bioassay to assess bull fertility in vitro. Anim. Reprod. Sci. 54, 159–168.

Campos-Chillón, L.F., Barcelo-Fimbres, M., Carnevale, E.M., Seidel, G.E.J., 2007. Use of bovine oocytes to evaluate in vitro fertilizing capacity of equine sperm. Theriogenology 68, 515–516.

Canovas, S., Coy, P., Gomez, E., 2007. First steps in the development of a functional assay for human sperm using pig oocytes. J. Androl. 28, 273–281.

Choi, Y.H., Landim-Alvarenga, F.C., Seidel Jr., G.E., Squires, E.L., 2003. Effect of capacitation of stallion sperm with polyvinylalcohol or bovine serum albumin on penetration of bovine zona-free or partially zona-removed equine oocytes. J. Anim. Sci. 81, 2080–2087.

Davis, B.K., 1981. Timing of fertilization in mammals: sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. Proc. Natl. Acad. Sci. U.S.A. 78, 7560–7564.

Eaglesome, M.D., Miller, S.A., 1989. Prediction of fertility of bovine semen: preliminary studies with the hamster egg penetration test. Theriogenology 31, 643–651.

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.

Fetterolf, P.M., Rogers, B.J., 1990. Prediction of human sperm penetrating ability using computerized motion parameters. Mol. Reprod. Dev. 27, 326–331.

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

Gadea, J., Selles, E., Marco, M.A., 2004. The predictive value of porcine seminal parameters on fertility outcome under commercial conditions. Reprod. Domest. Anim. 39, 303–308.

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

Graham, J.K., Foote, R.H., Parrish, J.J., 1986. Effect of dilauroylphosphatidylcholine on the acrosome reaction and subsequent penetration of bull spermatozoa into zona-free hamster eggs. Biol. Reprod. 35, 413–424.
Graham, J.K., Foote, R.H., 1987a. Dilauroylphosphatidylcholine liposome effects on the acrosome reaction and in vitro penetration of zona-free hamster eggs by bull sperm. II. A fertility assay for frozen-thawed semen. Gamete Res. 16, 147–158.
Graham, J.K., Foote, R.H., 1987b. Dilauroylphosphatidylcholine liposome effects on the acrosome reaction and in vitro penetration of zona-free hamster eggs by bull sperm. I. A fertility assay for fresh semen. Gamete Res. 16, 133–145.
Graham, J.K., Foote, R.H., Hough, S.R., 1987. Penetration of zona-free hamster eggs by liposome-treated sperm from the bull, ram, stallion, and boar. Biol. Reprod. 37, 181–188.
Ivanova, M., Mollova, M., 1993. Zona-penetration in vitro test for evaluating boar sperm fertility. Theriogenology 40, 397–410.
Kathiravan, P., Kalatharan, J., Edwin, M.J., Veerapandian, C., 2008. Computer automated motion analysis of crossbred bull spermatozoa and its relationship with in vitro fertility in zona-free hamster oocytes. Anim. Reprod. Sci. 104, 9–17.
Kouba, A.J., Atkinson, M.W., Gandolf, A.R., Roth, T.L., 2001. Species-specific sperm-egg interaction affects the utility of a heterologous bovine in vitro fertilization system for evaluating antelope sperm. Biol. Reprod. 65, 1246–1251.
Landim-Alvarenga, F.C., Alvarenga, M.A., Seidel Jr., G.E., Squires, E.L., Graham, J.K., 2001. Penetration of zona-free hamster, bovine and equine oocytes by stallion and bull spermatozoa pretreated with equine follicular fluid, dilauroylphosphatidylcholine or calcium ionophore A23187. Theriogenology 56, 937–953.
Liu, D.Y., Baker, H.W., 1992. Sperm nuclear chromatin normality: relationship with sperm morphology, sperm-zona pellucida binding, and fertilization rates in vitro. Fertil. Steril. 58, 1178–1184.
Martinez, E., Vazquez, J.M., Matas, C., Roca, J., Coy, P., Gadea, J., 1993. Evaluation of boar spermatozoa penetrating capacity using pig oocytes at the germinal vesicle stage. Theriogenology 40, 547–557.
McHugh, J.A., Rutledge, J.J., 1998. Heterologous fertilization to characterize spermatozoa of the genus Bos. Theriogenology 50, 185–193.
Miro, J., Lobo, V., Quintero-Moreno, A., Medrano, A., Pena, A., Rigau, T., 2005. Sperm motility patterns and metabolism in Catalonian donkey semen. Theriogenology 63, 1706–1716.
Okolski, A., Babusik, P., Tischner, M., Leitz, W., 1987. Evaluation of mare oocyte collection methods and stallion sperm penetration of zona-free hamster ova. J. Reprod. Fertil. 35, 191–196.
Papadopoulos, S., Hanrahan, J.P., Donovan, A., Duffy, P., Boland, M.P., Lonergan, P., 2005. In vitro fertilization as a predictor of fertility from cervical insemination of sheep. Theriogenology 63, 150–159.
Parks, J.E., Lynch, D.V., 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology 29, 255–266.
Samper, J.C., Behnke, E.J., Byers, A.P., Hunter, A.G., Crabo, B.G., 1989. In vitro capacitation of stallion spermatozoa in calcium-free Tyrode's medium and penetration of zona-free hamster eggs. Theriogenology 31, 875–884.
Schneider, C.S., Ellington, J.E., Wright Jr., R.W., 1999. Relationship between bull field fertility and in vitro embryo production using sperm preparation methods with and without somatic cell co-culture. Theriogenology 51, 1085–1098.
Suzuki, K., Mori, T., Shimizu, H., 1996. Effect of the duration of preincubation on the ability of pig spermatozoa to penetrate oocytes in vitro. Anim. Sci. Technol. Jpn. 67, 24–27.
Swanson, W.F., Wildt, D.E., 1997. Strategies and progress in reproductive research involving small cat species. Int. Zoo Yearbook 35, 152–159.
Terada, Y., Nakamura, S., Morita, J., Tachibana, M., Morito, Y., Ito, K., Murakami, T., Yaegashi, N., Okamura, K., 2004. Use of Mammalian eggs for assessment of human sperm function: molecular and cellular analyses of fertilization by intracytoplasmic sperm injection. Am. J. Reprod. Immunol. 51, 290–293.
Vazquez, J.M., Martinez, E., Roca, J., Coy, P., Pastor, L.M., 1993. Acrosome reaction of boar spermatozoa in homologous in vitro fertilization. Mol. Reprod. Dev. 36, 84–88.
Wilhelm, K.M., Graham, J.K., Squires, E.L., 1996. Comparison of the fertility of cryopreserved stallion spermatozoa with sperm motion analyses, flow cytometric evaluation, and zona-free hamster oocyte penetration. Theriogenology 46, 559–578.
Yanagimachi, R., Yanagimachi, H., Rogers, B.J., 1976. The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. Biol. Reprod. 15, 471–476.
Zhang, B.R., Larsson, B., Lundeheim, N., Rodriguez-Martinez, H., 1997. Relationship between embryo development in vitro and 56-day non-return rates of cows inseminated with frozen-thawed semen from dairy bulls. Theriogenology 48, 221–231.
Zhang, J., Boyle, M.S., Smith, C.A., Moore, H.D.M., 1990. Acrosome reaction of stallion spermatozoa evaluated with monoclonal antibody and zona-free hamster eggs. Mol. Reprod. Dev. 27, 152–158.

Discussió/ Discussion

Discussió /Discussion

Tal i com ja s'ha comentat a l'introducció, la finalitat d'aquesta tesi és l'estudi de les característiques reproductives de la femella i del mascle de l'ase Català per aplicar tecnologies reproductives com la conservació del semen (refrigeració o congelació) o la inseminació artificial i poder així, millorar la seva eficiència reproductiva, evitant l'extinció d'aquesta raça de burro.

Existeixen nombrosos estudis sobre el cicle estral, l'ovulació i el comportament sexual de les eugues i generalment es tendeix a l'extrapolació d'aquestes dades per a l'aplicació en el maneig reproductiu de les burres. Aquesta extrapolació pot conduir-nos a errors, degut a diferències presents en la fisiologia reproductiva entre espècies (Pukazhenthi i Wildt, 2004). En el primer article de la present Tesi es van avaluar els paràmetres reproductius de les burres. Vam observar els principals signes de zel: immobilitat, masticació amb les orelles estirades enrere, micció i vulveig, descrits anteriorment per altres autors (Trimeche i Tainturier, 1995; Blanchard *et al.*, 1999) i útils per la detecció dels zels. En base als nostres resultats, la durada del cicle estral de les burres Catalanes (25 dies) és similar a altres estudis (Vandeplassche *et al.*, 1981, Trimeche i Tainturier, 1995), tot i que alguns autors han descrit durades inferiors (Blanchard *et al.*, 1999; Nishikawa i Yamazaki, 1949). Tradicionalment s'ha classificat a les eugues i les burres com animals polièstrics estacionals. Malgrat això, en aquest estudi les burres ciclaven durant tot l'any, mostrant una duració del zel més perllongada durant l'hivern. Aquesta observació també s'havia obtingut prèviament en burres a Wisconsin, per la qual cosa sembla indicar-nos que les burres estan menys influenciades per l'estació, sobretot en zones properes a l'equador (Ginther *et al.*, 1987). L'ovulació és un altre punt clau per establir un protocol de inseminació artificial adequat i per obtenir una òptima eficiència reproductiva. Segons els nostres resultats, les burres Catalanes ovulaven amb una mida fol·licular preovulatòria de

45 mm, superior a mida descrita per la majoria d'estudis realitzats en burres (Vandeplassche *et al.*, 1981; Meira *et al.*, 1995; Dadarwal *et al.*, 2004), degut probablement a la gran mida corporal de la raça de burro Català. Les ovulacions múltiples eren molt freqüents en la burra Catalana (44%) i també es relacionaria amb la raça o amb l'estat nutricional dels animals, tal i com ja s'ha descrit en les eugues (Taylor *et al.* 2002). Degut a la importància d'una bona predicció del moment de l'ovulació, es van avaluar diferents paràmetres (comportament de zel, mida fol·licular, textura fol·licular, aparença ecogràfica del fol·licle i l'úter i to uterí) per establir quins eren els millors predictors, i entre ells es va escollir la mida fol·licular, la textura fol·licular i el comportament de zel, existeixen estudis en eugues amb resultats similars (Koskinen *et al.*, 1989; Miró *et al.*, 2004). Diverses combinacions d'aquests tres paràmetres aconseguien predir l'ovulació amb una probabilitat >75% durant les 24h següents.

La refrigeració del semen és una recurs molt útil a l'hora de planificar les inseminacions artificials perquè podem aconseguir la difusió del material genètic entre poblacions de rucs aïllades i permet l'ús de mascles d'alt valor genètic a un baix cost. Diversos autors han descrit que l'eliminació del plasma seminal millora els resultats de viabilitat i motilitat del semen refrigerat en diverses espècies (Pickett *et al.*, 1975; Pellicer-Rubio i Combarnous, 1998; Carver i Ball, 2002; Serres *et al.*, 2002). Així doncs, en l'Article 2 vam plantejar avaluar l'efecte de l'eliminació parcial del plasma seminal (mitjançant dilució o centrifugació) sobre la viabilitat i la motilitat, observant si existien variacions en l'estructura subpoblacional espermàtica del semen de ruc Català. En el nostre estudi, vam observar que la dilució amb ràtio elevada (1:10) i la centrifugació del semen eren els mètodes més eficaços per mantenir la viabilitat i la motilitat del semen, coincidint amb el que s'havia descrit també en cavalls (Brinsko *et al.*, 2000) i en burros Zamorano-Lleonesos (Serres *et al.*, 2002). En canvi, Rota *et al.* (2008) eliminant totalment el plasma seminal no va observar cap millora en la conservació de semen refrigerat de burro Amiata. Aquesta discrepància és probablement deguda a l'eliminació total del plasma

seminal, donat que en cavalls i toros s'ha descrit que el semen refrigerat es conserva millor si es manté entre 5-20% del plasma seminal (Jasko *et al.*, 1991; Pickett *et al.*, 1975). D'altra banda, en l'estudi observem que les 4 subpoblacions mòtils descrites en el semen de ruc Català (Miró *et al.*, 2005) es mantenen després de la refrigeració, conservant la mateixa proporció de cada subpoblació espermàtica en les mostres centrifugades i només a les 24h de refrigeració.

Després d'avaluar l'efecte del plasma seminal, vam decidir estudiar quina era la temperatura òptima de conservació dels espermatozoides de ruc Català. És freqüent que els animals no es trobin prop d'un centre capacitat per realitzar la inseminació artificial o la congelació de semen, per aquesta raó es va plantejar l'estudi de 3 temperatures de refrigeració del semen diluït (5, 15 i 20ºC) durant 2h i la seva posterior centrifugació. La refrigeració del semen pot comportar danys a la membrana plasmàtica dels espermatozoides, anomenats xoc tèrmic, sobretot entre els 18 i 8ºC (Amann i Pickett, 1987). Existeixen discrepàncies entre els diversos autors sobre la temperatura de refrigeració més òptima del semen de cavall i burro, en alguns estudis estableixen entre 4-6ºC mentre que altres aconsellen temperatures de 15 i 20ºC (Varner *et al.*, 1989; Moran *et al.*, 1992; Province *et al.*, 1995; Cottorello *et al.*, 2002). En el nostre estudi es va observar que la refrigeració a 5 i 20ºC mantenien millor les característiques mòtils dels espermatozoides, tot i que la viabilitat no mostrava diferències amb la refrigeració a 15ºC. D'altra banda, la posterior centrifugació a 5, 15 i 20ºC del semen, per eliminar el plasma seminal, va comportar una disminució de la viabilitat del semen centrifugat a 15ºC i una alteració de la distribució dels espermatozoides a les 4 subpoblacions espermàtiques del semen centrifugat a 5 i 15ºC. D'aquesta manera, es va establir que la millor temperatura de refrigeració i posterior centrifugació era 20ºC, en contradicció amb el descrit per Serres *et al.* (2002) que optaven per 4 o 15ºC. Les discrepàncies en els estudis poden ser degudes a l'ús de diluents diferents, a temps de refrigeració més curts, i una valoració de la motilitat més acurada en el nostre treball.

Un cop valorats els efectes de la temperatura de refrigeració i de l'eliminació del plasma seminal mitjançant la dilució o la centrifugació, vam centrar-nos en el punt clau de la conservació de semen congelat de ruc Català, que és el propi procés de congelació/descongelació. Aquest procés és perjudicial per l'espermatozoide de qualsevol mamífer perquè pot provocar danys a les estructures i orgànuls espermàtics, que es tradueixen amb alteracions de la funció espermàtica, sobretot alteracions de la motilitat dels espermatozoides (Curry, 2000; Watson *et al.*, 2000). Així doncs, en l'Article 4 vam estudiar com afecta la congelació/descongelació a l'estructura subpoblacional mòtil dels ejaculats de burro Català, comparant amb una altre espècie de mamífers com és el porc. Els nostres resultats estableixen que els canvis en la motilitat produïts per la congelació/descongelació es deuen a canvis en els paràmetres i els percentatges específics de cada subpoblació espermàtica mòtil present en els ejaculats tant de burro com de porc. Malgrat aquests canvis, en les dues espècies es va mantenir l'estructura de 4 subpoblacions espermàtiques, indicant la possible importància d'aquesta estructura per mantenir la funcionalitat de l'ejaculat. Aquesta estructura de l'ejaculat basat en subpoblacions està àmpliament descrit en diverses espècies de mamífers com el porc, la gasela, el cavall, el burro, el gos i el conill (Abaigar *et al.*, 1999; Rigau *et al.*, 2001, Miró *et al.*, 2005; Quintero-Moreno *et al.*, 2003 i 2007). Ara bé, el procés de congelació/descongelació produeix diferents respostes sobre la motilitat dels espermatozoides, en el porc vam observar un augment significatiu de les característiques de motilitat, al contrari que en el burro. Aquestes diferències poden explicar-se per característiques metabòliques i fisiològiques diferents, com l'activitat mitocondrial i la taxa glicolítica (Vidament *et al.*, 2000; Miró *et al.*,2005; Maldjian *et al.*, 2005).

Per últim, un altre punt important d'aquesta Tesi, ha sigut determinar si podíem valorar la funcionalitat del semen del burro Català utilitzant l'interacció amb oòcits bovins lliures de zona pel.lúcida, com un mètode alternatiu de valoració seminal. Aquest tindira com objectiu controlar la qualitat del semen (fresc, refrigerat o congelat) utilitzat en les

inseminacions artificials. El test de penetració en oòcits bovins lliures de zona pel·lúcida ha estat utilitzat per estudiar la capacitació *in vitro* i el potencial fèrtil dels espermatozoides de cavall (Choi *et al.*, 2003). Els nostres resultats mostren índexs de penetració elevats tant en el semen fresc (90.12%) com en el semen congelat (85.4%) de burro amb alta viabilitat en comparació amb els resultats obtinguts en test de penetració de semen de cavall (Choi *et al.*, 2003; Landim-Alvarenga *et al.*, 2001). Aquesta observació pot ser deguda a diferències en la capacitat de penetració dels espermatozoides d'espècies diferents en funció de l'origen de l'oòcit, ja que s'ha observat per exemple, que els espermatozoides de toro penetren més en oòcits de hàmster que els espermatozoides de cavalls (Graham *et al.*, 1986). D'altra banda, vam buscar si existia alguna correlació entre les característiques de motilitat i viabilitat i els índexs de penetració, tal com ja s'havia observat en altres treballs de fertilitat *in vitro* (Fetterolf i Rogers 1990; Wilhelm *et al.*, 1996; Kathiravan *et al.*, 2008). Vam observar correlacions positives de l'índex de penetració amb la viabilitat, la motilitat total, la VCL, la VAP i la ALH mitjana. Tot i això, hi ha estudis que no han observat correlacions entre la motilitat i l'índex de penetració, suggerint que el test de penetració mesura característiques diferents que un anàlisi seminal convencional (Suzuki *et al.*, 1996; Gadea, 2005).

Bibliografia

Amann RP, Pickett BW. (1987) Principle of cryopreservation and a review of cryopreservation of stallion spermatozoa. J Equine Vet Sci 7:145-173.

Abaigar T, Holt WV, Harrison R, 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.

Blanchard TL, Taylor TS, Love CL. (1999) Estrous cycle characteristics and response to estrus synchronization in mammoth asses (*Equus asinus americanus).* Theriogenology 52: 827-834.

Brinsko SP, Crockett EC, Squires EL. (2000) Effect of centrifugation and partial removal of seminal plasma on equine spermatozoalmotility after cooling and storage. Theriogenology 54:129–36.

Carver DA, Ball BA. (2002) Lipase activity in stallion seminal plasma and effect of lipase on stallion spermatozoa during storage at 5 8C. Theriogenology 58:1587–95.

Choi YH, Landim-Alvarenga FC, Seidel Jr GE, Squires EL. (2003) Effect of capacitation of stallion sperm with polyvinylalcohol or bovine serum albumin on penetration of bovine zona-free or partially zona-removed equine oocytes. J Anim Sci 81, 2080–2087.

Cottorello ACP, Amancio RC, Henry M, Borges I. (2002) Effect of storage temperature and extenders on “in vitro” activity of donkey spermatozoa. Theriogenology 58:325-328.

Curry MR. (2000) Cryopreservation of semen from domestic livestock. Rev Reprod 5:46–52.

Dadarwal D, Tandon SN, Purohit GN, Pareek PK. (2004) Ultrasonographic evaluation of uterine involution and postpartum follicular dynamics in French jennies (*Equus asinus*). Theriogenology 62: 257-264.

Fetterolf PM, Rogers BJ. (1990) Prediction of human sperm penetrating ability using computerized motion parameters. Mol Reprod Dev 27, 326–331.

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

Ginther OJ, Scraba ST, Bergfelt DR. (1987) Reproductive seasonality of the jenney. Theriogenology 27:587-592.

Graham JK, Foote RH, Parrish JJ. (1986) Effect of dilauroylphosphatidylcholine on the acrosome reaction and subsequent penetration of bull spermatozoa into zona-free hamster eggs. Biol Reprod 35, 413–424.

Jasko DJ, Moran DM, Farlin ME, Squires EL. (1991) Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. Theriogenology 35: 1059-1067.

Landim-Alvarenga FC, Alvarenga MA, Seidel Jr GE, Squires EL, Graham JK. (2001) Penetration of zona-free hamster, bovine and equine oocytes by stallion and bull spermatozoa pretreated with equine follicular fluid, dilauroylphosphatidylcholine or calcium ionophore A23187. Theriogenology 56, 937–953.

Maldjian A, Pizzi F, Gliozzi T, Cerolini S, Penny P, Noble R. (2005) Changes in sperm quality and lipid composition during cryopreservation of boar sperm. Theriogenology 63:411–21.

Miró J, Lobo V, Quintero-Moreno A, Medrano A, Peña A, Rigau T. (2005) Sperm motility patterns and metabolism in Catalonian donkey semen. Theriogenology 63:1706–16.

Miró J, Muñoz F, Piedrafita J, Quintero-Moreno A. (2004) Prediction of the day of ovulation in mares through physiological parameters measured during estrous. Revista Científica, FCV-LUZ XIV(1):54-58.

Meira C, Ferreira JCP, Papa FO, Tornero MTT, Bicudo SD. (1995) Study of the estrous cycle in donkeys (Equus asinus) using ultrasonography and plasma progesterone concentrations. Biol Reprod Mono 1:403-410.

Moran DM, Jasko DJ, Squires EL and Amann RP. (1992) Determination of temperature and cooling rate which induce cold shock in stallion spermatozoa. Theriogenology 38:999-1012.

Nishikawa Y, Yamazaki Y. (1949) Studies on reproduction in asses I. Breeding season, oestrous cycle and length of heat. Jpn J Zootech Sci 19:119-124.

Kathiravan P, Kalatharan J, Edwin MJ, Veerapandian C. (2008) Computer automated motion analysis of crossbred bull spermatozoa and its relationship with in vitro fertility in zona-free hamster oocytes. Anim Reprod Sci 104, 9–17.

Koskinen E, Kuntsi H, Lindeberg H, Katila T. (1989) Predicting ovulation in the mare on the basis of follicular growth and serum oestrone sulphate and progesterone levels. J Vet Med A 36: 299-304.

Pellicer-Rubio M, Combarnous Y. (1998) Deterioration of goat spermatozoa in skimmed milk-based extenders as a result of oleic acid released by the bulbourethral lipase BUSgp60. J Reprod Fertil 112; 95-105.

Pickett BW, Sullivan JJ, Byers WW, Pace MM, Remmenga EE. (1975) Effect of centrifugation and seminal plasmaonmotility and fertility of stallion and bull spermatozoa. Fertil Steril 26:167–74.

Province CA, Squires EL, Pickett BW, Amann RP. (1985) Cooling rates, storage temperatures and fertility of extended equine spermatozoa. Theriogenology 23: 925-934.

Pukazhenthi BS, Wildt DE. (2004) Which reproductive technologies are most relevant to studying, managing and conserving wildlife?. Reproduction, Fertility and Development 16: 33-46.

Quintero-Moreno A, Miró J, Rigau T, Rodríguez-Gil JE. (2003) Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 58:1973–90.

Quintero-Moreno A, Rigau T, Rodríguez-Gil JE. (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 42:312–9.

Rigau T, Farré M, Ballester J, Mogas T, Peña A, Rodríguez-Gil JE. (2001) Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology 56:801–15.

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 69:176–85.

Serres C, Rodríguez A, Alvarez AL, Santiago I,Gabriel J, Gómez-Cuétara C, Mateos E. (2002) Effect of centrifugation and temperature on the motility and plasma membrane integrity of Zamorano-Leonés donkey semen. Theriogenology 58:329–32.

Suzuki K, Mori T, Shimizu H. (1996) Effect of the duration of preincubation on the ability of pig spermatozoa to penetrate oocytes in vitro. Anim Sci Technol Jpn 67, 24–27.

Taylor TS, Matthews NS, Blanchard TL. (2002) An overview of reproduction in Donkeys. New England Journal of Large Animal Health 2: 35-37.

Trimeche A, Tainturier D. (1995) Étude échographique de la cinétique folliculaire des baudettes du Poitou pendant l'oestrus au cours du printemps et de l'été. Revue Méd Vét 146(11):743-748.

Vandeplassche M, Wesson JA, Ginther OJ. (1981) Behavioral, follicular and gonadotropin changes during the estroud cycle in donkeys. Theriogenology 16:239-249.

Varner DD, Blanchard TL, Meyers PJ and Meyers SA. (1989) Fertilizing capacity of equine spermatozoa stored for 24 hours at 5 and 20ºC. Theriogenology 32(4):515-525.

Vidament M, Ecot P, Noue P, Bourgeois C, Magistrini M, Palmer E. (2000) Centrifugation and addition of glycerol at 22ºC instead of 4ºC improve post-thaw motility and fertility of stallion spermatozoa. Theriogenology 54:907–19.

Watson PF. (2000) The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 60–61:481–92.

Wilhelm KM, Graham JK, Squires EL. (1996) Comparison of the fertility of cryopreserved stallion spermatozoa with sperm motion analyses, flow cytometric evaluation, and zona-free hamster oocyte penetration. Theriogenology 46, 559–578.

Conclusions generals
General conclusions

Conclusions finals / General conclusions

Els resultats presentats en aquesta Tesi Doctoral ens permeten arribar a les següents conclusions finals:

1. La burra Catalana durant el zel mostra comportaments homotípics davant el burro (mastegar, cap estirat endavant amb l'extensió de les orelles enrere, immobilitat, acceptació del mascle i ocasionalment, orinar i vulvejar) i comportaments heterotípics en vers altres burres en zel (perseguir-se, muntar-se, resposta de Flehmen i vocalitzar).

2. La durada del cicle sexual de la burra Catalana és de 24.90 dies, la durada del zel de 5.64 dies i la del diestre de 19.83 dies. Les burres Catalanes es mostren gairebé com a femelles polièstriques contínues.

3. El diàmetre mig del fol·licle preovulatori és de 44.9 mm amb un creixement mig de 3.7 mm/dia i sense diferències significatives en cas d'ovulació doble. La freqüència d'ovulacions múltiples és elevada (44.34%).

4. El diàmetre fol·licular, la simptomatologia de zel i la textura del fol·licle estadísticament són els únics paràmetres útils en la predicció de l'ovulació. Diverses combinacions en els valors obtinguts per aquests paràmetres ofereixen possibilitats d'ovulació superiors al 75% en les 24h següents.

5. L'eliminació del plasma seminal, a través de la dilució o la centrifugació incrementa la supervivència dels espermatozoides i manté les característiques de motilitat del semen de burro Català. A més, la centrifugació del semen és l'opció

que manté exactament la mateixa proporció de cada subpoblació espermàtica que en les mostres fresques.

6. El semen de ruc Català es pot mantenir en bones condicions durant 2h a 20ºC i després centrifugar amb una viabilitat i una estructura subpoblacional espermàtica similar al semen fresc. Tot i que, si fos necessari es podria refrigerar i centrifugar a 5ºC obtenint també bons resultats de conservació de les característiques seminals.

7. El procés de congelació/descongelació produeix canvis en la motilitat espermàtica lligats a canvis específics, tant en els paràmetres de motilitat com en els percentatges de cada subpoblació espermàtica, en dues espècies de mamífers com el burro i el porc. Ara bé, aquest procés no varia l'estructura de 4 subpoblacions espermàtiques, suggerint que aquesta juga un paper important a l'hora de mantenir la funcionalitat de l'ejaculat.

8. Els espermatozoides de ruc Català són capaços d'unir-se a oòcits bovins lliures de zona pel·lúcida i formar el pronucli masculí. Alguns paràmetres de motilitat (VAP, VCL, motilitat total, ALH mitjana) i la viabilitat estan correlacionats amb l'índex de fecundació *in vitro*. El test de penetració heteròloga en oòcits bovins ens aporta un model útil per valorar el potencial de penetració del semen congelat de ruc Català.