universidad de león

FACULTAD DE VETERINARIA

DEPARTAMENTO DE MEDICINA, CIRUGÍA Y ANATOMÍA VETERINARIA

ESTUDIO DE LA CONGELABILIDAD DE EYACULADOS DE OSO PARDO (*Ursus arctos*)

Study of freezability of brown bear ejaculated (*Ursus arctos*)

SUSANA CLÁUDIA GOMES ALVES

León, 2014

UNIVERSIDAD DE LEÓN

FACULTAD DE VETERINARIA

DEPARTAMENTO DE MEDICINA, CIRUGÍA Y ANATOMÍA VETERINARIA

ESTUDIO DE LA CONGELABILIDAD DE EYACULADOS DE OSO PARDO (*Ursus arctos*)

Study of freezability of brown bear ejaculated *(Ursus arctos)*

Memoria que presenta para optar al grado de Doctor la Licenciada en Medicina Veterinaria

SUSANA CLÁUDIA GOMES ALVES

León, 2014

universidad de león

Estudios de Doctorado

INFORME DE LOS DIRECTORES DE LA TESIS

(R.D. 534/2013 y Normativa de la ULE)

Los Doctores D. Luis Anel Rodríguez y Dª Mercedes Alvarez García, como Directores de la Tesis Doctoral titulada ***"Estudio de la congelabilidad de los eyaculados de oso pardo (Ursus arctos)"*** realizada por Dª Susana Cláudia Gomes Alves en el programa de doctorado 099 Sanidad Animal y Reproducción, en el Departamento de Medicina, Cirugía y Anatomía Veterinaria, informan favorablemente el depósito de la misma, dado que reúne las condiciones necesarias para su defensa.

Lo que firmamos, en León a 25 de OCTUBRE de 2014

Dr. Luis Anel Rodríguez	**Dra. Mercedes Álvarez García**

FINANCIACIÓN:

MICINN (CGL2010-19213/BOS) y MINECO (CGL2013-48255-R), CANTUR S.A y Ministerio Portugués de la Ciencia y Tecnología (SFRH/BD/45522/2008), cofinanciado por POPH-QREN-Tipología 4.1 (FEDER and MCTES)

AGRADECIMIENTOS

A mis directores, Luis Anel y Mercedes Álvarez, muchas gracias por haberme dirigido la Tesis, por vuestra ayuda y dedicación.

A Luis, te agradezco por incentivarme a hacer este proyecto, por proporcionarme todos los medios necesarios y por la confianza. Creo que aparte de todo esto (incluidas las broncas) me llevo un gran amigo. A Merce, que decirte…tantas horas, tantas alegrías y tantos disgustos compartidos…Muchas gracias amiga, por la compañía y ánimo, principalmente en esta fase final. A los dos, gracias por compartir vuestra sabiduría y experiencia profesional y personal conmigo, he aprendido mucho con vosotros!

A Paulino de Paz, por su disponibilidad, consejos y dedicación para que este trabajo fuera posible. Gracias por la paciencia.

A la Fundación para la Ciencia y Tecnología agradezco la financiación de la beca de doctorado SRFH/BD/45522/2008, sin la cual el desarrollo de este trabajo no hubiera sido posible.

A Santiago Borragán, por el cariño y apoyo demostrado a lo largo de estos años. Admiro tu fuerza, para mí es un orgullo disfrutar de tu amistad.

Marañón y Prieto, dos amigos que me encontré en Cabárceno, gracias por proporcionarme momentos únicos.

Patri…a ti te agradezco porque me has acogido, me has reñido, me has ayudado cuando lo he necesitado, o sea, como una “madre”! No te preocupes que lo nuestro no acabara aquí, eres una de las personas que me llevo para la vida….

A Juan Carlos Boixo, gracias por la disponibilidad, paciencia y compañía…Eres un buen amigo.

A todos los compañeros que me he encontrado en la universidad: Vanesa, Felipe, Jana, Maria Mata, Leticia, Manu, Julio, Patricia Manrique, recuerdo con cariño todos las horas que hemos pasado juntos, gracias por vuestra ayuda. A Enrique, por su ayuda y amistad. Nerea, fuiste de las primeras personas que me encontré en León y desde un primer momento intentaste que me sintiera "en casa"! Con paciencia y algunas bromas me has ayudado a soltarme con el español, a pesar de los "portuguesismos"…y a enterarme de las tareas del departamento. Gracias por tu amistad y hospitalidad. Luisete, no puedo llamarte de otra manera… gracias por hacer más amenas y divertidas las horas de trabajo que hemos compartido. A María Nicolás, por los buenos momentos que hemos pasado juntas, comidas, veladas, confidencias, inquietudes, consejos y horas al ordenador…gracias por la compañía y por tu ayuda. Elena te mereces un agradecimiento especial por la manera como intentas ayudar a los demás…Siempre de una forma tan desinteresada y paciente, me has ayudado a solucionar muchos de los problemas que me han surgido en esta Tesis. Muchas gracias! Carmen, gracias por tus palabras de ánimo y de confianza. Espero que la vida te sonría…

Ana Solas, nuestros caminos se han cruzado en León y partir de allí en otros sitios…gracias por tu amistad.

A los amigos veterinarios de campo, Jesús y Nico por su amistad y por compartir su experiencia profesional conmigo.

Al personal del IPB-Bragança agradezco la disponibilidad y el apoyo.

A mis compañeros de la Clínica en Bragança, agradezco su disponibilidad, su comprensión y sus palabras de ánimo. Gracias por aguantarme ahora en la recta final!

A todos mis otros amigos…A Susana Matias agradezco su amistad y cariño. A mis amigas de siempre, Nádia y Andrea quiero agradecer por hacerme sentir especial y no dejar que la distancia se imponga sobre nuestra amistad. Gracias por la seguridad y amistad incondicional.

A mis abuelos paternos, que recuerdo con mucha "saudade", quería agradecerles por hacerme vivir una infancia muy feliz. A mis abuelos maternos agradezco su preocupación, su dedicación y su cariño a lo largo de los años, me siento afortunada por poder disfrutar de vuestra compañía… Al resto de la familia gracias por aguantarme y por ayudarme… un beso a todos!

A mis hermanos, con quien tanto he compartido, dedico parte de este trabajo para demostrarles que en la vida nada es posible sin esfuerzo. Gracias por la complicidad y confianza, tengo mucho orgullo en ser vuestra "hermanita mayor".

A mi otra familia gracias por hacerme sentir tan querida. A Nucha, mi amiga y confidente en los momentos más difíciles de mi vida, gracias simplemente por saber escucharme, por tus palabras siempre tan reconfortantes. Al resto de la familia, Carolina, Bernardo y Zé Luis, gracias por vuestro cariño. A "Tia Lurdes" por su preocupación por los demás. Un agradecimiento muy especial al padre de Miguel, a quien recuerdo con "muita saudade" y admiración…y que siempre creyó en este proyecto.

A la familia Salgado agradezco su amistad y cariño desde el primer momento. Hay pocas personas como vosotros…

A mis padres a quien tanto tengo que agradecer… Gracias por proporcionarme mis estudios y una vida tan feliz! Gracias por los mimos que tantas veces me calman el corazón…Os admiro y os quiero mucho…

A Miguel, por el cariño, apoyo e insistencia…gracias por estar siempre a mi lado, por compartir buenos y malos momentos y ayudarme a superar esta etapa. Espero seguir luchando a tu lado por una vida más tranquila, creo que lo merecemos…

A todos, muchas gracias por hacer parte de mi vida, cada uno de vosotros, a su manera ha contribuido a que cierre esta etapa de mi vida.

A mis padres

A Miguel

TABLA DE CONTENIDO

INTRODUCCIÓN GENERAL

La influencia creciente de las actividades humanas sobre la biodiversidad del planeta Tierra es un hecho cada vez más patente que está alterando muchos de los ecosistemas. La ruptura de este equilibrio en la diversidad biológica, debido a unas interacciones sesgadas entre el hombre y el resto de las especies hace que muchas de ellas se encuentren en riesgo o muy seriamente amenazadas. El oso pardo *(Ursus arctos),* a pesar de no estar catalogado como especie en peligro a nivel mundial (IUCN 2008) debido a su extensa representación en Rusia, Canadá, Alaska y algunas regiones de Europa, en España está considerado en peligro de extinción (Real Decreto 439/1990 de la Ley Española, regulada en el Catálogo Nacional de las especies amenazadas) y catalogado como En Peligro Crítico, por el criterio D (IUCN 2006). Esta población de oso pardo se encuentra dividida en dos pequeños núcleos (200 osos en el núcleo occidental y 30 en el oriental (Fundación Oso Pardo -FOP-, 2014), que habitan en la Cordillera Cantábrica. Los factores decisivos que han contribuido a la situación crítica del oso pardo son el furtivismo y la pérdida de hábitat (Purroy, 2008). Con el fin de mejorar esta problemática se ha instaurado una Estrategia para la conservación del oso pardo cantábrico (Comisión Nacional de Protección de la Naturaleza, 2001).

En este sentido, los bancos de recursos genéticos (BRG) se presentan como una herramienta muy útil para la preservación de especies amenazadas (Holt and Pickard. 1999; Yoshida. 2000). El almacenamiento de biomateriales (espermatozoides, óvulos, embriones y células somáticas) en los BRG, así como su aplicación mediante técnicas de reproducción asistida suponen una estrategia fundamental en la conservación de los potenciales genéticos de determinadas especies que no se encuentran en riesgo de extinción, así como en el mantenimiento de la biodiversidad en poblaciones específicas

cuya supervivencia se encuentra amenazada. En este contexto, considerando la gran variabilidad que existe entre las características fisiológicas de los espermatozoides de las distintas especies de mamíferos salvajes (Leibo and Songsasen. 2002); en este trabajo nos planteamos estudiar diferentes aspectos de la congelabilidad de los espermatozoides del oso pardo, que permitan desarrollar estrategias de conservación eficaces para los gametos masculinos de esta especie, mejorando la calidad del material almacenado y en consecuencia el rendimiento del banco de germoplasma. Como punto de partida, se propone la adaptación de un diluyente de congelación estándar (TesT-Fructosa-Glicerol-Yema) a las particularidades de los espermatozoides de oso pardo. Dicho diluyente estándar, ha sido adaptado previamente a las características del semen de carnero por nuestro grupo de investigación (Anel et al. 2003).

Estudios previos indican que los espermatozoides de oso pardo son bastante resistentes al proceso de congelación (Anel et al. 2008); no obstante la adaptación de un protocolo de criopreservación implica el estudio de los diversos factores que pueden afectar el éxito del proceso de criopreservación (Gilmore et al. 1998), siendo particularmente prioritario en este contexto el diseño del medio de congelación.

Algunos autores han referenciado, con buenos resultados, el uso de diluyentes diseñados para otras especies en la conservación de semen de oso: Tris-buffered-yema de huevo en oso pardo de Hokkaido (Ishikawa et al. 2002), Tris-ácido cítrico-glucosa-yema de huevo en oso negro japonés (Okano et al. 2004), yema de huevo-lactosa (Seager et al. 1987) o TES-Tris-yema de huevo (Spindler et al. 2004; Zhang et al. 2005) en panda gigante. Sin embargo, dado el alto valor biológico y estratégico de las muestras espermáticas obtenidas de individuos de especies silvestres amenazadas, es

de extrema importancia optimizar el proceso de conservación, y entre otros aspectos fundamentales el rendimiento tras la descongelación de los medios de diluyoconservación empleados.

Un diluyente debe incluir en su composición soluciones tampón, crioprotectores, azúcares y otros aditivos que aparte de proporcionar sustrato energético, deben proteger a los espermatozoides de los cambios de osmolalidad y de pH a lo largo de las distintas fases del proceso de congelación espermática (Salamon and Maxwell. 2000). Además, se consideran como componentes básicos los protectores de membrana y crioprotectores, que van a jugar un papel esencial en la reversión eficaz del proceso de congelación espermática. En este sentido, tanto la yema de huevo como el glicerol son componentes habituales de los diluyentes usados en la mayor parte de los mamíferos. La yema de huevo es un estabilizador de membrana que ofrece protección a las células durante el proceso de congelación; sin embargo, algunos estudios revelan que este componente puede tener un efecto prejudicial sobre la movilidad e integridad acrosomal durante la criopreservación (Holt et al. 1996) y también en la viabilidad espermática (Ritar AJ. 1991). En la criopreservación de espermatozoides de úrsidos se han empleado concentraciones de yema entre el 10% (García-Macías et al. 2005) y el 20% (Okano et al. 2006; Anel et al. 2008) con resultados aceptables.

El glicerol es un crioprotector ampliamente utilizado en la criopreservación espermática; no obstante su empleo a altas concentraciones puede tener un efecto tóxico (Fahy. 1986) sobre las células, variable según la especie. En este sentido, es importante investigar el efecto de diferentes concentraciones de glicerol, en la conservación de espermatozoides de oso pardo para evaluar la tolerancia de esta especie al agente crioprotector. En la criopreservación de semen de oso, la

concentración de glicerol utilizada en los diluyentes varía entre el 4% y el 8% en las diferentes especies de oso (Seager et al. 1987; Anel et al. 1999; Ishikawa et al. 2002; Okano et al. 2004; Spindler et al. 2004; Zhang et al. 2005) con resultados aceptables.

La osmolalidad del medio es otro punto fundamental que puede ser decisivo para el éxito del proceso de congelación. El ajuste de la osmolalidad del diluyente es muy importante por su influencia en la deshidratación, rehidratación y formación de cristales intracelulares, procesos a los que son sometidos los espermatozoides durante la criopreservación espermática (Cheng et al. 2004).

Aún considerando como prioritaria la formulación de un diluyente específico para la preservación del semen de oso pardo, en el concepto de desarrollo de biotecnologías reproductivas (IA) en especies sin estudios previos, es importante la validación de diluyentes comerciales diseñados para otras especies; esta alternativa puede tener especial interés en situaciones imprevistas en las que puede no existir disponibilidad de un diluyente específico o la infraestructura necesaria para su elaboración. Diversos diluyentes comerciales diseñados para bovino y equino han sido utilizados en la criopreservción de semen de otras especies. En búfalo africano, Herold et al. (2004) han empleado el Triladyl® y el Andromed® en la congelación de espermatozoides epididimarios. Diluyentes comerciales de caballo (Gent®) y de bovino (Andromed® y Triladyl®) también han sido utilizados para congelar espermatozoides epididimarios de gato (Jimenez et al., 2013). En ambos estudios el Triladyl® presentó mejores resultados post-descongelación. Del mismo modo el uso de Andromed® en la congelación de espermatozoides epididimarios de perro mostró ser menos eficiente que el Biladyl suplementado con Equex STM Paste (Nothling, J.O. 2007). Martinez-Pastor et al (2006) demuestran que tanto el Andromed® como el Triladyl® pueden ser usados en ciervo

ibérico con resultados satisfactorios, al contrario de lo observado con el Bioxcell® que parece no ser efectivo en esta especie.

Además de la adecuación de los componentes básicos, y con el objetivo de desarrollar un diluyente optimizado, se deben considerar algunas de las particularidades de los eyaculados de oso pardo como son la espermioaglutinación y la contaminación con orina que se producen asociadas a la electroeyaculación en los machos de esta especie y que pueden inducir variaciones en la capacidad de resistencia espermática al proceso de congelación.

La aglutinación espermática, observada en un alto porcentaje de eyaculados de oso (Anel et al. 2008 en oso pardo, Kojima et al. 2001 en oso negro japonés) dificulta la manipulación de la muestra espermática (ej. cálculo de la concentración espermática) y podría afectar a la calidad post-descongelación de la misma. Sin embargo, estudios realizados en cerdo refieren que la aglutinación no interfiere con los resultados de fertilidad (Bollwein et al. 2004).

La aglutinación (cabeza-cabeza) puede resultar de una reacción ATP-dependiente de la superficie espermática activada por cationes divalentes (Ca^{2+}, Mg^{2+} y Mn^{2+}) y por la formación de cAMP. Alternativamente, también se ha sugerido que este fenómeno puede estar relacionado con la eliminación de anti-aglutinina de la superficie espermática inducida por la acción de la albumina sérica porcina y el methyl-beta-cyclodextrin (MBC) contenidos en el medio (Harayama et al., 1998, 2000).

Una de las alternativas para minimizar este problema sería la adición de un agente surfactante (Equex STM Paste) y de un agente quelante de calcio (EDTA) al diluyente de congelación. Estudios en diversas especies, demuestran que la incorporación de estos aditivos en el diluyente seminal aporta una mejoría en

diferentes parámetros de calidad de las muestras seminales. Los beneficios de la incorporación de Equex STM Paste en los diluyentes de congelación seminal se traducen por una mejoría en la movilidad (Alhaider and Watson. 2009), viabilidad (Rota et al. 1997) y longevidad (Pena et al. 2003) post-descongelación, en eyaculados de perro. Del mismo modo, Ponglowhapan and Chatdarong (2008) demuestran que el suplemento del diluyente seminal con Equex STM Paste confiere mejor protección al acrosoma frente a los daños producidos por la congelación y prolonga la movilidad espermática post-descongelación durante la incubación in vitro a 37ºC. La incorporación del Equex STM paste al diluyente de congelación, también protege los acrosomas de los espermatozoides epididimarios de gato durante el proceso de congelación-descongelación (Axner et al, 2004). Además Aisen et al. (2000), observan que el uso de diluyentes con EDTA en semen de carnero determina un porcentaje más elevado de espermatozoides con acrosomas intactos en post-descongelación.

Por otra parte, en estudios realizados en eyaculados de verraco, se ha observado que la aglutinación puede disminuir (o desaparecer) tras una dilución inicial (Bollwein et al. 2004) por lo que la recogida de semen sobre un diluyente específico podría ser una posibilidad para mejorar la calidad inicial con la disminución de la incidencia de la espermioaglutinación.

Otro de los problemas descritos en los eyaculados de oso obtenidos por electroeyaculación es la contaminación con orina y su importancia deriva de su interferencia en la congelabilidad espermática (Anel et al. 2008). Hay especies en que la contaminación del eyaculado con orina ocurre frecuentemente de forma espontánea (caballo: Althouse et al. 1989 y hombre: Chen et al. 1995), mientras que en otras la contaminación se presenta consecuentemente a la electroeyaculación (oso: Kojima et

al. 2001, Okano et al. 2004, Chen et al. 2007, Anel et al. 2008; felinos: Pukazhenthi et al. 2000; cabra montés: Santiago-Moreno et al. 2011). En estas situaciones, la urospermia es provocada por la estimulación involuntaria de las terminaciones nerviosas que controlan la micción (funcionalidad de la vejiga) durante la electroeyaculación (Santiago-Moreno et al. 2011).

Diversos estudios revelan una elevada incidencia de contaminación con orina en los eyaculados de oso obtenidos por electroyaculación. En oso pardo, en experiencias previas de nuestro grupo, se observó la contaminación con orina en un 70% de los eyaculados, sin embargo la aplicación de algunas medidas preventivas permitieron reducir esta incidencia hasta un 35% (Anel et al. 2008). Estudios en otros úrsidos confirman la elevada frecuencia de este problema: oso negro Japonés, 77% (Kojima et al. 2001) ó 100% (Okano et al. 2004) de los eyaculados contaminados con orina; oso negro Asiático se observó contaminación en un 58% los eyaculados (Chen et al. 2007).

Los efectos nocivos de la orina sobre las células espermáticas están relacionados con los cambios de osmolalidad y pH, así como con los efectos tóxicos de la urea y otros componentes urinarios (Griggers et al. 2001). La contaminación con orina perjudica la movilidad espermática (Kim and Kim. 1998), la integridad acrosomal (Santos et al. 2011) y reduce la capacidad fecundante del semen (Santos et al. 2011).

Algunos estudios en diferentes especies, demuestran que las condiciones hiposmóticas son más nocivas que las condiciones hiperosmóticas en cuanto a la movilidad (carnero, toro y humano: Blackshaw and Emmens. 1951) y a la viabilidad espermática (caballo: Pommer et al. 2002).

Estudios llevados a cabo en caballo muestran que la exposición de los espermatozoides a condiciones no isosmóticas induce daños irreversibles en la

membrana plasmática y en las mitocondrias tras el retorno a condiciones isotónicas (Gonzalez-Fernandez et al. 2012).

La tolerancia al estrés osmótico, así como la capacidad de recuperación de la movilidad espermática tras la exposición a condiciones no isosmóticas es muy variable según la especie (toro: Guthrie et al. 2002; antílope bongo: Wirtu et al. 2008; caballo: Griggers et al. 2001).

Okano et al. (2004) refiere que la contaminación de los eyaculados de oso con orina expone los espermatozoides a condiciones hiperosmóticas y que la reversión de esas condiciones produce daños poco significativos a nivel de la membrana espermática. Contrariamente, otros autores han encontrado serios daños en la integridad de la membrana espermática tras revertir la hipotonicidad (gato: Pukazhenthi et al. 2000).

En los casos de urospermia, son los cambios de osmolalidad los que más perjudican a la movilidad espermática, no obstante las alteraciones de pH también pueden ser nocivas. La reducción de la motilidad espermática es más acentuada en condiciones ácidas que en las alcalinas (Makler et al. 1981), pero este efecto es reversible en pH ácidos que vuelven a niveles normales y sin embargo irreversible para pH alcalinos.

Teniendo en cuenta la elevada incidencia de la urospermia en los eyaculados de oso pardo y el alto valor de las muestras seminales de esta especie amenazada, es de primordial importancia definir estrategias que permitan revertir los efectos deletéreos de la contaminación del semen con orina. Por otra parte, la utilización de un diluyente de selección espermática podría mejorar la calidad de las muestras contaminadas. Estudios previos de nuestro grupo, con eyaculados de oso pardo no contaminados con

orina demostraron la eficiencia del Puresperm® en la selección de poblaciones espermáticas de mejor calidad en semen fresco y semen congelado-descongelado (Nicolas et al. 2012b) y recongelado (Alvarez-Rodriguez et al. 2013b).

En resumen, la eficacia de un protocolo de criopreservación pasa por el desarrollo de un diluyente específico adaptado a las características de los espermatozoides de oso pardo (en este caso); no obstante la validación de diluyentes comerciales en esta especie supone una alternativa muy útil y práctica en situaciones de emergencia en las que no se disponga de la infraestructura adecuada para la preparación del diluyente específico. Además hay que tener en cuenta otros factores que se observan frecuentemente en los eyaculados de oso pardo obtenidos mediante electroeyaculación, como son la urospermia y la espermaglutinación, que pueden afectar a la congelabilidad de las muestras y por ello es importante desarrollar estrategias que permitan minimizar su efecto deletéreo sobre la calidad de las muestras seminales descongeladas.

OBJETIVOS

En el contexto de la creación de un banco de recursos genéticos para el oso pardo (de las montañas Cantábricas), el objetivo general de esta tesis doctoral es el estudio de diferentes aspectos de la congelabilidad seminal en esta especie con el fin de desarrollar una metodología de criopreservación espermática adaptada a las características específicas de los eyaculados de oso pardo obtenidos mediante electroeyaculación.

Para ello, se proponen los siguientes objetivos específicos que han sido desarrollados en las publicaciones que se indican:

1. Desarrollo de un diluyente específico para la criopreservación de eyaculados de oso pardo.

"Effect of basic factors of extender composition on post-thawing quality of brown bear electroejaculated spermatozoa". Theriogenology 74 (2010) 643-651.

2. Evaluación de diferentes diluyentes comerciales de congelación espermática empleados en otras especies como alternativa al diluyente específico.

"Use of commercial extenders and alternatives to prevent sperm agglutination for cryopreservation of brown bear semen". Theriogenology 82 (2014) 469–474.

3. Estudio de estrategias para mejorar la calidad de las muestras problemáticas de semen de oso pardo (urospermia y espermaglutinación).

"Use of commercial extenders and alternatives to prevent sperm agglutination for cryopreservation of brown bear semen". Theriogenology 82 (2014) 469–474

"Salvaging urospermic ejaculates from brown bear (Ursus arctos)". Animal Reproduction Science (2014). http://dx.doi.org/10.1016/j.anireprosci.2014.09.007

PUBLICACIONES

Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 74 (2010) 643–651
Theriogenology
www.theriojournal.com

Effect of basic factors of extender composition on post-thawing quality of brown bear electroejaculated spermatozoa

L. Anel[a], S. Gomes-Alves[a], M. Alvarez[a], S. Borragan[c], E. Anel[a], M. Nicolas[a], F. Martinez-Pastor[d], P. de Paz[b,*]

[a] *Animal Reproduction and Obstetrics, University of León, 24071 León, Spain*
[b] *Cell Biology, University of León, 24071 León, Spain*
[c] *Cabarceno Park, Cantabria, Spain*
[d] *INDEGSAL and Molecular Biology, University of León, 24071 León, Spain*

Received 28 November 2009; received in revised form 1 March 2010; accepted 7 March 2010

Abstract

The improvement of freezing extenders is critical when defining sperm cryopreservation protocols for wild species, in order to create germplasm banks. The aim of this study was to evaluate the effect of additives (Equex Paste and EDTA) supplementation, egg-yolk (10 and 20%) and glycerol (4 and 8%) concentrations and extender osmolality (300 and 320 mOsm/kg) on the post-thawing quality of brown bear semen. Semen was obtained from 20 adult males by electroejaculation, and centrifugated individually (600 × g for 6 min). The pellets were diluted 1:1 in the corresponding extender TTF (TES-Tris-Fructose with the aforementioned variants) and cooled to 5 °C. Then, it was diluted down to 100×10^6 spz/mL, loaded in 0.25 mL straws and frozen at −20°C/min. After thawing (in water at 65 °C for 6s), the semen samples were assessed for motility (CASA), viability (SYBR-14 with propidium iodide), acrosomal status (PNA-FITC with propidium iodide) and mitochondrial activity (JC-1). Extender supplementation with additives rendered significantly higher results for these sperm parameters. Comparing the two percentages of egg yolk, 20% egg yolk showed the highest motility results, percentages of viable spermatozoa and viable spermatozoa with intact acrosome. No differences were detected among samples frozen using 4 or 8% glycerol. For extender osmolality, 300 mOsm/kg showed higher values of VAP, VCL, VSL, and ALH than 320 mOsm/kg. Based on the best performance of sperm motility, viability and acrosome status, we conclude that the most suitable extender to cryopreserve brown bear spermatozoa was TTF adjusted to 300 mOsm/kg, supplemented with 20% egg yolk, 4–8% glycerol, and the additives 1% Equex paste and 2% EDTA.

Keywords: Brown bear; Cryopreservation; Spermatozoa; Extender; Germplasm banks

1. Introduction

Genetic resource banks (GRBs) are a valuable tool for the conservation of threatened or endangered species and valuable breeds [1,2]. Some populations of brown bear are currently endangered, and are a possible target for germplasm banking in order to aid their protection. This could be the case of grizzly bears (*Ursus arctos horribilis*), considered threatened outside of Alaska by the Threatened Species Conservation Act of the USA and Cantabrian brown bear (*Ursus arctos*), which is considered to be at risk of extinction (Royal Decree 439/1990 of the Spanish law, regulation of the

* Corresponding author: Tel/fax: +34 987291320.
E-mail address: ppaze@unileon.es (P. de Paz).

0093-691X/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2010.03.004

National Catalogue of Endangered Species), and only survives in two small isolated populations in the Cantabrian mountains (Northern Spain).

Sperm cryopreservation is usually the first approach in establishing a germplasm bank for the preservation of this species [3]. However, the most important challenge for establishing GRBs is the lack of knowledge on specific sperm traits, which are critical for an adequate protocol design [4].

According to Anel et al. [5], brown bear sperm can be considered that freezes well. The adjustment of a cryopreservation protocol requires the study of several factors affecting the success of the freezing process [6] particularly with regard to the formulation of a specific extender to the characteristics of brown bear ejaculates. There are a few reports on bear semen cryopreservation employing extenders already used in other species, which rendered good results: Tris-buffered-egg-yolk in Hokkaido brown bear sperm [7], Tris-citric acid–glucose-egg yolk in Japanese black bear sperm [8], egg yolk-lactose [9] or TES-Tris-egg yolk [10,11] in giant panda sperm. Nevertheless, due to the great biological value and scarcity of sperm samples obtained from wild, endangered animals, it is necessary to optimize these extenders for the species, in order to improve post-thawing sample quality [5].

Extender composition includes buffer systems, cryoprotectants, sugars, and other additives, which are supposed to protect spermatozoa, providing energy substrates and preventing deleterious effects of changes in pH and osmolality [12,13]. Among the most common components of semen diluents for mammals are egg yolk and glycerol. Egg yolk is beneficial to sperm cryopreservation because it protects against cold shock [12], and has been included routinely in most extenders for frozen semen from domestic animals as well as several wild species at different concentrations (in Cuvier's gazelle semen from 5% to 20% [14]). However, several studies revealed that higher egg yolk concentrations in some species negatively affected preservation of motility and acrosome integrity during cryopreservation [15] or reduce post-thawing viability [16]. In bear sperm, egg yolk has been used at different concentrations: 15% [7] or 20% [17].

Concentrations of glycerol ranging from 2 to 10% have been used to cryopreserve mammalian spermatozoa. Glycerol, despite its value as cryoprotectant, can be toxic to spermatozoa at high concentrations [18]. For bear sperm cryopreservation, glycerol is the main cryoprotectant used in extenders and the cryoprotective effect of concentrations from 4% to 8% has been assayed in different bear species [7,8,9,10,11,19].

To develop an optimal extender, we must also consider the special characteristics of brown bear ejaculates. A high incidence of sperm agglutination was documented by Kojima et al. [20] and Anel et al. [5]. Agglutination makes semen assessment and handling difficult and can influence sperm freezability [5], although it has been reported that it did not interfere with fertility results in boars [21]. Therefore, the extender composition needs to be improved in order to reduce this problem. One of the options explored in the present study was the addition of a surfactant (Equex paste) and a calcium chelator (EDTA) to the freezing diluents. Studies in other species have shown the beneficial effects of these substances on semen: dog [22–25], cat [26], boar [27], stallion [28], bull [29], and deer [30].

Media osmolality is another important factor that can influence the outcome of the cryopreservation process. The adjustment of extender osmolality is of great importance because of its influence on the dehydration, rehydration, and intracellular crystal formation to which spermatozoa are subjected during the cryopreservation process [30].

Anel et al. [32] suggested that an extender used for ram semen freezing (TES-Tris-fructose-10% egg-yolk-4% glycerol) rendered good results for motility and viability of brown bear spermatozoa. In fact, this extender was successfully used for freezing semen from different wild species: somalia wild ass [33], Iberian red deer [34], blue wildebeest [35]. In this regard, based on a preliminary study carried out by our group in brown bear ejaculates with the above mentioned extender, we propose to assay four variants of that extender, aiming at adjusting its composition to the characteristics of brown bear ejaculates. In a first experiment we conducted three different assays to evaluate: the use of Equex paste and EDTA, two concentrations of egg-yolk (10 and 20%) and two glycerol concentrations (4 and 8%). In a second experiment we carried out a study of the use of two extenders with different osmolality (300 mOsm/kg and 320 mOsm/kg).

2. Material and methods

All the chemicals were at least of reagent grade, and they were obtained from Sigma (Madrid, Spain), unless otherwise stated.

2.1. Animals and sample collection

Animal handling was performed in accordance with Spanish Animal Protection Regulation RD223/1998, which conforms to European Union Regulation 86/609. Sixty semen samples from 20 sexually mature male brown bears were obtained by electroejaculation during the breeding season (end of April to early July). The animals were housed in a half-freedom regime in Cabarceno Park (Cantabria, Spain; 43° 21′ N, 3° 50′ W; altitude: 143 m), and fed on a diet based on chicken meat, bread, and fruits.

The males were immobilized by teleanaesthesia, using zolazepam HCl, tiletamine HCl (Zoletil100®; Virbac, Carros, France) 7 mg/kg, and ketamine (Imalgene 1000®; Rhone-Mérieux, Lyon, France) 2 mg/ kg. After immobilization, the males were weighed and monitored (pulse, oxygen saturation, and respiration). Prior to electroejaculation, the prepucial area was shaved and washed with physiological saline serum, and the rectum was emptied of feces. The bladder was catheterized during semen collection to prevent urine contamination. Electroejaculation was carried out with a PT Electronics® electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were given until ejaculation (10 V and 250 mA, on average). The ejaculates were collected by fractions in graduated glass tubes. The fresh semen samples of brown bear yielded an average number of spermatozoa of 423.0 ± 53.7 × 10^6 (mean ± SD). All ejaculates used in the present study have been selected to be urine-free, as explained below.

2.2. Experimental design

Semen cryopreservation was performed according to Anel et al. [32]: extender TES-Tris-Fructose with egg yolk, glycerol and antibiotics; cooling rate: −0.25 °C/min and freezing rate: −20 °C/min.

First, three different assays were carried out to test the addition of additives (Equex paste and EDTA), two glycerol concentrations and two egg-yolk concentrations, respectively. The effect of osmolarity was then evaluated in a fourth assay. The scarce number of total spermatozoa obtained by ejaculated, due to the loss of cells during centrifugation and the number of cells necessary for semen evaluation, made it impossible to design a multifactorial experiment to study these four parameters (additives, glycerol, egg-yolk, and osmolality). All semen samples were evaluated and frozen individually.

2.2.1. Experiment 1

This experiment was based on previous data that we obtained for brown bear ejaculates and on bibliographic reports [7,8,10,22], therefore we used as base an extender composed by TES-Tris–Fructose [32]. In each assay, we used this extender analyzing two different combinations of each basic component: assay 1.1: addition or not of additives (1% Equex paste and 2% EDTA); assay 1.2: two percentages of egg-yolk (10-20%) and assay 1.3: two glycerol concentrations (4-8%). Antibiotics (penicillin and streptomycin) were added to each extender formulation. In all the assays, semen was divided in two aliquots to evaluate each combination.

Assay 1.1: Additive supplementation

Fifteen ejaculates obtained from eleven different bears were studied to evaluate the benefits of Equex paste (Minitüb, Germany) and EDTA added to the sperm cryopreservation extender. Ejaculates were divided in two aliquots (in all assays) and frozen either in the TTF extender [with 20% egg-yolk and 8% glycerol at 320 mOsm/kg] supplemented with additives or without additives (A and NA, respectively).

Assay 1.2: Percentages of egg-yolk

To determine the best concentration of egg-yolk in the cryopreservation extender we used fourteen brown bear semen samples from seven males, which were frozen in the TTF extender [with 8% glycerol and 1% Equex paste + 2% EDTA at 320 mOsm/kg] supplemented with 10% egg-yolk (Y10) or with 20% egg-yolk (Y20).

Assay 1.3: Glycerol concentrations

The effect of two glycerol concentrations on the post-thawing quality of brown bear spermatozoa was evaluated. Eleven ejaculated sperm samples were collected from 10 different bears and cryopreserved with each of two extenders: TTF [with 20% egg-yolk and 1% Equex paste + 2% EDTA at 320 mOsm/kg] supplemented with 4% glycerol (G4) or with 8% glycerol (G8).

2.2.2. Experiment 2

According to our results in Experiment 1, we defined an optimal extender: TTF, 20% egg-yolk, 8% glycerol and 1% Equex paste + 2% EDTA, which was applied in a final assay to test the effect of osmolality.

Assay 2.1: Extender osmolality

Twenty ejaculates from fifteen males were used to test the effect of extender osmolality on brown bear sperm cryopreservation. Samples were processed with TTF above mentioned either adjusted to 300 or to 320 mOsm/kg (E300 and E320, respectively).

2.3. Semen cryopreservation

Immediately after collection, the volume of each ejaculate was recorded, osmolality was measured using a cryoscopic osmometer (Osmomat-030, Gonotec ™; Berlin) and the pH value was determined by pH meter (CG 837-Schott; Germany). For each ejaculate, sample motility was assessed with a phase contrast microscope and urospermia was evaluated by means of a rapid urea test (Merckgnost Urea Rapid Screening test, Merck, Barcelona, Spain). Samples of low motility (<50%) or urine contaminated samples (>80 mg urea/dL) were rejected [36]. The selected samples were divided into two aliquots and centrifuged at 600 × g during 6 min. The supernatant was discarded and each pellet was diluted with an equal volume of the corresponding extender, depending on the experiment, at room temperature. Tubes with the diluted semen were put in glasses containing 100 ml of water at room temperature and transferred to refrigerated container to 5 °C, so temperature decreased slowly to 5 °C (70–80 min). Once at 5 °C, the samples were diluted again 1:1 with the same diluents prepared with a higher glycerol concentration (6% for the G4 assay and 12% for the others), in order to achieve a final glycerol concentration for each extender (4% and 8%, respectively). The samples were diluted with the original extender (4% glycerol for the G4 assay and 8% for the others) to yield a final concentration of 100 × 10^6 spermatozoa/mL. After equilibration for 1 h at 5 °C, the semen was packaged into 0.25 mL plastic straws, and frozen in a programmable biofreezer (Kryo 10-16 II PlanerTM) at −20 °C/min down to −100 °C, and then transferred to liquid nitrogen containers. The cryopreserved samples were stored in liquid nitrogen for a minimum of one week. Thawing was performed by plunging the straws in water at 65 °C for 6 s and the sample are evaluated immediately at room temperature.

2.4. Semen evaluation

Before freezing and after thawing, the semen samples were analyzed for sperm agglutination, motility, viability, acrosomal status and mitochondrial activity.

Sperm agglutination was evaluated on a microscope slide by subjective observation under a phase contrast microscope and rated on a scale from 0 to 4: 0-absence of agglutination; 1-low degree of agglutination (<10% agglutinated sperm); 2-intermediate level of agglutination (10–30%); 3-high degree of agglutination (30–50%) and 4-very high degree of agglutination (>50%).

Motility parameters were assessed by means of a computer-assisted sperm analysis system (CASA) (Sperm Class Analyzer; Microptic, Barcelona, Spain). Samples were diluted (10–20 ×10^6 cells/mL) in an 1% buffer (HEPES 20 mmol/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose; pH 7; 300mOsm/kg), and warmed on a 37 °C plate for 5 min. Then, 5 μL sperm sample was placed in a Makler counting cell chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel) and analyzed using a negative phase objective (×10) in a contrast microscope with a warmed stage (38 °C). The standard parameter settings were as follows: 25 frames/s; 5 to 80 μm2 for head area; VCL>10 μm/s to classify a spermatozoon as motile. At least fields and 200 spermatozoa were saved and analyzed afterwards. Reported parameters were total motility (TM), progressive motility (PM; VCL>25, STR>80), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s) and amplitude of lateral head displacement (ALH, μm). A detailed explanation of these motility parameters has been provided elsewhere [37].

To evaluate sperm viability, the double stain SYBR-14 with propidium iodide (LIVE/DEAD Sperm Viability Kit; Invitrogen, Barcelona, Spain) using flow cytometer was applied. Sperm samples were diluted with PBS down to 5 × 10^6 spermatozoa/ml, and 300 μL were transferred to a polypropylene tube to which we added 3 μL PI (24 μM) and 1.5 μL SYBR-14 (100 nM). The tubes were kept at 37 °C for 20 min in the dark. We detected three populations corresponding to percentage of live spermatozoa (green), moribund spermatozoa (red + green) and dead spermatozoa (red). Only live spermatozoa (named VIAB) were recorded.

For acrosomal status, we used the double stain PNA-FITC (PNA-FITC) and PI in a stock solution in PBS at 1 μg/mL and 24 μM, respectively. PNA is a lectin from *Arachis hypogaea* that binds to beta-galactose moieties associated with the outer acrosomal membrane of spermatozoa, indicating acrosome damaged cells. Sperm samples were diluted in PBS (5 × 10^6 spermatozoa/mL), and 300 μL were transferred to a polypropylene tube to which we added PI (24 μM) and PNA-FITC (1 μg/mL). Flow cytometer rendered the percentage of

viable spermatozoa with intact acrosome (non red and non green fluorescence, ACR).

JC-1 (Invitrogen, Barcelona, Spain) was used to assed mitochondrial status, identifying mitochondria with high mitochondrial potential. Samples were diluted in 300 μL of PBS (5×10^6 spermatozoa/mL), adding JC-1 at 6.8 μM. After 30 min at 37 °C, we obtained by flow cytometer the percentage of orange stained sperm (high membrane mitochondrial) named MIT.

Evaluation of flow cytometer parameters (viability, acrosomal status and mitochondrial status) was carried out using a FACScalibur flow cytometer (Becton Dickinson Systems, San Jose, CA, USA), equipped with standard optics and an argon ion laser, tuned at 488 nm, and running at 200 mW. Calibration was carried out periodically using standard beads (Calibrites; Becton Dickinson). 10,000 events with a flow rate of 200 cells/s were analyzed per sample.

2.5. Statistical analysis

Data were analyzed using the SAS™ V.9.0. package. The parameters of semen quality were the dependent variables and a GLM procedure was used to evaluate the effect of various factors: addition or not of additives, egg-yolk concentration, glycerol concentration and extender osmolality. Least-squares means were computed for each effect listed and P-values for differences of the multiple comparisons were calculated by Tukey's test. Changes on the frequencies of the agglutinated spermatozoa classification depending on additive supplementation were analyzed using a FREQ procedure. Values were considered to be statistically significant at $P < 0.05$.

Table 1
Post-thawing semen quality (mean ± SEM) for supplementation or not with additives (Equex paste® and EDTA); (TTF* with or without additives corresponds to A and NA, respectively).

Parameters	A	NA	P
TM (%)	53.9 ± 3.5	36.5 ± 3.9	0.021
PM (%)	28.1 ± 3.3	17.4 ± 2.9	0.018
VAP (μm/s)	60.0 ± 3.4	45.2 ± 3.6	0.005
VCL (μm/s)	107.2 ± 6.3	81.2 ± 7.1	0.010
VSL (μm/s)	45.3 ± 3.3	33.4 ± 3.0	0.011
ALH (μm)	4.5 ± 0.2	3.5 ± 0.3	0.011
VIAB (%)	60.0 ± 1.4	44.1 ± 2.4	<0.001
ACR (%)	63.5 ± 1.9	46.5 ± 2.3	<0.001
MIT (%)	62.35 ± 4.6	45.6 ± 4.3	0.011

TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: the straight line velocity; ALH: amplitude of lateral head displacement. VIAB: sperm viability; ACR: sperm with no damage acrosome within VIAB; MIT: spermatozoa mitochondrial membrane potential.
P indicates the difference between columns.
* TTF extender with 20% egg-yolk and 8% glycerol at 320 mOsm/kg.

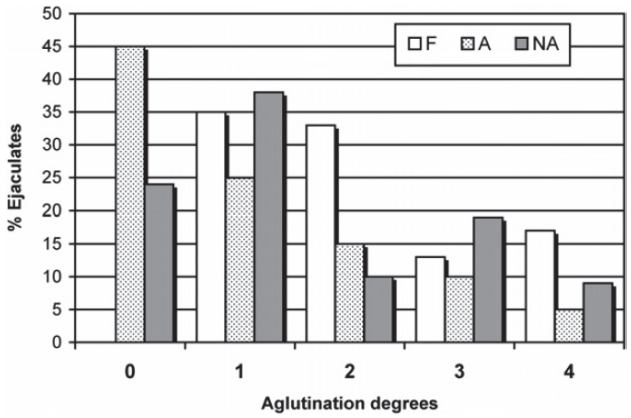

Fig. 1. Distribution (%) of agglutination degrees (0–4) in fresh semen (F) and post-thawed semen in extender with or without additives (A and NA, respectively).

Scale from 0–4: 0-absence of agglutination, 1-low degree of agglutination (<10% agglutinated sperm), 2-intermediate level of agglutination (10–30%), 3-high degree of agglutination (30–50%) and 4-very high degree of agglutination (>50%).

3. Results

3.1. Experiment 1

The quality of post-thawed sperm was improved by extender supplementation with additives. Table 1 show that TTF with additives (A) rendered significantly higher results for all sperm parameters analyzed. Moreover, when we studied the effect of additives we observed that all of the fresh samples (F) showed agglutination (Fig. 1): most of the samples presented grades 1 or 2 of agglutination (68%) and the rest of the ejaculates showed grades 3 or 4 (32%). A gradual trend to decreasing agglutination levels in post-thawed samples was observed when we used the extender with additives (A), thus 45% of the samples did not present agglutination and 25% manifested grade 1 agglutination. However, most of post-thawed samples frozen with the extender without additives (NA) showed grade 1 agglutination (38.1%).

As in the case of supplementation with additives, there were significant differences between Y10 and Y20 (Table 2). Samples frozen with Y20 showed better post-thawing mean values for all motility parameters, being significantly higher for all of them except: VCL ($P = 0.057$) and ALH ($P = 0.155$). With regard to fluorescence parameters, Y20 showed significantly higher results for VIAB and ACR, whereas significance

Table 2
Post-thawing semen quality (mean ± SEM) for the two egg-yolk concentrations (TTF * with 10% or 20% egg-yolk; Y10 and Y20, respectively).

Parameters	Y10	Y20	P
TM (%)	22.2 ± 3.5	53.6 ± 4.6	<0.001
PM (%)	7.4 ± 1.6	25.2 ± 3.0	<0.001
VAP (μm/s)	42.8 ± 5.3	61.2 ± 3.7	0.009
VCL (μm/s)	87.5 ± 10.6	112.4 ± 6.7	0.057
VSL (μm/s)	28.6 ± 3.9	42.3 ± 3.1	0.010
ALH (μm)	3.8 ± 0.4	4.5 ± 0.3	0.155
VIAB (%)	31.1 ± 5.1	58.4 ± 3.8	<0.001
ACR (%)	33.7 ± 4.5	62.5 ± 3.7	<0.001
MIT (%)	30.5 ± 8.3	55.8 ± 9.3	0.053

TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: the straight line velocity; ALH: amplitude of lateral head displacement. VIAB: sperm viability; ACR: sperm with no damage acrosome within VIAB; MIT: spermatozoa mitochondrial membrane potential.
P indicates the difference between columns.
* TTF extender with 8% glycerol and 1% Equex paste + 2% EDTA at 320 mOsm/kg.

was not found for MIT (P = 0.053). Table 3 shows no difference between G4 and G8.

3.2. Experiment 2

For the extender osmolality experiment, osmolality of electroejaculated samples was 308 ± 38 mOsm/kg (mean ± SD).

The comparison of the effect of the two extenders, E300 and E320, with different osmolalities, on quality parameters of post-thawed brown bear semen is shown in Table 4. We observed that post-thawed semen parameters were significantly lower for E320 than for E300 extender: VAP (P = 0.019), VCL (P = 0.022), VLS (P = 0.021) and ALH (P = 0.032). For the other parameters, the results were similar for both extenders.

Table 3
Post-thawing semen quality (mean ± SEM) for the two glycerol concentrations (TTF* with 4% or 8% glycerol, G4 and G8, respectively).

Parameters	G4	G8	P
TM (%)	45.8 ± 7.1	48.6 ± 5.4	0.758
PM (%)	25.3 ± 4.6	25.1 ± 4.6	0.976
VAP (μm/s)	87.5 ± 6.5	84.5 ± 8.0	0.769
VCL (μm/s)	158.3 ± 12.3	164.7 ± 14.9	0.747
VSL (μm/s)	72.0 ± 6.0	67.7 ± 7.4	0.661
ALH (μm)	6.3 ± 0.5	7.0 ± 0.6	0.412
VIAB (%)	34.9 ± 3.0	43.7 ± 4.1	0.097
ACR (%)	41.2 ± 2.9	48.3 ± 3.8	0.152
MIT (%)	43.6 ± 3.0	40.9 ± 5.6	0.670

TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: the straight line velocity; ALH: amplitude of lateral head displacement. VIAB: sperm viability; ACR: sperm with no damage acrosome within VIAB; MIT: spermatozoa mitochondrial membrane potential.
p indicates the difference between columns.
* TTF extender with 20% egg-yolk and 1% Equex paste + 2% EDTA at 320 mOsm/kg.

Table 4
Post-thawing semen quality (mean ± SEM) for the two extender osmolalities (E300 and E320 correspond to TTF* adjusted to 300 or 320 mOsm/kg).

Parameters	E300	E320	P
TM (%)	55.5 ± 2.9	51.5 ± 3.2	0.360
PM (%)	28.8 ± 2.2	23.7 ± 2.1	0.095
VAP (μm/s)	73.1 ± 3.7	60.7 ± 3.5	0.019
VCL (μm/s)	133.3 ± 7.0	111.4 ± 6.2	0.022
VSL (μm/s)	53.5 ± 3.5	42.8 ± 2.8	0.021
ALH (μm)	5.4 ± 0.3	4.6 ± 0.2	0.032
VIAB (%)	61.6 ± 2.4	56.7 ± 3.2	0.218
ACR (%)	63.5 ± 2.3	61.1 ± 2.6	0.483
MIT (%)	55.9 ± 5.8	56.7 ± 5.9	0.923

TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: the straight line velocity; ALH: amplitude of lateral head displacement. VIAB: sperm viability; ACR: sperm with no damage acrosome within VIAB; MIT: spermatozoa mitochondrial membrane potential.
p indicates the difference between columns.
* TTF extender with 8% glycerol, 20% egg-yolk and 1% Equex paste + 2% EDTA.

4. Discussion

The choice of adequate extenders and freezing protocols is a crucial point for the success of semen cryopreservation in any species. Extender composition includes buffer systems, cryoprotectants, sugars, and other additives, which interact with the cell membrane during the freezing-thawing process in a very specific way. Because of this, several combinations were proposed for the different species.

Different extenders formulated for other species were used for bear sperm cryopreservation [7,8,9,10,19]. Although the results of sperm quality obtained were satisfactory, it is necessary to adjust the extender composition to bear semen characteristics. In the present work, the use of an extender manufactured in our laboratory with a known composition allowed the levels of its constituents to be easily adjusted [32].

To our knowledge, no studies have been carried out to analyze the effect of additives on bear semen quality during cryopreservation. In order to improve the post-thawing quality of these samples we proposed that the extender be supplemented with additives (1% Equex paste and 2% EDTA) and we observed a significant

improvement in sperm sample quality. Equex paste is a commercially available additive for use with semen extenders. It contains a detergent, sodium dodecyl sulphate (SDS), which might interact with the egg-yolk structure and could increase its protective effect against cold shock and freezing injury [27]. It has been proposed that Equex STM Paste improves post-thaw survival of spermatozoa by acting as a surfactant to stabilize cell membranes, particularly acrosome membranes, and to protect spermatozoa against the toxic effects of glycerol during the freezing-thawing process [38]. This substance, added to diluents at 0.5–1.5% v/v, disperses egg yolk components and allows better interaction with the sperm plasma membrane surface [39]. Addition of EDTA to semen extenders as a chelating agent blocks the action of calcium as a mediator of sperm capacitation and the acrosome reaction [23].

Previous studies have reported that the use of Equex STM Paste on semen freezing extenders benefits post-thaw motility [25], viability [22], and longevity [24] of ejaculated canine spermatozoa. Also, addition of Equex to freezing extenders protects the acrosomes of cat epididymal spermatozoa during the freezing-thawing process. In this regard, Ponglowhapan and Chatdarong [38], reported that supplementation with Equex Paste in the semen extender was effective for cryopreserving canine epididymal spermatozoa because it protected acrosome integrity against damage induced by freezing and prolonged post-thaw sperm motility during *in vitro* incubation at 37 °C. Moreover, Aisen et al. [23], reported higher percentages of cells with intact acrosome in the post-thawing evaluation of ram spermatozoa when extenders containing EDTA was used.

We observed that the use of additives could also reduce the agglutination level. In boar, the incidence and degree of agglutination was affected by individual, ejaculatory and seasonal variations. It was also reported that sperm agglutination exerted a negative effect on motility but was largely reduced after dilution in semen extender, although it did not interfere with fertility results [21].

Egg yolk was routinely included in semen crypreservation protocols of domestic as well as wild species and seemed to help spermatozoa in resisting against cold shock [40,41].

Egg yolk concentrations of 15% (Hokkaido brown bear [7]) and 20% (Japanese black bear [42]) have been used for freezing the bears semen. In our study, 20% egg yolk appeared as the most suitable egg yolk concentration for preserving brown bear spermatozoa, since it improves sperm motility, viability and acromosomal status. Although some authors have reported negative effects of high concentrations of egg yolk on the preservation of motility and acrosome integrity (in Mohor gazelle [16]) we noted that it has been suggested that egg yolk's effects differ depending on the composition of the extender buffer [43] .Thus, the higher egg yolk concentration in a raffinose-based diluent resulted in a better preservation of motility and membrane integrity during the cooling of spermatozoa in Cuvier's gazelle [14] or in red deer [44].

Concentrations of glycerol ranging from 2 to 10% have been applied to cryopreserve mammalian spermatozoa [31]. For ungulates, optimal glycerol concentration for sperm cryopreservation usually ranges between 4% and 8% [39]. Glycerol concentrations used in sperm of different bear species vary between 4–8% [7,8,9,10,11,19]. In this regard, our results coincide with those for the glycerol concentrations used in other ursids. Frozen-thawed sperm motility was influenced by different glycerol concentrations in some species, (stallion [44]; boar [45], ram [46] and Rhesus monkeys [47]. However, in Japanese black bear sperm motility was not affected by different glycerol concentrations (4–12%) but the percentage of viability and intact acrosomes were higher for sperm frozen with 4 and 6% glycerol [17]. In our study, sperm motility, viability, acrosome integrity and mitochondrial activity were not significantly affected by the two glycerol concentrations. This suggested that the adequate glycerol concentration for freezing brown bear sperm could be between 4–8%.

The role of extender osmolality on the quality of frozen-thawed brown bear spermatozoa was analyzed in present study. Once we observed the benefits of using additives and the most suitable glycerol and egg yolk concentrations (additives supplementation, glycerol–8%, egg yolk–20%), we prepared two extenders with this composition adjusted to 300 and 320 mOsm/kg (E300 and E320). For brown bear spermatozoa, extender E300 showed significantly higher results for some kinetic parameters (VAP, VCL, VSL, and ALH).

Environment osmolality is a significant factor when cryopreserving semen. Not only do anisoosmotic solutions induce cellular stress, but they also influence the outcome of the cryopreservation process. However, this influence could be positive since the osmolality of the extender modifies water flux through spermatozoa membrane [48]. In this regard, we must consider the average osmolality of the sperm samples used in this experiment (308 ± 38 mOsm/kg) to interpret our results.

According to this, the use of an E300 extender with an osmolality similar to that expected for the sample might be the best option. So, the hypothesis that a nearly isoosmotic extender could provide the best environment for brown bear spermatozoa was confirmed. This finding did not agree with reports documented in other species (bull [49], ram [23], and deer [34]) which showed that moderately hyperosmotic extenders might protect spermatozoa better than isoosmotic ones. In this context, spermatozoa of each species are affected in a different way by changes in the osmolality of the media, so this is a decisive factor to obtain acceptable cryopreservation results [49]. For instance, whereas osmotic tolerance of ram sperm in hypoosmotic conditions is much lower than bull sperm, it is higher than boar sperm [50]. Several reports have revealed information on the osmotic effects of glycerol addition and removal on post-thawing motility and acrosome integrity of ram spermatozoa [51].

Considering the lack about knowledge of sperm cryobiology in bear, our findings can contribute to developing specific extenders for an efficient cryopreservation of brown bear spermatozoa.

In conclusion, we found that the best extender for cryopreserving brown bear sperm was a TES-Tris-Fructose buffer with an osmolality of 300mOsm/kg, and supplemented 20% egg-yolk, 4–8% glycerol and additives (EDTA and Equex paste). In order to further refine extender composition, new studies must be developed in order to improve cryopreservation results.

Acknowledgments

This work was supported in part by CICYT (CGL 2004-02178/BOS), CICYT (CGL 2007-63748/BOS) and CANTUR S.A. Felipe Martínez-Pastor was supported by the Ramón y Cajal Program (MICINN, Spain).

References

[1] Holt WV, Pickard AR. Role of reproductive technologies and genetic resource banks in animal conservation. Rev Reprod. 1999;4:143–150.

[2] Yoshida M. Conservation of sperms: current status and new trends. Anim Reprod Sci. 2000;6061:349–55.

[3] Anel L, Martinez-Pastor F, Alvarez M, Garcia-Macias V, Borragan S, Celada M, Martinez F, Chamorro CA, Herraez P, Anel E, Paz P. Basis for the establishment of germplasm banks for brown bear: spermatology. Reprod Domest Anim 2005;40:335.

[4] Leibo SP, Songsasen N. Cryopreservation of gametes and embryos of non-domestic species. Theriogenology 2002;57: 303–26.

[5] Anel L, Alvarez M, Martinez-Pastor F, Gomes S, Nicolas M, Mata M, Martinez AF, Borragan S, Anel E, de PP. Sperm cryopreservation in brown bear (*Ursus arctos*): preliminary aspects. Reprod Domest Anim1 2008a;43:(Suppl 4)9–17.

[6] Gilmore JA, McGann LE, Ashworth E, Acker JP, Raath JP, Bush M, Critser JK. Fundamental cryobiology of selected African mammalian spermatozoa and its role in biodiversity preservation through the development of genome resource banking. Anim Reprod Sci 1998;53:277–97.

[7] Ishikawa A, Matsu M, Sakamoto H, Katagiri S, Takahashi Y. Cryopreservation of the semen collected by electroejaculation from the Hokkaido brown bear (*Ursus arctos yesoensis*). J Vet Med Sci 2002;64:373–6.

[8] Okano T, Murase T, Tsubota T. Electroejaculation and semen cryopreservation of free-ranging Japanese black bears (*Ursus thibetanus japonicus*). J Vet Med Sci 2004;66:1371–6.

[9] Seager SWJ, Dulensek EP, He G, Schaller GB. Giant panda (*Ailuropoda melanoleuca*) semen collection, evaluation and freezing in Southwest China. In: Proceedings of the 15th Annual Meeting of the Society for Cryobiology. Tokyo, Japan, pp. 123–126. 1987.

[10] Spindler RE, Huang Y, Howard JG, Wang P, Zhang H, Zhang G, Wildt DE. Acrosomal integrity and capacitation are not influenced by sperm cryopreservation in the giant panda. Reproduction 2004;127:547–56.

[11] Zhang M, Tang H, Shen G, Zhou B, Wu Z, Peng Z, Zhang J, Yan J, Xia G. Atrial natriuretic peptide induces an acrosome reaction in giant panda spermatozoa and enhances their penetration of salt-stored porcine oocytes. Theriogenology 2005;64: 1297–308.

[12] Salamon S, Maxwell WM. Storage of ram semen. Anim Reprod Sci 2000;62:77–111.

[13] Verstegen JP, Onclin K, Iguer-Ouada M. Long-term motility and fertility conservation of chilled canine semen using egg yolk added Tris-glucose extender: in vitro and in vivo studies. Theriogenology 2005;64:720–33.

[14] Garde JJ, del Olmo A, Soler AJ, Espeso G, Gomendio M, Roldan ERS. Effect of egg yolk, cryoprotectant, and various sugars on semen cryopreservation in endangered Cuvier's gazelle (*Gazella cuvieri*). Animal Reprod Sci 2008;108:384–401.

[15] Holt WV, Abaigar T, Jabbour HN. Oestrus synchronization, semen preservation and arti.cial insemination in the Mohor Gazelle (*Gazella dama mhorr*) for the establishment of a genome resource bank programme. Reprod Fertil Dev 1996;8: 1215–22.

[16] Ritar AJ, Salamon S. Effects of month of collection, method of processing, concentration of egg yolk and duration of frozen storage on viability of Angora goat spermatozoa. Small Rumin Res 1991;4:29–37.

[17] Okano T, Nakamura S, Komatsu T, Murase T, Miyazawa K, Asano M, Tsubota T. Characteristics of frozen-thawed spermatozoa cryopreserved with different concentrations of glycerol in captive Japanese black bears (*Ursus thibetanus japonicus*). J Vet Med Sci 2006b;68:1101–4.

[18] Fahy GM. The relevance of cryoprotectant "toxicity" to cryobiology. Cryobiology 1986;23:1–13.

[19] Anel L, Martinez F, Alvarez M, Anel E, Boixo JC, Kaabi M, Paz P, Chamorro C, Herraez P. Post-mortem spermatozoa recovery and freezing in Cantabric brown bear (*Ursus arctos*): a preliminary report. Theriogenology 1999;51:277.

[20] Kojima E, Tsuruga H, Komatsu T, Murase T, Tsubota T, Kita I. Characterization of semen collected from beagles and captive Jap-

anese black bears (*Ursus thibetanus japonicus*). Theriogenology 2001;55:717–31.
[21] Bollwein H, Petschow K, Weber F, Leiding C, Stolla R. The incidence of agglutination and its influence on sperm quality and fertility of boar semen. Berl Munch Tierarztl Wochenschr 2004;117:327–33.
[22] Rota A, Strom B, Linde-Forsberg C, Rodriguez-Martinez H. Effects of equex STM paste on viability of frozen-thawed dog spermatozoa during in vitro incubation at 38 degrees C. Theriogenology 1997;47:1093–101.
[23] Aisen EG, Alvarez HL, Venturino A, Garde JJ. Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. Theriogenology 2000;53:1053–61.
[24] Peña AI, Lugilde LL, Barrio M, Herradon PG, Quintela LA. Effects of Equex from different sources on post-thaw survival, longevity and intracellular Ca2+ concentration of dog spermatozoa. Theriogenology 2003;59:1725–39.
[25] Alhaider AK, Watson PF. Cryopreservation of dog semen: the effects of Equex STM paste on plasma membrane fluidity and the control of intracellular free calcium. Anim Reprod Sci 2009;110:147–61.
[26] Axner E, Hermansson U, Linde-Forsberg C. The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. Anim Reprod Sci 2004;84:179–91.
[27] Pursel VG, Schulman LL, Johnson LA. Effect of Orvus ES Paste on acrosome morphology, motility and fertilizing capacity of frozen-thawed boar sperm. J Anim Sci 1978;47:198–202.
[28] Martin JC, Klug E, Gunzel AR. Centrifugation of stallion semen and its storage in large volume straws. J Reprod Fertil. 1979; Suppl 47–51.
[29] Arriola J, Foote RH. Glycerolation and thawing effects on bull spermatozoa frozen in detergent-treated egg yolk and whole egg extenders. J Dairy Sci. 1987:70:1664–70.
[30] Cheng FP, Wu JT, Chan JP, Wang JS, Fung HP, Colenbrander B, Tung KC. The effect of different extenders on post-thaw sperm survival, acrosomal integrity and longevity in cryopreserved semen of Formosan Sika deer and Formosan Sambar deer. Theriogenology 2004;61:1605–16.
[31] Agca Y, Critser JK. Cryopreservation of spermatozoa in assisted reproduction. Semin. Reprod. Med. 2002;20:15–23.
[32] Anel L, de PP, Alvarez M, Chamorro CA, Boixo JC, Manso A, Gonzalez M, Kaabi M, Anel E. Field and in vitro assay of three methods for freezing ram semen. Theriogenology 2003; 60:1293–308.
[33] Alvarez M, Martinez-Pastor F, Garcia-Macias V, Borragan S, Celada M, Bernardo J, Gonzalez N, Alves S, Anel L. Epididymal sperm cryopreservation of one Somalia wild ass (*Equus africanus somaliensis*) using 6 different extenders. Reprod Fertil Dev 2006;18:215.
[34] Martinez-Pastor F, Martinez F, Garcia-Macias V, Esteso MC, Anel E, Fernandez-Santos MR, Soler AJ, de PP, Garde J, Anel L. A pilot study on post-thawing quality of Iberian red deer spermatozoa (*epididymal and electroejaculated*) depending on glycerol concentration and extender osmolality. Theriogenology 2006;66:1165–72.
[35] Anel E, Borragan S, Alvarez M, Nicolas M, Gomes-Alves S, Mata M, Boixo JC, Anel L. Sperm charateristics in ejaculated and epididymal Blue wildebeest (*Connochaetes taurinus*). Reprod Domest Anim 2008b;43:Suppl 3 209.
[36] Garcia-Macias V, Martinez-Pastor F, Alvarez M, Borragan S, Chamorro CA, Soler AJ, Anel L, de Paz P. Seasonal changes in sperm chromatin condensation in ram (*Ovis aries*), Iberian red deer (*Cervus elaphus hispanicus*), and brown bear (Ursus arctos). J Androl 2006;27:837–46.
[37] Mortimer D. Computerized semen analysis. Fertil Steril 1988; 49:182–5.
[38] Ponglowhapan S, Chatdarong K. Effects of Equex STM Paste on the quality of frozen-thawed epididymal dog spermatozoa. Theriogenology 2008;69:666–72.
[39] Watson PF, Holt WV. Cryobanking the Genetic Resource: Wildlife Conservation for the Future? London: Taylor and Francis. 2001.
[40] White IG. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. Reprod Fertil Dev 1993; 5:639–58.
[41] Fernandez-Santos MR, et al. Effects of egg yolk and cooling rate on the survival of refrigerated red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa. Reprod Domest Anim 41.2 (2006):114–18.
[42] Okano T, Murase T, Yayota C, Komatsu T, Miyazawa K, Asano M, Tsubota T. Characteristics of captive Japanese black bears (*Ursus thibetanus japonicus*) semen collected by electroejaculation with different voltages for stimulation and frozen-thawed under different conditions. Anim Reprod Sci 2006a;95:134–43.
[43] Watson PF. Electroejaculation, semen characteristics and semen preservation of the brindled gnu. J Reprod Fertil 1976;47: 123–26.
[44] Cochran JD, Amann RP, Froman DP, Pickett BW. Effects of centrifugation, glycerol level, cooling to 5 degrees C, freezing rate and thawing rate on the post-thaw motility of equine sperm. Theriogenology 1984;22:25–38.
[45] Buhr MM, Fiser P, Bailey JL, Curtis EF. Cryopreservation in different concentrations of glycerol alters boar sperm and their membranes. J Androl 2001;22:961–9.
[46] Fiser PS, Fairfull RW. The effect of glycerol concentration and cooling velocity on cryosurvival of ram spermatozoa frozen in straws. Cryobiology 1984;21:542–51.
[47] Si W, Zheng P, Li Y, Dinnyes A, Ji W. Effect of glycerol and dimethyl sulfoxide on cryopreservation of rhesus monkey (*Macaca mulatta*) sperm. Am J Primatol 2004;62:301–6.
[48] Fernandez-Santos MR, Martinez-Pastor F, Garcia-Macias V, Esteso MC, Soler AJ, de Paz P, Anel L, Garde JJ. Extender osmolality and sugar supplementation exert a complex effect on the cryopreservation of Iberian red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa. Theriogenology 2007;67: 738–53.
[49] Liu Z, Foote RH, Brockett CC. Survival of bull sperm frozen at different rates in media varying in osmolarity. Cryobiology 1998;37:219–30.
[50] Varisli O, Uguz C, Agca C, Agca Y. Motility and acrosomal integrity comparisons between electro-ejaculated and epididymal ram sperm after exposure to a range of anisosmotic solutions, cryoprotective agents and low temperatures. Anim Reprod Sci 2009;110:256–68.
[51] Gao DY, Liu J, Liu C, McGann LE, Watson PF, Kleinhans FW, Mazur P, Critser ES, Critser, JK. Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. Hum Reprod 1995;10:1109–22.

Theriogenology 82 (2014) 469–474

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Use of commercial extenders and alternatives to prevent sperm agglutination for cryopreservation of brown bear semen

S. Gomes-Alves [a,b], M. Alvarez [a,b], M. Nicolas [a,b], E. Lopez -Urueña [a,b], C. Martínez-Rodríguez [a,d], S. Borragan [c], P. de Paz [a,d,*], L. Anel [a,b]

[a] ITRA-ULE, INDEGSAL, University of León, León, Spain
[b] Animal Reproduction and Obstetrics, University of León, León, Spain
[c] Cabárceno Park, Cantabria, Spain
[d] Molecular Biology, University of León, León, Spain

ARTICLE INFO

Article history:
Received 6 November 2013
Received in revised form 9 May 2014
Accepted 12 May 2014

Keywords:
Brown bear ejaculates
Spermatozoa freezability
Cryopreservation extender
Sperm agglutination

ABSTRACT

The objective of this study was to evaluate different bovine and canine commercial semen extenders for cryopreservation of brown bear ejaculates and the effect of semen collection directly into extender on sperm agglutination. Semen samples were obtained by electro-ejaculation from 13 adult males. In experiment 1, eleven ejaculates from eight bears were used to evaluate Bioxcell and Andromed as extenders, whereas in experiment 2, nine ejaculates from six bears were used to evaluate Triladyl canine, CaniPro, and Extender 2 as extenders. An extender specifically developed for brown bears (Test-Tris-fructose-egg yolk-glycerol, TTF-ULE/bear) served as a control extender in both experiments. After thawing, total and progressive sperm motility and sperm viability were greater ($P < 0.05$) for TTF-ULE/bear and Andromed extenders than for Bioxcell in experiment 1 and greater ($P < 0.05$) for TTF-ULE/bear extender than for Triladyl Canine, CaniPro, and Extender 2 in experiment 2. In experiment 3, addition of handling extender (TTF-H) to the semen collection tube for eight ejaculates from seven bears resulted in less ($P < 0.05$) sperm agglutination in fresh samples (score 0.5 ± 0.2 vs. 1.8 ± 0.4 in diluted and control samples, respectively) with no effect on pre-freeze and post-thawing semen quality. In conclusion, TTF-ULE/bear is the most suitable extender for brown bear semen cryopreservation, but comparable results can be obtained with the commercial extender Andromed. In addition, collection of ejaculates directly in TTF-H extender decreases sperm agglutination in fresh samples.

1. Introduction

The Cantabrian brown bear (*Ursus arctos*) is considered at risk of extinction (Real Decreto 439/1990, regulation of the National Catalog of Endangered Species), and the availability to cryopreserve sperm could help preserve biodiversity in this wild species [1,2]. The success of semen cryopreservation depends on several factors, including the initial quality of the semen sample, cryopreservation protocol, and freezing extenders used [3,4]. It is fundamental to develop specific extender and cryopreservation protocols adapted to the characteristics of a particular species [5]. Our group has conducted studies in brown bear to develop optimal sperm cryopreservation technique, including developing a specific semen extender [6],

* Corresponding author. Tel.: +0034 987 291320; fax: +0034 987 291322.
E-mail address: ppazc@unileon.es (P. de Paz).

0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.05.015

evaluating different freezing and glycerol rates [7], timing of glycerol addition [8], and testing different centrifugation protocols [9–11]. Although a bear-specific extender has been successfully used for cryopreservation of sperm [7], assessing the suitability of commercial extenders designed for other species could provide a practical solution for unexpected situations when the specific extenders may not be available.

To optimize semen cryopreservation protocols, other particularities of brown bear ejaculates must also be taken into account. Sperm agglutination is a problem that occurs frequently with brown bear ejaculates [5,6,12], and this phenomenon can interfere with semen assessment and pre-freezing handling and possibly affect freezability [5]. In brown bear, high or very high level of agglutination is observed in 32% of the ejaculates [6]. Thus, on the basis of the fact that agglutination may disappear in boar semen after diluting in extender [13], dilution during ejaculate collection might be used as a strategy to improve the initial quality of seminal samples in brown bears.

The objective of the present study was to evaluate different bovine and canine commercial semen extenders for cryopreservation of brown bear ejaculates. In addition, the effect of semen collection directly into extender on sperm agglutination was also evaluated.

2. Materials and methods

2.1. Animals and ejaculate collection

All experiments were performed after obtaining ethical committee approval from the Ethical Committee for Experimentation with Animals of León University, Spain (03-02/2010), and all procedures were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. This study was performed at the Cabarceno Park (Cantabria, Spain; 43° 21′ N, 3° 50′ W; altitude: 143 m). The animals were housed in a half-freedom regime and fed chicken meat, bread, and fruits.

Ejaculates from 13 sexually mature male brown bears were obtained by electroejaculation during the breeding season (end of April to early July). The males were immobilized using 750 mg of zolazepam HCl and tiletamine HCl (Zoletil100; Virbac, Carros, France) and 6 mg of medetomidine (Zalopin, Orion Pharma Animal Health, Finland, 10 mg/mL), concurrently in the same dart. After immobilization, the males were weighed and monitored (pulse, oxygen saturation and breathing) using a portable veterinary monitor and were transported to a collection room (18 °C–24 °C). Before electroejaculation, the prepucial area was shaved and washed with physiological saline, and the rectum was emptied of feces. The bladder was catheterized during semen collection to prevent urine contamination. Electroejaculation was carried out with a PT Electronics electroejaculator (PT Electronics, Boring, OR, USA) using a transrectal probe 320 mm in length and 26 mm in diameter. Electric stimuli were given until ejaculation (10 V and 250 mA, on average) [14]. The ejaculates were collected in graduated glass tubes protected in a double-walled conservation tube filled with water at 30 °C. In experiment 3, each ejaculate was collected in a funnel and a piping system in the form of an "inverted Y" distributing the sample into two tubes with approximately equal volume. The collection of ejaculate was performed as isolated fractions to prevent urine contamination or low sperm concentration. The sample tubes were transferred to the laboratory (22 °C) for analysis and processing. Fractions of reduced concentration ($<200 \times 10^6$ sperm/mL), low motility (<50%), or contaminated urine (>80 mg urea/dL; DiaSysEcoline GmbH, Holzheim, Germany) were discarded. All valid fractions from the same electroejaculation were mixed to constitute a single ejaculate.

2.2. Semen extenders and cryopreservation

All the chemicals were at least of reagent grade, and they were acquired from Sigma (Madrid, Spain), unless otherwise stated.

The Test-Tris-fructose (TTF) base extender was prepared from a N-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid solution (300 mOsm/kg) and Tris solution (300 mOsm/kg) mixed to pH 7.1, with 4% final volume of D-fructose solution (300 mOsm/kg). The TTF base solution was double filtered through a cellulose acetate membrane (0.22 μm pore), supplemented with 20% egg yolk and centrifuged (3000× *g*, 30 minutes), discarding the sediment. Then, 6% glycerol (final concentration) and 2% EDTA and 1% Equex Paste (Minitüb, Tiefenbach, Germany) were added. The extender was supplemented with 0.302 mg/mL penicillin G sodium salt and 0.625 mg/mL dihydrostreptomycin sesquisulfate; this constituted the control extender (TTF-egg yolk-glycerol [ULE]/bear [6,7]). The agglutination handling extender (TTF-H) was prepared from the TTF base extender supplemented with 1% egg yolk.

The commercial bovine extenders used in this study included Bioxcell (IMV, L'AigleCedex, France) and Andromed (Minitüb). The commercial canine extenders included Triladyl canine (Minitüb), CaniPro (Minitüb), and Extender 2 [15]. CaniPro was supplied as two separate fractions, one with and one without glycerol; a mixture of equal parts of the two fractions was used in this study. All other extenders were supplied as a single fraction containing glycerol.

Ejaculates were centrifuged at 600× *g* for 6 minutes, the supernatant was removed, and the pellet was divided into aliquots according to the number of treatments. Each aliquot was diluted (1:1, vol/vol) with semen extenders at room temperature; accordingly, glycerol concentration was 3% to 3.5% at this stage. Tubes with diluted semen were placed in beakers containing 100 mL of water at room temperature and transferred to a refrigerator, where they were slowly cooled to 5 °C during approximately 75 minutes. Once at 5 °C, the samples were again diluted 1:1 (vol/vol) with the respective extender to which additional glycerolated extender was added to obtain the final concentration recommended by manufacturer after dilution: TTF-ULE/bear = 6%, Bioxcell = 6.4%, Andromed = 7%, Triladyl canine = 7%, CaniPro = 6%, and Extender 2 = 6%. Finally, the samples were diluted again with the respective original extender to adjust the cell concentration to 100×10^6 sperm/mL. After equilibration at 5 °C for 1 hour, the semen

was packaged into 0.25-mL plastic straws, the straws were transferred to a programmable biofreezer (Kryo560–16 Planer; Planer PLC, Sunbury-On-Thames, UK), the temperature was lowered from 5 to −100 °C at −20 °C/min, and the straws were then plunged into liquid nitrogen. Cryopreserved samples were stored in the liquid nitrogen for a minimum of 1 month. Thawing was performed by placing the straws in water at 65 °C for 6 seconds.

2.3. Semen evaluation

Immediately after collection, the volume of each ejaculate was recorded, osmolality was measured using a cryoscopic osmometer (Osmomat-030; Gonotec TM, Berlin, Germany), and the pH value was determined by a pH meter (CG 837; Schott, Mainz, Germany).

Sperm concentration was determined using a Bürker hemocytometer and computer-assisted sperm analysis (Integrated Semen Analyzer System; Proiser, Valencia, Spain). Sperm agglutination was evaluated on a microscope slide by subjective observation under phase contrast and rated on a scale from 0 to 4: 0 = absence of agglutination, 1 = less than 10% agglutination, 2 = 10% to 30% agglutination, 3 = 30% to 50% agglutination, and 4 = greater than 50% agglutination [6].

Sperm motility and kinematics parameters were determined using computer-assisted semen motility analysis system. Samples were diluted (10–20 × 10^6 cells/mL) in a 1% buffer (HEPES 20 mmol/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose; pH 7; 300 mOsm/kg), warmed to 37 °C plate for 5 minutes, then 5 μL of the semen sample was placed in a Makler cell counting chamber, and analyzed using a negative phase objective (×10) in a contrast microscope with a heated stage at 38 °C. At least five fields and 200 sperm were saved and analyzed. Results included total motility, progressive motility, average path velocity (VAP), curvilinear velocity (VCL), linearity, and amplitude of lateral head displacement. The standard settings were 25 frames/s, 5 to 50 μm^2 for head area, VCL greater than10 μm/s, and straightness greater than 65 to classify a spermatozoon as motile.

A flow cytometer (Becton Dickinson Systems, San Jose, CA, USA) equipped with standard optics and an argon ion laser, tuned at 488 nm, and running at 200 mW was used to determine sperm viability, acrosome integrity, and mitochondrial status. A flow rate of 200 sperm/s was used, and 10,000 sperm were analyzed per sample. Sperm viability was evaluated using SYBR-14 and propidium iodide (PI) (LIVE/DEAD Sperm Viability Kit; Invitrogen, Barcelona, Spain) using flow cytometer. Semen samples were diluted with PBS to 5 × 10^6 sperm/mL, and 300 μL were transferred to a polypropylene tube to which 3 μL PI (24 μM) and 1.5 μL SYBR-14 (100 nM) were added. Sperm with positive SYBR-14 fluorescence and negative PI fluorescence were considered live. Acrosome integrity was evaluated using Peanut agglutinin-Fluorescein isothiocyanate and PI in a stock solution in PBS at 1 mg/mL and 1.5 mM, respectively. Semen samples were diluted in PBS to 1 to 2 × 10^6 sperm/mL, and 300 μL were transferred to a polypropylene tube to which 0.3 μL of stock solution was added. The samples were analyzed after 10 minutes at room temperature. Sperm with negative Peanut agglutinin-Fluorescein isothiocyanate and PI fluorescence were considered live with intact acrosome. Mitochondrial status was evaluated using JC-1 stain (Invitrogen). Semen samples were diluted in PBS to 1 to 2 × 10^6 sperm/mL, and 300 μL were transferred to a polypropylene tube to which 0.3 μL of stock solution (3 mM JC-1 in DMSO) was added. Sperm emitting orange fluorescence were considered to be positive (high mitochondrial membrane potential). Stained samples were incubated at 37 °C for 20 to 30 minutes before evaluation of sperm viability and mitochondrial status and for 10 minutes at room temperature before evaluation of acrosome integrity.

2.4. Experimental design and statistical analysis

In experiment 1, 11 ejaculates from eight bears were used to evaluate Bioxcell and Andromed as extenders, whereas in experiment 2, nine ejaculates from six bears were used to evaluate Triladyl canine, CaniPro, and Extender 2 as extenders; TTF-ULE/bear served as the control extender in both experiments. In experiment 3, eight ejaculates from seven bears were used to evaluate the effect of adding 0.5 mL of handling extender (TTF-H) to the semen collection tube. In experiments 1 and 2, semen samples were evaluated just before freezing (pre-freezing) and within 10 minutes after thawing (post-thawing). In experiment 3, semen samples were also evaluated within 10 minutes after collection (fresh).

Statistical analysis was conducted using the SAS, v.9.1 package. The effects of treatment (extenders in experiments 1 and 2, collection method in experiment 3) were determined using mixed models with ejaculate included in the models as random effect. Significant fixed effects were located using multiple comparisons of means with Tukey's contrasts.

3. Results

The volume of ejaculates obtained in this study was 2.2 ⊥ 0.2 mL (mean ⊥ SEM). Fresh semen samples had an osmotic pressure of 299.3 ± 4.3 mOsm/kg, pH of 8.3 ± 0.1, and sperm concentration of 288.5 ± 44.6 × 10^6 sperm/mL.

In experiment 1, greater ($P < 0.05$) pre-freezing total and progressive motility were observed for TTF-ULE/bear extender than for Bioxcell. After thawing, total and sperm progressive motility and sperm viability were greater ($P < 0.05$) for TTF-ULE/bear and Andromed extenders than for Bioxcell. In addition, Bioxcell resulted in lower ($P < 0.05$) VAP and VCL than Andromed and less ($P < 0.05$) acrosome-intact sperm than TTF-ULE/bear (Table 1).

In experiment 2, pre-freezing VAP was lower ($P < 0.05$) for TTF-ULE/bear than for CaniPro, but the proportion of live sperm was greater ($P < 0.05$) for the former than in the latter. After thawing, total and sperm progressive motility and sperm viability were greater ($P < 0.05$) for TTF-ULE/bear extender than for Triladyl Canine, CaniPro, and Extender 2. In addition, VAP and VCL were also greater ($P < 0.05$) for TTF-ULE/bear than for Triladyl Canine. Sperm linearity was greater for CaniPro than for Triladyl Canine (Table 2).

Table 1
Sperm motility, kinetics, viability, acrosome integrity, and mitochondrial status (mean ± SEM) in brown bear semen according to the type of extender (n = 11).

Type of extender	Total motility (%)	Progressive motility (%)	Average path velocity (μm/s)	Curvilinear velocity (μm/s)	Linearity (%)	Live (%)	Acrosome intact (%)	High mitochondria potential (%)
Pre-freezing								
TTF-ULE/bear	74.7 ± 4.3[a]	47.6 ± 5.1[a]	65.2 ± 5.8	109.2 ± 10.0	42.7 ± 2.0	67.1 ± 4.7	67.6 ± 4.7	56.3 ± 9.4
Andromed	63.3 ± 7.6[a]	39.6 ± 7.0[ab]	79.2 ± 8.3	135.8 ± 14.8	37.9 ± 2.4	67.3 ± 4.8	68.1 ± 3.8	57.3 ± 7.9
Bioxcell	50.1 ± 7.8[b]	29.3 ± 6.5[b]	75.2 ± 7.8	121.5 ± 13.1	40.7 ± 2.5	63.8 ± 5.6	67.0 ± 4.1	51.8 ± 9.8
Post-thawing								
TTF-ULE/bear	55.5 ± 6.2[a]	23.8 ± 2.9[a]	44.3 ± 4.4[ab]	79.8 ± 8.7[ab]	35.0 ± 1.1	44.4 ± 4.1[a]	46.1 ± 3.4[a]	29.1 ± 8.0
Andromed	54.5 ± 5.2[a]	27.9 ± 3.3[a]	58.5 ± 5.4[a]	110.7 ± 11.5[a]	35.6 ± 1.0	42.1 ± 3.9[a]	41.1 ± 4.4[a]	38.5 ± 8.0
Bioxcell	17.8 ± 5.3[b]	7.2 ± 2.3[b]	36.3 ± 5.8[b]	64.2 ± 10.2[b]	36.7 ± 2.0	29.4 ± 3.8[b]	33.3 ± 4.9[b]	20.9 ± 4.7

[a,b]Rows with different superscripts differ ($P < 0.05$).

In experiment 3, collection of semen directly into TTF-H extender resulted in less ($P < 0.05$) sperm agglutination in fresh samples (score 0.5 ± 0.2 vs. 1.8 ± 0.4 in diluted and control samples, respectively). However, there were no effects of collection method on sperm agglutination score, motility, kinetics characteristics, viability, acrosome integrity, and mitochondrial status in pre-freezing and post-thawing samples (data not shown).

4. Discussion

Both Andromed and Bioxcell are soybean-based extenders and free from animal compounds (egg yolk or milk), which is becoming a widespread recommendation to avoid the risk of disease transmission [16]. Use of TTF-ULE/bear extender supplemented with 5% type A soybean lecithin for cryopreservation of brown bear semen resulted in similar sperm motility to that of the same extender supplemented with egg yolk, but sperm viability or acrosomal integrity were lower [17]. In the present, use of Andromed resulted in similar semen quality after cryopreservation compared with TTF-ULE/bear (egg yolk based). Andromed has been previously used in other species with varying results. Some studies in rams, red deer, and gazelle have demonstrated that results obtained with Andromed were similar to those obtained with egg yolk extenders [18–20], whereas others have observed lower post-thaw semen quality with Andromed compared with egg yolk extenders for cryopreservation of African buffalo, dog, and cat semen [21–24]. The differences observed might be either because of differences in protective effect of soybean lecithin in semen cryopreservation across species or because of the source of sperm (epididymal or ejaculated).

Bioxcell has been used for cryopreservation of bull [25], ram [26,27], and caprine [28] semen with results similar to those obtained using egg yolk or milk extenders. In rams, Bioxcell was more effective than Andromed in preserving the fertilizing potential of chilled or frozen ram semen [29]; however, Bioxcell resulted in fewer motile sperm after freezing of red deer [18]. In the present study, Bioxcell was less effective in protecting brown bear sperm from damage induced by cryopreservation than TTF-ULE/bear and Andromed extenders. These results indicate that Bioxcell is more specifically indicated to ruminants and suggest that the unknown compounds present in the extender have only minor protective effect on cell structures of brown bear sperm during the cryopreservation process.

Triladyl canine, CaniPro, and Extender 2 are egg yolk–based diluents used for cryopreservation of canine sperm. The results of the present study indicate that these extenders were not as effective TTF-ULE/bear extender for cryopreservation of brown bear sperm. Although the fractions of CaniPro and Extender 2 extenders containing glycerol are usually only added after cooling, post-thaw sperm motility and viability were similar when glycerol was added at room temperature or at 5 °C to TTF-ULE/bear extender in a previous study [8], thus indicating that brown bear sperm tolerant well the addition of glycerol at room temperature. All egg yolk–based extenders used in the present study have the same concentration of egg yolk and very similar concentration of glycerol, suggesting that the differences among extenders are related to other extender components.

Table 2
Sperm motility, kinetics, viability, acrosome integrity, and mitochondrial status (mean ± SEM) in brown bear semen according to the type of extender (n = 9).

Type of extender	Total motility (%)	Progressive motility (%)	Average path velocity (μm/s)	Curvilinear velocity (μm/s)	Linearity (%)	Live (%)	Acrosome intact (%)	High mitochondria potential (%)
Pre-freezing								
TTF-ULE/bear	77.6 ± 5.1	46.8 ± 6.8	61.0 ± 6.1[a]	116.1 ± 13.7	37.5 ± 2.5	71.8 ± 4.1[a]	76.2 ± 3.2	29.6 ± 8
Triladyl Canine	72.0 ± 6.8	41.5 ± 4.5	69.5 ± 4.9[ab]	119.2 ± 11.2	40.9 ± 2.0	59.2 ± 5.6[b]	68.3 ± 5	40.4 ± 9.7
CaniPro	69.4 ± 5.3	38.3 ± 3.7	75.9 ± 6.2[b]	131.8 ± 11.8	39.4 ± 2.0	66.8 ± 5.9[ab]	71.7 ± 5.5	29.1 ± 7
Extender 2	75.0 ± 5.5	35.8 ± 6.4	70.3 ± 5.7[ab]	122.1 ± 11.0	35.5 ± 1.8	66.5 ± 5.6[ab]	71.1 ± 5.1	38.8 ± 8.7
Post-thawing								
TTF-ULE/bear	71.8 ± 8.2[a]	35.7 ± 4.3[a]	52.4 ± 5.2[a]	98.8 ± 9.8[a]	35.0 ± 1.8[ab]	47.2 ± 4.6[a]	50.2 ± 4.9	29.1 ± 10
Triladyl Canine	48.6 ± 6.1[b]	15.7 ± 2.7[b]	30.6 ± 5.3[b]	53.0 ± 8.3[b]	32.9 ± 1.1[b]	31.9 ± 3.6[b]	41.5 ± 3.4	36.9 ± 5.9
CaniPro	46.3 ± 8.8[b]	21.4 ± 5.3[b]	46.1 ± 8.1[a]	83.0 ± 15.4[ab]	37.2 ± 1.3[a]	32.9 ± 1.4[b]	41.8 ± 6.4	32 ± 5.5
Extender 2	47.9 ± 8.3[b]	20.6 ± 4.5[b]	42.3 ± 7.6[ab]	73.7 ± 12.2[ab]	35.3 ± 1.4[ab]	33.9 ± 5.2[b]	41.6 ± 5.4	33.8 ± 6.2

[a,b]Rows with different superscripts differ ($P < 0.05$).

Sugars also play an important role in semen extenders, providing energy substrates and acting as osmotic and cryoprotective agents [30]. Okano et al. [31] referred to studies that demonstrated that efficient sugar supplementation varies across the species. However, Japanese black bear semen could be frozen with any of the three examined diluents that contained different sugars (glucose, fructose, and fructose-lactose-raffinose). Two extenders used in the present study contained fructose (TTF-ULE/bear and Andromed) and two contained glucose (CaniPro and Extender 2); the other extenders were unknown. These observations seem to suggest that fructose is a better alternative to glucose for brown bear semen extenders.

Sperm agglutination can occur among sperm (head-head or head-tail) or with other particles present in the sample. Monga and Roberts [32] provided evidence that fimbriated bacteria can adhere to the glycoproteins of spermatozoa surface inducing agglutination of motile sperm. In addition, specific sperm surface proteins appear to be involved in sperm agglutination [33,34]. Collecting brown bear ejaculates directly into extender significantly reduced the degree of sperm agglutination in fresh samples without affecting pre-freezing and post-thawing semen quality. Reduced agglutination might be related to reduction of bacterial activity and/or masking sperm surface proteins.

In conclusion, TTF-ULE/bear is the most suitable extender for brown bear semen cryopreservation, but comparable results can be obtained with the commercial extender Andromed. In addition, collection of ejaculates directly in TTF-H extender decreases sperm agglutination in fresh samples.

Acknowledgments

The authors thank Miguel Angel Marañon, Miguel Angel Prieto, and other gamekeepers of Cabárceno Park (Cantabria, Spain) and Maria Mata Campuzano, Manuel Alvarez Rodríguez, Patricia Manrique Revuelta, Carmen Martinez Rodriguez, and Luis Anel-Lopez. This work was supported by MICINN (CGL2010-19213/BOS) and CANTUR S.A. Susana Gomes-Alves was supported by a PhD fellowship (SFRH/BD/45522/2008, co-founded by Programa Operacional Potencial Humano-Quadro de Referência Estratégico Nacional).

References

[1] Holt WV, Pickard AR. Role of reproductive technologies and genetic resource banks in animal conservation. Rev Reprod 1999;4:143–50.
[2] Leibo SP, Songsasen N. Cryopreservation of gametes and embryos of non-domestic species. Theriogenology 2002;57:303–26.
[3] Gilmore JA, McGann LE, Ashworth E, Acker JP, Raath JP, Bush M, et al. Fundamental cryobiology of selected African mammalian spermatozoa and its role in biodiversity preservation through the development of genome resource banking. Anim Reprod Sci 1998;53:277–97.
[4] Yoshida M. Conservation of sperms: current status and new trends. Anim Reprod Sci 2000;60-61:349–55.
[5] Anel L, Alvarez M, Martinez-Pastor F, Gomes S, Nicolas M, Mata M, et al. Sperm cryopreservation in brown bear (Ursus arctos): preliminary aspects. Reprod Domest Anim 2008;43(Suppl 4):9–17.
[6] Anel L, Gomes-Alves S, Alvarez M, Borragan S, Anel E, Nicolas M, et al. Effect of basic factors of extender composition on post-thawing quality of brown bear electroejaculated spermatozoa. Theriogenology 2010;74:643–51.
[7] de Paz P, Alvarez-Rodriguez M, Nicolas M, Alvarez M, Chamorro C, Borragan S, et al. Optimization of glycerol concentration and freezing rate in the cryopreservation of ejaculate from brown bear (Ursus arctos). Reprod Domest Anim 2012;47:105–12.
[8] Alvarez-Rodriguez M, Alvarez M, Gomes-Alves S, Borragan S, Martinez-Pastor F, de Paz P, et al. Quality of frozen-thawed semen in brown bear is not affected by timing of glycerol addition. Theriogenology 2011;75:1561–5.
[9] Nicolas M, Alvarez M, Gomes-Alves S, Mata-Campuzano M, Borragan S, Martinez-Pastor F, et al. Effects on brown bear (Ursus arctos) spermatozoa freezability of different extender and dilution ratios used for prefreezing centrifugation. Eur J Wild Res 2011;57:259–66.
[10] Nicolas M, Alvarez M, Anel E, Martinez F, Borragan S, Martinez-Pastor F, et al. Spermatozoa recovery and post-thawing quality of brown bear ejaculates is affected for centrifugation regimes. Eur J Wild Res 2012;58:77–84.
[11] Nicolas M, Alvarez M, Borragan S, Martinez-Pastor F, Chamorro CA, Alvarez-Rodriguez M, et al. Evaluation of the qualitative and quantitative effectiveness of three media of centrifugation (Maxifreeze, Cushion fluid equine, and PureSperm 100) in preparation of fresh or frozen-thawed brown bear spermatozoa. Theriogenology 2012;77:1119–28.
[12] Kojima E, Tsuruga H, Komatsu T, Murase T, Tsubota T, Kita I. Characterization of semen collected from beagles and captive japanese black bears (Ursus thibetanus japonicus). Theriogenology 2001;55:717–31.
[13] Bollwein H, Petschow K, Weber F, Leiding C, Stolla R. The incidence of agglutination and its influence on sperm quality and fertility of boar semen. Berl Munch Tierarztl Wochenschr 2004;117:327–33.
[14] Garcia-Macias V, Martinez-Pastor F, Alvarez M, Borragan S, Chamorro CA, Soler AJ, et al. Seasonal changes in sperm chromatin condensation in ram (Ovis aries), Iberian red deer (Cervus elaphus hispanicus), and brown bear (Ursus arctos). J Androl 2006;27:837–46.
[15] Rota A, Strom B, Linde-Forsberg C, Rodriguez-Martinez H. Effects of equex STM paste on viability of frozen-thawed dog spermatozoa during in vitro incubation at 38 degrees C. Theriogenology 1997;47:1093–101.
[16] de Ruigh L, Bosch JC, Brus MC, Landman B, Merton JS. Ways to improve the biosecurity of bovine semen. Reprod Domest Anim 2006;41:268–74.
[17] Alvarez-Rodriguez M, Alvarez M, Anel-Lopez L, Martinez-Rodriguez C, Martinez-Pastor F, Borragan S, et al. The antioxidant effects of soybean lecithin- or low-density lipoprotein-based extenders for the cryopreservation of brown-bear (Ursus arctos) spermatozoa. Reprod Fertil Dev 2013;25:1185–93.
[18] Martinez-Pastor F, Martinez F, Alvarez M, Maroto-Morales A, Garcia-Alvarez O, Soler AJ, et al. Cryopreservation of iberian red deer (Cervus elaphus hispanicus) spermatozoa obtained by electroejaculation. Theriogenology 2009;71:628–38.
[19] Fukui Y, Kohno H, Togari T, Hiwasa M, Okabe K. Fertility after artificial insemination using a soybean-based semen extender in sheep. J Reprod Dev 2008;54:286–9.
[20] Saragusty J, Gacitua H, King R, Arav A. Post-mortem semen cryopreservation and characterization in two different endangered gazelle species (Gazella gazella and Gazella dorcas) and one subspecies (Gazella gazelle acaiae). Theriogenology 2006;66:775–84.
[21] Herold FC, De Haas K, Cooper D, Colenbrander B, Nothling JO, Theunisen W, et al. Comparison of three different media for freezing of epididymal sperm from the African buffalo (Syncerus caffer) and influence of equilibration time on the post-thaw sperm quality. Onderstepoort J Vet Res 2004;71:203–10.
[22] Herold FC, de Haas K, Colenbrander B, Gerber D. Comparison of equilibration times when freezing epididymal sperm from african buffalo (Syncerus caffer) using triladyl or AndroMed. Theriogenology 2006;66:1123–30.
[23] Nothling JO, Gerber D, Colenbrander B, Dijkstra M, Bakker T, De Cramer K. The effect of homologous prostatic fluid on motility and morphology of dog epididymal spermatozoa extended and frozen in biladyl with equex STM paste or andromed. Theriogenology 2007;67:264–75.
[24] Jimenez E, Perez-Marin CC, Vizuete G, Millan Y, Aguera EI. Effect of different extenders on in vitro characteristics of feline epididymal sperm during cryopreservation. Reprod Domest Anim 2013;48:665–72.

[25] Stradaioli G, Noro T, Sylla L, Monaci M. Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: comparison between two extenders. Theriogenology 2007;67:1249–55.
[26] Gil J, Lundeheim N, Soderquist L, Rodriiuez-Martinez H. Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. Theriogenology 2003a;59: 1241–55.
[27] Gil J, Rodriguez-Irazoqui M, Lundeheim N, Soderquist L, Rodriguez-Martinez H. Fertility of ram semen frozen in bioexcell and used for cervical artificial insemination. Theriogenology 2003b;59: 1157–70.
[28] Roof DJ, Bowley S, Price LL, Matsas DJ. Comparison of two commercial extenders for cryopreservation of goat semen without sperm washing. Theriogenology 2012;77:412–20.
[29] Khalifa T, Lymberopoulos A, Theodosiadou E. Association of soybean-based extenders with field fertility of stored ram (Ovis aries) semen: a randomized double-blind parallel group design. Theriogenology 2013;79:517–27.
[30] Salamon S, Maxwell WM. Storage of ram semen. Anim Reprod Sci 2000;62:77–111.
[31] Okano T, Murase T, Yayota C, Komatsu T, Miyazawa K, Asano M, et al. Characteristics of captive Japanese black bears (Ursus thibetanus japonicus) semen collected by electroejaculation with different voltages for stimulation and frozen-thawed under different conditions. Anim Reprod Sci 2006;95:134–43.
[32] Monga M, Roberts JA. Spermagglutination by bacteria: receptor-specific interactions. J Androl 1994;15:151–6.
[33] Fornes MW, Burgos MH. Epididymal glycoprotein involved in rat sperm association. Mol Reprod Dev 1994;38:43–7.
[34] Flaherty SP, Swann NJ, Primakoff P, Myles DG. A role for the WH-30 protein in sperm-sperm adhesion during rouleaux formation in the guinea pig. Dev Biol 1993;156:243–52.

G Model
ANIREP-5056; No. of Pages 10
ARTICLE IN PRESS

Animal Reproduction Science xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Animal Reproduction Science

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

Salvaging urospermic ejaculates from brown bear (*Ursus arctos*)

S. Gomes-Alves[a,b], M. Alvarez[a,b], M. Nicolas[a,b], C. Martínez-Rodríguez[a,d], S. Borragán[c], C.A. Chamorro[a], L. Anel[a,b], P. de Paz[a,d,*]

[a] *ITRA-ULE, INDEGSAL, University of León, 24071 León, Spain*
[b] *Animal Reproduction and Obstetrics, University of León, 24071 León, Spain*
[c] *Cabárceno Park, Cantabria, Spain*
[d] *Cell Biology, University of León, 24071 León, Spain*

ARTICLE INFO

Article history:
Received 15 May 2014
Received in revised form 9 September 2014
Accepted 10 September 2014
Available online xxx

Keywords:
Urospermic ejaculates
Osmotic stress reversion
PureSperm®
Brown bear
Spermatozoa

ABSTRACT

The objective of this study was to reverse the osmotic stress of sperm in urine contaminated bear ejaculates that were obtained by electroejaculation using pre-freezing washing or density gradient centrifugation isolation. In Experiment 1, ejaculates were divided into six aliquots, five were diluted in each washing extender: 200, 300, 400, 500 and 700 mOsm/kg (prepared from a Tes–Tris–Fructose base, adding water or fructose as corresponds), at a 1:2 ratio (raw semen: washing solution, v/v); and the other aliquot was handled without washing (Control group). Samples were centrifuged at 600 × *g* for 6 min prior to freezing. In Experiment 2, ejaculates were divided into two aliquots: one was diluted 1:1 with TCG (Tris–Citric acid–Glucose) and centrifuged at 600 × *g* for 6 min (Centrifugation Control; C-Control); the other was treated with PureSperm® density gradient column. After treatments, samples were cryopreserved. Sperm motility, viability (SYBR-14/propidium iodide (PI)) and acrosomal status (peanut agglutinin-fluorescein isothiocyanate (PNA-FITC)/PI) were analyzed before and after freezing. Ejaculates with an initial osmolality of less than 120 mOsm/kg treated with pre-freezing washing, and the Control sample had greater pre-freezing sperm motility than the raw ejaculate, but sperm viability was not different among these groups. The samples washed with 700 mOsm/kg solutions had the least pre-freezing viability. In the post-thawing evaluation, pre-freezing washing treatments did not provide any improvement in comparison with the Control sample, and treatment with 700 mOsm/kg extender had deleterious effects in all urospermic samples. PureSperm® density gradient centrifugation applied to urospermic raw semen was suitable for improving sperm motility and viability of pre-freezing samples and the selected spermatozoa had greater freezing capacity.

1. Introduction

Brown bear ejaculates obtained by electroejaculation have certain characteristics that can interfere with sperm freezing capacity (Anel et al., 2008). Spontaneous contamination of ejaculates with urine occurs frequently in many species (horse, Althouse et al., 1989; man, Chen et al., 1995). In some species, the urine contamination occurs

* Corresponding author at: Cell Biology, University of León, 24071 León, Spain. Tel.: +34 9872911320.
E-mail address: ppazc@unileon.es (P. de Paz).

http://dx.doi.org/10.1016/j.anireprosci.2014.09.007
0378-4320/

when the sample is obtained by electroejaculation (bear: Kojima et al., 2001; Okano et al., 2004; Chen et al., 2007; Anel et al., 2008; felines: Pukazhenthi et al., 2000; Iberian ibex: Santiago-Moreno et al., 2011). An elevated incidence of urine contamination in ejaculates had been reported (brown bear: Anel et al., 2008; Japanese black bears: Kojima et al., 2001 and Okano et al., 2004; Asiatic black bears: Chen et al., 2007).

The deleterious effects of urine on spermatozoa are due to changes in pH and osmolality, as well as to the toxic effect of urea and other urine components (Griggers et al., 2001; Wirtu et al., 2008). Urine contamination was detrimental to sperm motility (Kim and Kim, 1998) and membrane and acrosomal integrity (Santos et al., 2011), and reduces the sperm fertilizing capacity (Santos et al., 2011). Some authors have reported that hypoosmotic solutions are more harmful than hyperosmotic solutions to sperm (ram, bull and human spermatozoa: Blackshaw and Emmens, 1951; horse: Pommer et al., 2002); but others concluded that sperm was affect by exposure to either hypo- or hyperosmotic diluents (bull: Guthrie et al., 2002; stallion: Pommer et al., 2002 and Gonzalez-Fernandez et al., 2012; dog: Santos et al., 2011).

The loss of sperm motility brought about by changes in osmolality was irreversible when bull spermatozoa were returned to an isosmotic diluent (Guthrie et al., 2002); but the spermatozoa of others species are more tolerant to osmotic stress and differ in the ability to recover motility under these conditions (bongo antelope: Wirtu et al., 2008; bear: Okano et al. (2004). In horses, the recovery of progressive sperm motility, reduced by urine contamination, depends on the extender used to dilute semen (Griggers et al., 2001). Human spermatozoa survival exposed to hyperosmotic solutions was affected by temperature and by time of exposure to anisosmotic environments (Gao et al., 1993). Others found serious damage in sperm membrane integrity when hypertonicity was reversed (cats: Pukazhenthi et al., 2000).

Density gradient centrifugation has been suggested as a means of selecting animal spermatozoa for artificial breeding (Morrell et al., 2009). The PureSperm® (Nidacon, Gothenburg, Sweden) density gradient centrifugation technique is designed to select viable and morphologically intact human spermatozoa for assisted reproductive technologies (Soderlund and Lundin, 2000). In previous studies with brown bear semen without urine contamination, it was reported that PureSperm® improved the quality of fresh semen and frozen-thawed semen, but did not improve the freezing capacity of spermatozoa (Nicolas et al., 2012b).

The brown bear population in the Cantabric Mountains is considered as an endangered species (Spanish law: Real Decreto 439/1990; European Union: Council Directive 92/43/EEC, annexes II and IV). A genetic resources bank is necessary to protect this species and this requires the design of specific procedures to reverse the detrimental effects of urine contamination in brown bear sperm cryopreservation.

The aims of this study were: (1) to evaluate the ejaculated brown bear sperm with semen extenders of a different osmolality in an attempt to reduce the deleterious effects of urospermia in post-thawed sperm, and (2) to assess the application of density gradient centrifugation in urine contaminated brown bear spermatozoa to improve freezing capacity.

2. Materials and methods

2.1. Materials

All chemicals were of at least reagent grade and were acquired from Sigma (Madrid, Spain), unless otherwise specified.

2.2. Animals and sample collection

Urospermic ejaculates ($n = 49$) from 13 sexually mature male brown bears were obtained by electroejaculation during the breeding season (end of April to early July) for 4 year. This study was performed at the Cabarceno Park (Cantabria, Spain; 43°21′ N, 3°50′ W; altitude: 143 m). The animals were housed in a partial-freedom regimen and diet consisted of chicken meat, bread and fruits. Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. All experiments were performed after obtaining ethical committee approval from the Ethical Committee for Experimentation with Animals of León University, Spain (03-02/2010).

The animals were immobilized by teleanaesthesia with 750 mg zolazepam HCl+ tiletamine HCl (Zoletil 1001; Virbac, Carros, France, 100 mg/mL) and 6 mg of medetomidine (Zalopin®, Orion Pharma Animal Health, Finland, 10 mg/mL). After immobilization, animals were weighed and monitored during anaesthesia (pulse, saturation of peripheral oxygen and breathing). Prior to electroejaculation, the pubic region was cleaned, the penis washed with sterile physiological saline and the rectum was emptied of faeces. The bladder was catheterized during semen collection. Electroejaculation was conducted with a PT Electronics electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were given until ejaculation (10 V and 250 mA, on average). The ejaculates were considered urine-contaminated when urea >80 mg/dL.

2.3. Experimental design

The different extenders were prepared from TTF-base extender (Tes–Tris–Fructose 300 mOsm/kg, pH 7.1). In Experiment 1, washing extenders were obtained by adding distilled water (200 mOsm/kg) or fructose (400, 500 and 700 mOsm/kg) at TTF-base. For cryopreservation we used the TTF-ULE extender (Tes–Tris–Fructose 300 mOsm/kg, pH 7.1, 6% glycerol, 20% egg yolk, 2% EDTA and 1% Equex Paste; supplemented with 0.302 mg penicillin G sodium salt/mL and 0.625 mg dihydrostreptomycin sesquisulfate/mL; de Paz et al., 2012).

Table 1
Urospermic ejaculates from brown bear: characteristics of different classes of raw semen grouped according to its initial osmolality (OS) in Experiment 1 and raw semen for PureSperm® in Experiment 2 (mean ± SEM).

Class	OS (mOsm/kg)	pH	Urea (ng/dL)
A (n = 8)	96.1 ± 4.5[a]	6.7 ± 0.2[a]	152.4 ± 18.9
B (n = 15)	157.3 ± 5.4[b]	7.1 ± 0.2[ab]	148.8 ± 23.1
C (n = 13)	266.0 ± 17.0[c]	7.5 ± 0.1[b]	163.6 ± 27.1
PureSperm®	146.8 ± 17.2	6.9 ± 0.2	170.5 ± 29.9

Class: A (initial OS < 120 mOsm/kg; pH < 7), B (120 mOsm/kg < initial OS < 200 mOsm/kg; pH > 7), C (initial OS > 200 mOsm/kg; pH > 7).
Different letters in each column indicate significant differences (P < 0.05) between Classes A, B and C.

2.3.1. Experiment 1: pre-freezing washing

The ejaculates were treated with an isosmotic or hyperosmotic medium compared to original osmotic values to dilute urine contamination and/or restore normal osmotic conditions. The pre-freezing washing of brown bear urospermic sperm with extenders of different osmolality (200, 300, 400 and 500 mOsm/kg) and its effects on post-thawing quality of brown bear spermatozoa were evaluated. The negative control was included by washing the ejaculate with hyperosmotic solutions (700 mOsm/kg) to evaluate the effect of osmotic increases on bear spermatozoa. The aim was to restore normal osmotic conditions but some urospermic ejaculate – washing medium combinations produced hyperosmotic conditions and were also tested.

Ejaculates (n = 36) from 13 brown bears were used in this experiment. Immediately after collection, fresh semen was divided into six aliquots; five were diluted with each previously described washing extender (1 fresh semen: 2 extender, v/v) and centrifuged at 600 × g for 6 min (Nicolas et al., 2011); and the other was centrifuged without dilution and was used as the Control. After centrifugation, the supernatant was removed and the pellet was re-suspended in TTF-ULE extender at 20–22 °C and cryopreserved (Section 2.4).

The ejaculates used in this experiment were classified into three classes according to osmotic characteristics and pH of raw semen (Table 1): Class A (osmolality less than 120 mOsm/kg and pH < 7; n = 8), B (osmolality between 121 and 200 mOsm/kg and pH > 7; n = 15) and C (osmolality greater than 201 mOsm/kg and pH > 7; n = 13). Osmolality of urospermic brown bear semen after dilution (1:2, v/v) in each washing extender and centrifugation was calculated as follows: [osmolality of each sample + (2 × osmolality of extender in treatment)]/3.

2.3.2. Experiment 2: PureSperm® gradient applied to urospermic samples

Ejaculates (n = 13) from 13 brown bears were used in this experiment. Immediately after collection, fresh urospermic semen was divided into two aliquots: one aliquot (Centrifugation Control; C-Control) was diluted with the same volume of TCG (Tris, Citric acid, glucose extender) (Nicolas et al., 2011) and centrifuged at 600 × g for 6 min; the other was treated with PureSperm® (Nidacon Laboratories AB, Goteborg, Sweden).

The bottom fraction of the PureSperm® gradient (90%) was prepared by mixing 9 mL of commercially supplied PureSperm® solution with 1 mL of TCG medium. The upper fraction (45%) was obtained by dilution of the 90% solution with an equal volume of TCG medium and stored at 5 °C until used. To prepare the gradient for sperm purification, 3.5 mL of 90% PureSperm® was pipetted into the bottom of the centrifuge tube and 2.2 mL of 45% PureSperm® was carefully layered over the bottom fraction.

The semen sample was placed on top of the upper layer of the PureSperm® gradient and centrifuged at 600 × g for 20 min. The resulting pellet, placed in the bottom of the tube, was aspirated and diluted 1:4 in TCG and centrifuged at 600 × g for 6 min (PureSperm® pellet; PS-Pellet). Semen placed in the interface, between the bottom and upper fraction, was handled in the same way as the previous sample (PureSperm® interface; PS-Interface). The samples for the Centrifugation Control, PS-Pellet and PS-Interface were re-suspended in TTF-ULE/bear extender at 20–22 °C and frozen (see Section 2.4).

2.4. Spermatozoa cryopreservation

Tubes with diluted semen in TTF-ULE/bear extender at 3% glycerol were placed in glass dishes containing 100 mL of water at 20 °C and transferred to a refrigerator at 5 °C, the temperature decreasing slowly to 5 °C (0.25 °C/min, ~70 min). A second 1:1 dilution was then performed at 5 °C, using the TTF extender at 9% glycerol to reach a final concentration of 6% glycerol. Then, more TTF-ULE/bear extender at 6% glycerol was added to obtain a final concentration of 100 × 10^6 spz/mL. After 1 h of equilibration at 5 °C, the semen was packaged into 0.25 mL plastic straws, and the samples were frozen in a programmable biofreezer (Kryo 560-16 Planer™, Planer plc. Sunbury-On-Thames, UK) at −20 °C/min down to −100 °C, and then transferred to liquid nitrogen containers. The cryopreserved samples were stored in liquid nitrogen for a minimum of 1 mo. Thawing was performed by plunging the straws in water at 65 °C for 6 s.

2.5. Semen evaluation

Immediately after collection, the volume of each ejaculate was recorded, osmolality was measured using a cryoscopic osmometer (Osmomat-030, Gonotec TM, Berlin, Germany) and the pH value was determined by pH meter (CG 837-Schott; Germany). For each ejaculate, sample motility was assessed with an E200 phase contrast microscope (Nikon, Tokyo, Japan) and urospermia was evaluated by means of a rapid urea test (Urea test strips, DiaSys Ecoline® GmbH, Holzheim, Germany).

Sperm concentration was assessed using a Bürker hemocytometer (Marienfeld GmbH, Marienfeld, Germany) by Computer-Assisted Semen Analysis (CASA) (ISAS, Integrated Semen Analyser System; Proiser, Valencia, Spain). In Experiment 2, concentration was determined before and after centrifugation and the quantitative recovery rate was calculated (recovered spermatozoa in the pellet/spermatozoa in pre-centrifugation sample, %).

In Experiment 1, the percentage of swollen spermatozoa in raw and washed samples was calculated analysing

100 cells of each sample, previously diluted and fixed in a glutaraldehyde solution (2%), using a phase contrast microscope (400×). The quality of the samples was evaluated before and after freezing/thawing. Recovery rates of different variables were calculated as the post-thawing value/pre-freezing value × 100.

The motility variables were assessed using CASA (Integrated Semen Analyser System; Proiser, Valencia, Spain). The samples were diluted (10–20 × 10^6 cells/mL) in a buffer (HEPES 20 mmol/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose; pH 7; 300 mOsm/kg) with 1% egg yolk, and warmed on a 38 °C plate for 5 min. Then, a 5 μl sperm sample was placed in a Makler cell counting chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel) and examined using a negative phase contrast microscope (100×) with a warmed (38 °C) stage. The general settings of dog spermatozoa for CASA defined by ISAS were used. The settings to define progressive motility in CASA are specific to bears and are defined in a previous experiment (Anel et al., 2010). The settings were as follows: 25 frames/s; 5–50 μm^2 for head area. At least five fields or 200 spermatozoa were saved and analyzed afterwards. Reported variables were total motility (TM, %; spermatozoa were considered motile if curvilinear velocity VCL > 10 μm/s), progressive motility (PM, %; spermatozoa were considered progressive if VCL > 10 and STR > 65), average path velocity (VAP, μm/s) and linearity (LIN, %).

Sperm viability and acrosomal status were analyzed by flow cytometry according to García-Macías et al. (2006). Sperm viability was estimated using the double stain SYBR-14 with PI (LIVE/DEAD Sperm Viability Kit). Sperm samples were diluted with PBS down to 5 × 10^6 sperm/mL, and 300 μL was transferred to a polypropylene tube to which 3 μL PI (24 mM in water) and 1.5 μL SYBR-14 (1 mM in dimethyl sulfoxide (DMSO) were added. The tubes were kept at 37 °C for 20 min in the dark. Three populations were detected and the percentage of viable spermatozoa (SYBR-14+/PI−; VIAB) was recorded.

Triple staining with PNA-FITC (Sigma–Aldrich, Madrid, Spain), propidium iodide (PI; Sigma–Aldrich, Madrid, Spain), and Hoechst 33342 (Sigma–Aldrich, Madrid, Spain) were used to evaluate viability and acrosomal status. Staining was performed by diluting the sperm sample (2 million spermatozoa/mL) in 300 μL of PBS with 1 μg/mL PNA-FITC, 1.5 μM PI, and 5 μM Hoechst 33342. The samples were analyzed by flow cytometry after 10 min at room temperature in the dark. The spermatozoa (Hoechst+) were classified into three sperm subpopulations: IP+ (not viable), IP−/PNA+ (viable with damaged acrosome; dACRO), and IP−/PNA− (viable with intact acrosome).

Flow cytometry analysis was performed using a CyAn ADP flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with 405 nm and 488 nm lasers. The PNA-FITC and PI were excited at 488 nm, and Hoechst 33342 was excited at 405 nm. The fluorescence emitted by PNA-FITC was quantified using the FL1 photodetector (530/40 band pass filter), PI fluorescence was analyzed using the FL3 photodetector (670 long pass filter), and Hoechst 33342 fluorescence was analyzed using the FL6 photodetector (450/50 band pass filter). The signals, forward scatter/side scatter (FSC/SSC), and Hoechst 33342 fluorescence was used to discriminate the sperm population from other events. For each sample, 10,000 spermatozoa were utilized using Summit v 4.3 software (Beckman Coulter). Calibration was conducted periodically using standard beads (Calibrite; Becton Dickinson BioSciences). Flow cytometry data were analyzed using the Weasel v.2.6 program (the Walter and Eliza Hall Institute of Medical Research, Victoria, Australia).

Table 2
Osmolality (OS)[a] of brown bear urospermic semen for each initial osmolality Class (A, B, C) after treatment with washing extender at different osmolality (dilution: 1 fresh semen: 2 extender, v/v).

Washing extender (mOsm/kg)	Class A ($n = 8$)	Class B ($n = 15$)	Class C ($n = 13$)
200	165.0 ± 1.6	185.4 ± 2.1	235.5 ± 6.9
300	232.8 ± 1.5	253.1 ± 1.9	288.7 ± 5.7
400	297.9 ± 1.8	318.8 ± 2.1	363.4 ± 7.9
500	365.4 ± 1.9	386.4 ± 2.4	430.1 ± 7.9
700	499.9 ± 2.1	520.1 ± 3.9	542.7 ± 3.1

Class: A (initial OS < 120 mOsm/kg; pH < 7), B (120 mOsm/kg < initial OS < 200 mOsm/kg; pH > 7), C (initial OS > 200 mOsm/kg; pH > 7).
[a] Calculation: [initial osmolality of each sample + (2 × osmolality of extender in treatment)]/3.

2.6. Statistical analysis

Data were analyzed using the SASTM V.9.0 package (SAS Institute Inc., Cary, NC, USA). Results are shown as means and standard errors of the mean. The effects of different factors on variables of sperm quality were analyzed using linear mixed-effects models (MIXED procedure), considering ejaculate as a random effect. Significant fixed effects were further analyzed using multiple comparisons of means with Tukey contrasts. Differences among groups were considered significant when $P < 0.05$.

3. Results

The fresh brown bear semen samples in this study, had the following values (mean ± SD): volume, 4.0 ± 0.4 mL; cell concentration, 176.2 ± 38.7 × 10^6 spermatozoa/mL; pH, 7.0 ± 0.1; osmolality 161.9 ± 10.9 mOsm/kg.

3.1. Pre-freezing washing

General characteristics of different classes of raw semen samples are shown in Table 1. The urea concentration was similar among all classes but the initial osmolality was different ($P < 0.05$) between Classes A and C. Osmolality values after the washing treatment are included in Table 2, and values for percentage of swollen spermatozoa for each treatment group are included in Table 3. Changes in cell volume (swelling) observed in urospermic samples were reduced after washing and the reduction has a linear relationship with increased washing extender osmolality.

The samples washed with 200 mOsm/kg had the greatest recovery rates of progressively motile sperm after thawing for any of the osmolality classes (Fig. 1), and this value was greater ($P < 0.05$) than that of samples

Table 4
Quality of Class A brown bear spermatozoa (initial osmolality < 120 mOsm/kg; pH < 7) in three samples: Urospermic ejaculates (raw), Control semen (sample centrifuged without pre-freezing washing) and semen after pre-freezing washing with TTF (Tes–Tris–Fructose) extenders of different osmolality (200–700 mOsm/kg); samples were analyzed before freezing and after thawing; data are shown as means ± SEM ($n = 8$).

Class A	TM (%)	PM (%)	VAP (μm/s)	LIN (%)	dACRO	VIAB
Pre-freezing						
Raw	1.8 ± 6.3 a	0.1 ± 4.4 a	5 ± 6.1 a	20.1 ± 5.9 a	6.1 ± 0.9	59.6 ± 5.5
Control	36.7 ± 6.6 b	20.2 ± 4.6 b	40.4 ± 6.1 b	37.9 ± 5.9 b	5.5 ± 1.1	65.3 ± 5.8
200	35.7 ± 6.6 b	17.7 ± 5 b	38.5 ± 6.4 b	38.1 ± 4.9 b	6.1 ± 1.0	60.8 ± 7.5
300	28.8 ± 6.4 b	14.1 ± 4.5 b	37.3 ± 6 b	38.6 ± 5.8 b	5.3 ± 1.1	58.8 ± 5.6
400	34.8 ± 6.8 b	16.9 ± 4.7 b	38.8 ± 6.2 b	36.2 ± 6.1 b	4.7 ± 1.1	61.8 ± 6.0
500	31.7 ± 7.0 b	18.2 ± 4.9 b	37.6 ± 6.5 b	40.1 ± 6.3 b	6.9 ± 1.3	64.1 ± 6.2
700	27.1 ± 7.6 b	17.4 ± 5.3 b	40.4 ± 7.1 b	42.7 ± 6.8 b	5.1 ± 1.4	59.5 ± 7.0
Post-thawing						
Control	34.1 ± 5.5 a	15.3 ± 2.2 b	28.6 ± 2.5	34 ± 1.6 a	10.3 ± 2.1	58 ± 2.5
200	30.7 ± 6.7 ab	14.9 ± 3.2 ab	32.5 ± 3.6	33.7 ± 1.7 a	11.2 ± 1.4	51.3 ± 6.9
300	25.1 ± 5.4 ab	12.5 ± 2.2 ab	33.6 ± 2.4	36.4 ± 1.6 b	11.6 ± 1.5	51.3 ± 2.4
400	25.3 ± 5.7 ab	11.4 ± 2.3 ab	32.8 ± 2.5	36.9 ± 1.7 b	11.1 ± 1.5	52.2 ± 2.6
500	34.8 ± 5.9 a	14.7 ± 2.4 ab	30.6 ± 2.6	34.3 ± 1.7 a	12.2 ± 1.7	55.4 ± 2.7
700	20.4 ± 6.5 b	10 ± 2.6 a	32.9 ± 2.9	36.6 ± 1.9 b	10.6 ± 1.4	55.5 ± 3.0

TM: total motility (%), PM: progressive motility (%), VAP: average path velocity (μm/s), LIN: linearity of the curvilinear trajectory, dACRO: percentage of acrosome-damaged spermatozoa peanut agglutinin-fluorescein isothiocyanate+) and VIAB: percentage of viable spermatozoa (SYBR14+/propidium iodide−).
[a,b] For pre-freezing or post-thawing, different letters in each column indicate differences between treatments ($P < 0.05$).

extender was less ($P < 0.05$) than with the raw, Control, and 200 mOms samples. Post-thawing analysis (Table 5) showed that the 700 mOms treatment resulted in lesser values for PM than 200 and 400 mOms samples; and lesser values of VAP than the rest of the treatments groups and Control group. Samples washed with 700 mOms extender had a greater percentage of damaged acrosomes than the Control. Using of 500 and 700 mOms extenders impairs viability compared to the Control ($P < 0.05$).

For Class C samples: pre-freezing washed sperm samples were not different in motility compared with the Control sperm sample (Table 6). The VAP and LIN decreased in all treatments as compared to those with the raw semen. There were no differences in acrosomal damage. The 700 mOms samples had the least viability ($P < 0.05$; Table 6).

Samples of 700 mOms/kg had the least post-thawing motility and were different in comparison with most of the other sperm samples (Table 6). Acrosomal damage and viability assessments did not indicate any differences between sperm samples.

3.2. PureSperm® applied to urospermic samples

The proportion of spermatozoa recovered from the bottom of the PureSperm® gradient (PS) after centrifugation (31.5 ± 5.9%) was less ($P < 0.05$) than the total number of sperm recovered in the C-Control sample (75.5 ± 4.1%).

The urospermic raw sample improved after the treatment with PureSperm® (Table 7). In pre-freezing, the PS-Pellet sample had greater values ($P < 0.05$) for TM and PM, and lesser values for dACRO than the raw sample. The

Table 5
Quality of Class B brown bear spermatozoa (initial osmolality > 120 mOsm/kg and <200 mOsm/kg; pH > 7) in three samples: Urospermic ejaculates (raw), Control semen (sample centrifuged without pre-freezing washing) and sperm after pre-freezing washing with TTF extenders of different osmolality (200–700 mOsm/kg); samples were analyzed before freezing and after thawing; data are shown as means ± SEM ($n = 15$).

Class B	TM (%)	PM (%)	VAP (μm/s)	LIN (%)	dACRO	VIAB
Pre-freezing						
Raw	37.5 ± 5.3	12.6 ± 2.8	30.8 ± 3.9	33.7 ± 3.5	10.6 ± 1.8	62.6 ± 1.9 a
Control	35.5 ± 5.7	14.9 ± 3	32 ± 4.2	35.1 ± 3.7	10.1 ± 1.8	64.3 ± 1.9 a
200	35.6 ± 5.6	13.3 ± 2.6	30.1 ± 3.7	29 ± 2.9	9.2 ± 2.2	63.4 ± 3.1 a
300	38.4 ± 5.5	16 ± 2.9	34 ± 4.1	31.6 ± 3.6	9.6 ± 1.9	58.9 ± 1.9 ab
400	34.9 ± 5.6	17.1 ± 3	37 ± 4.2	35.6 ± 3.7	10.7 ± 1.9	59.1 ± 2.0 ab
500	35.6 ± 5.9	14.8 ± 3.1	32.1 ± 4.4	32.9 ± 3.9	10.7 ± 2.0	57.5 ± 2.1 ab
700	26.2 ± 8.2	12.7 ± 4.3	34.7 ± 6.1	40.5 ± 5.3	9.9 ± 2.8	56.1 ± 2.7 b
Post-thawing						
Control	30.3 ± 4.4	10.4 ± 1.4 ab	30.2 ± 2.2 a	32.5 ± 1.3	13.7 ± 1.9 a	54 ± 1.9 a
200	34.3 ± 4.9	12.5 ± 1.5 a	31.9 ± 2.3 a	31.7 ± 1.2	16.1 ± 2.5 ab	50.1 ± 2.6 ab
300	30.7 ± 4.3	10.4 ± 1.3 ab	32.2 ± 2.2 a	33.1 ± 1.3	15.7 ± 1.8 ab	50 ± 1.9 ab
400	31.6 ± 4.3	12.4 ± 1.3 a	32.6 ± 2.2 a	33.1 ± 1.3	15 ± 1.9 ab	49.6 ± 1.9 ab
500	29.8 ± 4.6	9.8 ± 1.4 ab	31.9 ± 2.3 a	32.2 ± 1.4	19.4 ± 2.0 ab	45.7 ± 2.0 b
700	25.5 ± 6.6	6.8 ± 2 b	22.6 ± 3.3 b	30 ± 1.9	22.1 ± 2.8 b	46.7 ± 2.9 b

TM: total motility (%), PM: progressive motility (%), VAP: average path velocity (μm/s), LIN: linearity of the curvilinear trajectory, dACRO: percentage of acrosome-damaged spermatozoa (peanut agglutinin-fluorescein isothiocyanate+) and VIAB: percentage of viable spermatozoa (SYBR14+/propidium iodide−).
For pre-freezing or post-thawing, different letters (a, b) in each column indicate differences between treatments ($P < 0.05$).

Table 6
Quality of Class C brown bear spermatozoa (initial osmolality > 200 mOsm/kg; pH > 7) in three samples: urospermic ejaculates (raw), Control semen (sample centrifuged without pre-freezing washing) and sperm after pre-freezing washing with TTF extenders of different osmolality (200–700 mOsm/kg); samples were analyzed before freezing and after thawing; data are shown as means ± SEM (n = 13).

Class C	TM (%)	PM (%)	VAP (μm/s)	LIN (%)	dACRO	VIAB
Pre-freezing						
Raw	52.1 ± 6.4	22.4 ± 4.5	65.7 ± 6.8 a	44.9 ± 3 a	7.7 ± 2.2	63.9 ± 2.7 a
Control	51.2 ± 6.5	22.1 ± 4.6	47.6 ± 6.9 b	37.3 ± 3.1 b	10.3 ± 2.3	58.5 ± 2.7 a
200	53.1 ± 7.9	18.9 ± 4.6	47.3 ± 6 b	32 ± 2.7 b	9.6 ± 2.7	58.2 ± 5.2 a
300	49.8 ± 6.4	20.8 ± 4.5	51.2 ± 6.8 b	34.4 ± 3 b	9.7 ± 2.3	56.6 ± 2.7 a
400	48.9 ± 6.8	17.5 ± 4.8	49.4 ± 7.3 b	32.6 ± 3.2 b	9.6 ± 2.4	55.7 ± 2.9 a
500	44.1 ± 6.9	16.9 ± 4.1	45.9 ± 7.3 b	34.2 ± 3.2 b	10.6 ± 2.4	56.8 ± 2.9 a
700	37.3 ± 8.4	19.6 ± 5.8	49.1 ± 8.7 b	37.7 ± 3.9 b	11.2 ± 3.2	44.6 ± 3.8 b
Post-thawing						
Control	30.8 ± 5.3 a	10.5 ± 3 ab	34.2 ± 6.4 ab	27.5 ± 5.8	20.5 ± 4.1	39.6 ± 3.9
200	35 ± 6.3 a	13.9 ± 2.8 a	38.1 ± 5.7 ab	31.4 ± 4.6	17.2 ± 4.5	38.4 ± 6.6
300	31.1 ± 5.3 a	10.5 ± 3 ab	33.3 ± 6.3 ab	35.4 ± 5.7	19.8 ± 4.1	38.0 ± 3.9
400	28.4 ± 5.7 ab	8.6 ± 3.3 bc	33.6 ± 7 ab	29.2 ± 6.5	20.7 ± 4.1	33.6 ± 3.8
500	25.1 ± 5.3 ab	11.6 ± 2.9 ab	40.1 ± 7 b	31.5 ± 6.5	18.0 ± 4.4	38.0 ± 4.1
700	19.3 ± 6.5 b	6.7 ± 2.6 c	27.4 ± 7.7 a	30.7 ± 6.5	18.7 ± 5.0	34.5 ± 4.8

TM: total motility (%), PM: progressive motility (%), VAP: average path velocity (μm/s), LIN: linearity of the curvilinear trajectory, dACRO: percentage of acrosome-damaged spermatozoa (peanut agglutinin-fluorescein isothiocyanate+) and VIAB: percentage of viable spermatozoa (SYBR14+/propidium iodide−).
For pre-freezing or post-thawing, different letters (a, b) in each column indicate differences between treatments (P < 0.05).

PS-Interface sample had the least values for all variables (P < 0.05) except for dACRO.

Post-thawing analysis (Table 7) indicated that the PS-Pellet sample had more desirable TM and PM values (P < 0.05) than C-Control and PS-Interface. The PS-Pellet had greater values for viability than the C-Control and less acrosomal damage than the PS-Interface (P < 0.05).

4. Discussion

Urine contamination occurs frequently in sperm samples collected by electroejaculation in brown bears producing a detrimental effect on spermatozoa quality. Urine-contaminated samples are usually rejected unless the contamination is minor or there is acceptable motility during the initial evaluation. In brown bears, considering the biological value of ejaculates, methods for salvaging urine-contaminated ejaculates were investigated. Effectiveness of pre-freezing washing with extenders of different osmolality to reverse the deleterious effects of urospermia on the post-thawing quality of brown bear sperm was investigated. Also, a centrifugation gradient selection to improve quality of urospermic brown bear ejaculates was analyzed.

Urospermia causes deleterious effects on semen in several species and these effects are due to changes in pH and osmolality and to the toxic effect of urine components (Griggers et al., 2001). Sensitivity of spermatozoa to osmotic damage differs markedly among species and this determines the ability of cells to recover motility after osmotic stress. The present study on brown bear spermatozoa in anisosmotic conditions showed that the sperm motility is the variable that is most affected. In the present study, motility of urospermic brown bear sperm could be partially restored with pre-freezing washing. Similarly, in bongo antelope sperm (Wirtu et al., 2008) and in stallions sperm (Griggers et al., 2001) it has been reported that the addition of washing extender restored the motility of urine contaminated samples. In humans, conception

Table 7
Quality of spermatozoa in urospermic brown bear semen (raw), semen after dilution with TCG (Tris–Citric acid–Glucose) (C-Control) and centrifugation (C-Control), and semen selected using PureSperm (PS) (two samples were acquired: PS-Pellet and PS-Interface); samples were analyzed before and after freezing; data are shown as means ± SEM (n = 13).

	TM (%)	PM (%)	VAP (μm/s)	LIN (%)	dACRO	VIAB
Pre-freezing						
Raw	35.1 ± 6.7 b	17.4 ± 3.3 b	46.6 ± 7.9 ab	34.6 ± 4.7	9.2 ± 1.1 a	68.1 ± 2.7 a
C-Control	50.5 ± 7.4 a	24.1 ± 6.3 b	46.1 ± 5.1 ab	39.3 ± 1.5	5.2 ± 1.5 b	65.9 ± 3.7 ab
PS-Pellet	61.7 ± 5.9 a	56.9 ± 2.1 a	57.3 ± 3.2 a	45.7 ± 2	4.7 ± 1.4 b	76.8 ± 2.7 a
PS-Interface	20.4 ± 3.0 b	10.5 ± 1.7 b	38.0 ± 3.4 b	38.9 ± 2.4	3.5 ± 1.1 b	57.4 ± 3.5 b
Post-thawing						
C-Control	36.8 ± 5.3 a	15.8 ± 2.2 a	41.2 ± 5.5	32.1 ± 1.4	7.2 ± 1.5 ab	42.3 ± 5.2 a
PS-Pellet	55.2 ± 5.5 b	32.1 ± 3.8 b	56.0 ± 5.5	32.1 ± 2.7	6.2 ± 1.1 a	56.8 ± 3.7 b
PS-Interface	11.1 ± 1.5 c	5.9 ± 1.1 c	49.6 ± 7.5	23.9 ± 4.4	10.1 ± 1.4 b	49.3 ± 4.8 ab

TM: total motility (%), PM: progressive motility (%), VAP: average path velocity (μm/s), LIN: linearity of the curvilinear trajectory, dACRO: percentage of acrosome-damaged spermatozoa (peanut agglutinin-fluorescein isothiocyanate+) and VIAB: percentage of viable spermatozoa (SYBR14+/propidium iodide−).
For pre-freezing or post-thawing, different letters (a, b) in each column indicate differences between treatments (P < 0.05).

was achieved with urospermic semen applying a washing treatment to the semen with a buffer solution followed by centrifugation before artificial insemination (Crich and Jekier, 1978). Nevertheless, centrifugation did not seem to be useful for washing urine contaminated horse semen because there was no benefit in sperm motility reported when comparisons were made with the simple addition of extender. This can be explained by the sedimentation of urine crystals inside the pellet with the spermatozoa which could produce deleterious effects on cells (Griggers et al., 2001). Centrifugation is a necessary step to increase the concentration of brown bear semen obtained by electroejaculation and particularly in urospermic ejaculates that usually have lesser cell concentrations. Previous studies assessing the deleterious effects of centrifugation indicated that it is not a harmful procedure for brown bear spermatozoa (Nicolas et al., 2011; 2012a). In the present study, the Control sample treated only with centrifugation was amongst those with the most desirable values for sperm motility, viability and acrosomal status, and the toxic effect of urine was not evident.

In humans, a progressive loss of sperm motility has been observed when the pH and osmolality deviated from standard values (Makler et al., 1981). The pH effect on motility could be restored shortly after pH was restored to normal values. In the present study, the pH of urospermic semen was not a problem because the deviations from normality were small and the urea concentration was not significantly different in the three classes of raw urospermic semen from bears. This suggests that the most important factor in loss of sperm motility was the deviation of osmolality from isosmotic values.

Several studies have reported that the motility of spermatozoa exposed to anisosmotic conditions and returned to an isosmotic environment was significantly reduced (bull: Guthrie et al., 2002; horse: Pommer et al., 2002). In this respect, hypoosmolality produces more harmful effects on sperm (human, ram and bull: Blackshaw and Emmens, 1951; horse: Pommer et al., 2002 and Macias-Garcia et al., 2012) than hyperosmotic conditions. Considering the restoration of motility in urine contaminated semen with very low osmolality (Class A) after centrifugation or washing, it might suggest that brown bear spermatozoa are more tolerant to hypoosmotic stress than other species. Such differences between species have already been observed by other authors (Curry and Watson, 1994).

An important difference among the three semen classes depending on osmolality was observed in the present study: the sperm motility of the raw semen was negatively affected by hypoosmolality but the viability and acrosomal status did not suffer this negative effect. Consequently, sperm motility is an important variable for evaluating the recovery of urospermic samples. Also, spermatozoa swell more noticeably in Class A than in others sperm classes and this correlates with a greater loss of motility.

Bull sperm undergoes damage in anisosmotic solutions and the mechanisms responsible for sperm motility were seriously affected in extreme ranges of osmolality than was the sperm plasma membrane (Liu and Foote, 1998). Pommer et al. (2002) showed that cellular swelling or shrinkage caused irreversible damage to plasma membranes and cellular organelles of horse spermatozoa, which resulted in an alteration in sperm function. Horse spermatozoa behaved as linear osmometers and responded to anisosmotic conditions by regulating cellular volume but irreversible damage occurs to the spermatozoa plasmalemma and mitochondria (Gonzalez-Fernandez et al., 2012). In the present study, the sperm viability does not differ between classes so it is assumed that sperm plasma membrane integrity was not greatly affected by the hypoosmotic conditions of urine contamination. In stallion spermatozoa exposed to changes in osmolality, it was documented that mitochondria were more sensitive to injuries induced by osmotic changes than was the sperm plasmalemma (Macias-Garcia et al., 2012). Similarly, the mitochondrial function of mouse spermatozoa incubated in hyperosmotic solutions decreased markedly after return to isosmotic solution (Willoughby et al., 1996). This greater sensitivity of mitochondria to osmotic damage could explain the more abrupt loss of motility observed in the present study.

Spermatozoa were more sensitive to osmotic deviations (from hypoosmotic or hyperosmotic to isosmotic conditions) than to a simple exposure to a given non-physiological osmolality (human: Gao et al., 1993; horse: Macias-Garcia et al., 2012). There are no previous studies on osmotic tolerance in brown bear spermatozoa. Results of the present study showed that brown bear spermatozoa can suffer osmotic changes (e.g. Class A from 96 to 499 mOsm/kg) as evidenced by the percentage of swollen cells observed without sperm motility and viability being significantly impacted. Sperm viability was decreased by the osmotic stress in Class B and C samples when the urospermic sperm was washed with 700 mOsm/kg medium and final osmolality was greater than 520. This result is similar to that reported by Songsasen et al. (2002) for dog spermatozoa. These authors reported that sperm motility and plasma membrane integrity of dog spermatozoa was substantially reduced after exposure to solutions with over 500 mOsm/kg osmolality and in the present study only sperm viability was affected. The effects of osmotic changes on the functionality of sperm seems to vary among animal species and in the present study it was demonstrated that plasma membranes of brown bear spermatozoa seem to respond to osmotic stress in a similar way to that of dog spermatozoa.

In the present study, the post-thaw viability of urospermic semen washed in medium with different osmolality was not influenced and this indicates the resistance of the plasma membrane to changes induced by freezing is not affected by osmotic changes induced during treatment. However, results of the present study indicate that a 200 mOsm/kg solution is most desirable for progressive motility whereas osmotic deviations induced by a 700 mOsm/kg solution significantly reduces the progressive motility of post-thaw brown bear semen. The osmolality of the solutions employed in washing the urospermic ejaculates, therefore, affects sperm motility.

An important observation in the present study was the least post-thaw viability and progressive motility was achieved in the Class C semen for all treatments. This

cannot be easily explained based on the differences in osmolality and pH observed between classes. These results may indicate that the washing protocol should be tailored to each sample according to osmotic status. The recovery of sperm motility in horse semen with osmolality altered by urine contamination depends on the extender used to dilute semen (Griggers et al., 2001). This fact suggests that further research should be performed to determine if other diluents are required to improve the recovery of Class C samples.

Bear ejaculates contaminated with urine are usually of poor quality and as previously detected, motility is the variable affected to the greatest extent. The centrifugation gradients allow motile spermatozoa to be selected and thus improve urospermic ejaculates before freezing. Gradient centrifugation techniques have been widely used in several species to improve the quality of semen samples (human: Claassens et al., 1998; Esteves et al., 2000; bull: Rodriguez-Martinez et al., 1997; ram: García-Álvarez et al., 2010; marmoset: Hernández-López et al., 2005; stallion: Morrell et al., 2009 and bongo bull: Wirtu et al., 2008). Previous studies reported that PureSperm® enhanced the motility, viability and acrosomal status of bear sperm when applied to fresh semen (Nicolas et al., 2012b) or post-thawed semen in a double freezing procedure (Alvarez-Rodríguez et al., 2013).

In the present study, the post-thawing motility and viability of urospermic ejaculates treated with PureSperm® were substantially greater than the untreated samples; and the PureSperm® selected spermatozoa had greater freezing capacity than non-selected samples. In agreement with these results, the centrifugation of dog semen in PureSperm® density-gradient improved the motility and viability of frozen-thawed spermatozoa (Dorado et al., 2011). It has also been documented that PureSperm® could be considered to select human fresh and frozen-thawed spermatozoa used in assisted reproductive techniques (Mousset-Simeon et al., 2004). However, Centola et al. (1998) observed that sperm separated by the PureSperm® was not different in motility as compared with fresh specimens but had the greatest percentage of hyper-active sperm. In bongo antelope, Wirtu et al. (2008) demonstrated that processing semen by column gradient centrifugation improved the membrane integrity of post-thaw spermatozoa; however the processed semen showed a proportional motility loss greater than in the sample frozen without column gradient.

Puresperm® gradient could be a viable alternative to improve the quality of urine-contaminated brown bear ejaculates but its efficiency is limited by the loss of a large number of spermatozoa on the gradient. In the present study, a small proportion of spermatozoa were recovered in the PS-Pellet (31.5%) and these data were inferior to those obtained in previous studies without density gradients (~60%: Nicolas et al., 2012a; 58.3%: Alvarez-Rodríguez et al., 2013). When the PureSperm® gradient was applied to post-thawed semen, Maxwell et al. (2007) obtained a 35.8% recovery of freeze–thawed bull spermatozoa, a rate greater than that obtained in a previous study with post-thawed brown bear sperm (12.5%, Nicolas et al., 2012b). This demonstrates that the efficacy of centrifugation gradients varies among species. In this sense, further studies might be conducted to optimize those selection techniques in brown bear semen to recover a greater number of spermatozoa, nevertheless maintaining a high quality selected cells.

In conclusion, contamination of brown bear ejaculates with urine substantially reduces sperm motility but the viability of spermatozoa is not compromised. When the urospermic bear ejaculates were washed during pre-freezing in media of different osmolalities, the most desirable values for sperm motility and viability were obtained with 200 mOsm/kg media and was similar to those obtained with the dilution employed in a standard cryopreservation procedure (without pre-freezing washing). The most effective washing media for the cryopreservation of urospermic ejaculates seems to depend on the initial osmolality of semen. PureSperm® density gradient centrifugation is a suitable method for improving the motility and viability of urine contaminated ejaculates and the selected spermatozoa had a greater resistance to the detrimental impacts of freezing.

Conflict of interest

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

Acknowledgements

This research was supported in part by project CGL2013-48255-R (MINECO, Spain) and CANTUR. S.A. Susana Gomes-Alves was supported by a Ph.D. grant from the Portuguese Ministry of Science and Technology (SFRH/BD/45522/2008), cofounded by POPH–QREN-Tipologia 4.1 (FEDER and MCTES). The authors thank Miguel Angel Marañon, Miguel Angel Prieto and the game keepers of the Cabarceno Nature Park, María Mata Campuzano, Julio Tamayo Canul, Elena Lopez Urueña and Patricia Manrique Revuelta.

References

Althouse, G.C., Seager, S.W., Varner, D.D., Webb, G.W., 1989. Diagnostic aids for the detection of urine in the equine ejaculate. Theriogenology 31, 1141–1148.

Alvarez-Rodríguez, M., Alvarez, M., Lopez-Urueña, E., Martinez-Rodriguez, C., Borragan, S., Anel-Lopez, L., de Paz, P., Anel, L., 2013. Brown bear sperm double freezing: effect of elapsed time and use of PureSperm® gradient between freeze–thaw cycles. Cryobiology 67, 339–346.

Anel, L., Alvarez, M., Martinez-Pastor, F., Gomes, S., Nicolas, M., Mata, M., Martinez, A.F., Borragan, S., Anel, E., de Paz, P., 2008. Sperm cryopreservation in brown bear (*Ursus arctos*): preliminary aspects. Reprod. Domest. Anim. 43 (Suppl. 4), 9–17.

Anel, L., Gomes-Alves, S., Alvarez, M., Borragan, S., Anel, E., Nicolas, M., Martinez-Pastor, F., de Paz, P., 2010. Effect of basic factors of extender composition on post-thawing quality of brown bear electroejaculated spermatozoa. Theriogenology 74, 643–651.

Blackshaw, A.W., Emmens, C.W., 1951. The interaction of pH, osmotic pressure and electrolyte concentration on the motility of ram, bull and human spermatozoa. J. Physiol. 114, 16–26.

Centola, G.M., Herko, R., Andolina, E., Weisensel, S., 1998. Comparison of sperm separation methods: effect on recovery, motility, motion parameters, and hyperactivation. Fertil. Steril. 70, 1173–1175.

Chen, D., Scobey, M.J., Jeyendran, R.S., 1995. Effects of urine on the functional quality of human spermatozoa. Fertil. Steril. 64, 1216–1217.
Chen, L.M., Hou, R., Zhang, Z.H., Wang, J.S., An, X.R., Chen, Y.F., Zheng, H.P., Xia, G.L., Zhang, M.J., 2007. Electroejaculation and semen characteristics of Asiatic black bears (*Ursus thibetanus*). Anim. Reprod. Sci. 101, 358–364.
Claassens, O.E., Menkveld, R., Harrison, K.L., 1998. Evaluation of three substitutes for Percoll in sperm isolation by density gradient centrifugation. Hum. Reprod. 13, 3139–3143.
Crich, J.P., Jekier, A.M., 1978. Infertility in men with retrograde ejaculation: the action of urine on sperm motility, and a simple method for achieving antegrade ejaculation. Fertil. Steril. 30, 572–576.
Curry, M.R., Watson, P.F., 1994. Osmotic effects on ram and human sperm membranes in relation to thawing injury. Cryobiology 31, 39–46.
de Paz, P., Alvarez-Rodríguez, M., Nicolas, M., Alvarez, M., Chamorro, C., Borragan, S., Martinez-Pastor, F., Anel, L., 2012. Optimization of glycerol concentration and freezing rate in the cryopreservation of ejaculate from brown bear (*Ursus arctos*). Reprod. Domest. Anim. 47, 105–112.
Dorado, J., Alcaráz, L., Duarte, N., Portero, J.M., Acha, D., Demyda, S., Muñoz-Serrano, A., Hidalgo, M., 2011. Centrifugation on PureSperm® density-gradient improved quality of spermatozoa from frozen-thawed dog semen. Theriogenology 76, 381–385.
Esteves, S.C., Sharma, R.K., Thomas Jr., A.J., Agarwal, A., 2000. Improvement in motion characteristics and acrosome status in cryopreserved human spermatozoa by swim-up processing before freezing. Hum. Reprod. 15, 2173–2179.
Gao, D.Y., Ashworth, Y., Watson, P.F., Kleinhans, F.W., Mazur, P., Critser, J.K., 1993. Hyperosmotic tolerance of human spermatozoa: separate effects of glycerol, sodium chloride, and sucrose on spermolysis. Biol. Reprod. 49, 112–123.
García-Álvarez, O., Maroto-Morales, A., Ramon, M., del Olmo, E., Montoro, V., Dominguez-Rebolledo, A.E., Bisbal, A., Jimenez-Rabadan, P., Perez-Guzman, M.D., Soler, A.J., 2010. Analysis of selected sperm by density gradient centrifugation might aid in the estimation of in vivo fertility of thawed ram spermatozoa. Theriogenology 74, 979–988.
García-Macías, B., Moran, A.M., Fernandez, L.G., Ferrusola, C.O., Rodriguez, A.M., Bolanos, J.M., da Silva, C.M., Martinez, H.R., Tapia, J.A., Pena, F.J., 2012. The mitochondria of stallion spermatozoa are more sensitive than the plasmalemma to osmotic-induced stress: role of c-Jun N-terminal kinase (JNK) pathway. J. Androl. 33, 105–113.
Gonzalez-Fernandez, L., Morrell, J.M., Pena, F.J., Macias-Garcia, B., 2012. Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. Theriogenology 78, 415–422.
Griggers, S., Paccamonti, D.L., Thompson, R.A., Eilts, B.E., 2001. The effects of pH, osmolarity and urine contamination on equine spermatozoal motility. Theriogenology 56, 613–622.
Guthrie, H.D., Liu, J., Critser, J.K., 2002. Osmotic tolerance limits and effects of cryoprotectants on motility of bovine spermatozoa. Biol. Reprod. 67, 1811–1816.
Hernández-López, L., Umland, N., Mondragon-Ceballos, R., Nayudu, P.L., 2005. Comparison of the effects of Percoll and PureSperm on the common marmoset (*Callithrix jacchus*) semen. J. Med. Primatol. 34, 86–90.
Kim, S.C., Kim, H.W., 1998. Effects of nitrogenous components of urine on sperm motility: an in vitro study. Int. J. Androl. 21, 29–33.
Kojima, E., Tsuruga, H., Komatsu, T., Murase, T., Tsubota, T., Kita, I., 2001. Characterization of semen collected from beagles and captive Japanese black bears (*Ursus thibetanus japonicus*). Theriogenology 55, 717–731.
Liu, Z., Foote, R.H., 1998. Bull sperm motility and membrane integrity in media varying in osmolality. J. Dairy Sci. 81, 1868–1873.
Makler, A., David, R., Blumenfeld, Z., Better, O.S., 1981. Factors affecting sperm motility. VII. Sperm viability as affected by change of pH and osmolarity of semen and urine specimens. Fertil. Steril. 36, 507–511.
Maxwell, W.M., Parrilla, I., Caballero, I., Garcia, E., Roca, J., Martinez, E.A., Vazquez, J.M., Rath, D., 2007. Retained functional integrity of bull spermatozoa after double freezing and thawing using PureSperm density gradient centrifugation. Reprod. Domest. Anim. 42, 489–494.
Morrell, J.M., Dalin, A.M., Rodriguez-Martinez, H., 2009. Comparison of density gradient and single layer centrifugation of stallion spermatozoa: yield, motility and survival. Equine Vet. J. 41, 53–58.
Mousset-Simeon, N., Rives, N., Masse, L., Chevallier, F., Mace, B., 2004. Comparison of six density gradient media for selection of cryopreserved donor spermatozoa. J. Androl. 25, 881–884.
Nicolas, M., Alvarez, M., Gomes-Alves, S., Mata-Campuzano, M., Borragan, S., Martinez-Pastor, F., de Paz, P., Anel, L., 2011. Effects on brown bear (*Ursus arctos*) spermatozoa freezability of different extender and dilution ratios used for pre-freezing centrifugation. Eur. J. Wildl. Res. 57, 259–266.
Nicolas, M., Alvarez, M., Anel, E., Martinez, F., Borragan, S., Martinez-Pastor, F., de Paz, P., Anel, L., 2012a. Spermatozoa recovery and post-thawing quality of brown bear ejaculates is affected for centrifugation regimes. Eur. J. Wildl. Res. 58, 77–84.
Nicolas, M., Alvarez, M., Borragan, S., Martinez-Pastor, F., Chamorro, C.A., Alvarez-Rodríguez, M., de Paz, P., Anel, L., 2012b. Evaluation of the qualitative and quantitative effectiveness of three media of centrifugation (Maxifreeze, Cushion Fluid Equine, and PureSperm 100) in preparation of fresh or frozen-thawed brown bear spermatozoa. Theriogenology 77, 1119–1128.
Okano, T., Murase, T., Tsubota, T., 2004. Electroejaculation and semen cryopreservation of free-ranging Japanese black bears (*Ursus thibetanus japonicus*). J. Vet. Med. Sci. 66, 1371–1376.
Pommer, A.C., Rutllant, J., Meyers, S.A., 2002. The role of osmotic resistance on equine spermatozoal function. Theriogenology 58, 1373–1384.
Pukazhenthi, B., Noiles, E., Pelican, K., Donoghue, A., Wildt, D., Howard, J., 2000. Osmotic effects on feline spermatozoa from normospermic versus teratospermic donors. Cryobiology 40, 139–150.
Rodriguez-Martinez, H., Larsson, B., Pertoft, H., 1997. Evaluation of sperm damage and techniques for sperm clean-up. Reprod. Fertil. Dev. 9, 297–308.
Santiago-Moreno, J., Toledano-Diaz, A., Sookhthezary, A., Gomez-Guillamon, F., Salas de la Vega, R., Pulido-Pastor, A., Lopez-Sebastian, A., 2011. Effects of anesthetic protocols on electroejaculation variables of Iberian ibex (*Capra pyrenaica*). Res. Vet. Sci. 90, 150–155.
Santos, I.P., Cuhna, I.C.P., Melo, E.T.J., 2011. Effects of urine and NaCl solutions of different osmolarities on canine sperm. Anim. Reprod. 8, 73–79.
Soderlund, B., Lundin, K., 2000. The use of silane-coated silica particles for density gradient centrifugation in in-vitro fertilization. Hum. Reprod. 15, 857–860.
Songsasen, N., Yu, I., Murton, S., Paccamonti, D.L., Eilts, B.E., Godke, R.A., Leibo, S.P., 2002. Osmotic sensitivity of canine spermatozoa. Cryobiology 44, 79–90.
Willoughby, C.E., Mazur, P., Peter, A.T., Critser, J.K., 1996. Osmotic tolerance limits and properties of murine spermatozoa. Biol. Reprod. 55, 715–727.
Wirtu, G., Pope, C.E., MacLean, R.A., Godke, R.A., Paccamonti, D.L., Dresser, B.L., 2008. Reversal of motility loss in bongo antelope (*Tragelaphus eurycerus isaaci*) spermatozoa contaminated with hypoosmotic urine during electroejaculation. Anim. Reprod. Sci. 103, 392–397.

DISCUSIÓN GENERAL

Los bancos de recursos genéticos (BRG) en asociación con la aplicación de técnicas de reproducción asistida juegan un papel importante en la preservación de especies amenazadas, como es el caso del oso pardo en España. En este contexto, la crioconservación de espermatozoides se presenta como una herramienta básica para preservar recursos genéticos y evitar la extinción de especies amenazadas.

La optimización de protocolos de conservación espermática adaptados a esta especie, implica el estudio de varios factores que pueden influir en la calidad de las muestras seminales criopreservadas. En la presente tesis doctoral se han evaluado tres aspectos relacionados con la congelabilidad de eyaculados de oso pardo: 1) el desarrollo de un diluyente específico adaptado a las condiciones particulares de los espermatozoides de esta especie; 2) la cuantificación de la especificidad de los extender de criopreservación espermática en estos úrsidos, mediante la evaluación de alternativas comerciales definidas para otras especies, y 3) el empleo de estrategias para mejorar la congelabilidad de muestras problemáticas (espermioaglutinación y contaminación con orina).

La elección de un protocolo de congelación eficiente (diluyentes optimizados, así como un patrón de tratamiento térmico adecuado), es crucial para el éxito del proceso de criopreservación seminal en cualquier especie. En el primer estudio de la presente tesis doctoral se evalúan diferentes aspectos básicos de la composición del diluyente con el fin de encontrar la combinación más adecuada para la congelación de espermatozoides de oso pardo. En este trabajo se utiliza como base un diluyente diseñado en nuestro laboratorio (Anel et al. 2003) para la especie ovina, lo que permite un fácil ajuste de sus componentes.

La yema de huevo es uno de los protectores de membrana más utilizados en los protocolos de conservación de semen, tanto en especies domesticas como salvajes, y su principal función es proteger los espermatozoides contra los efectos deletéreos provocados por el frío (White. 1993; Fernandez-Santos et al. 2006, Gil et al. 2011). La yema de huevo actúa sobre la membrana celular, preservando la movilidad, viabilidad y integridad de los espermatozoides tras la descongelación (Salamon and Maxwell. 2000). A pesar de sus efectos beneficiosos, la yema de huevo puede producir efectos nocivos en los espermatozoides tanto en la integridad acrosomal (Holt et al. 1996) como en la viabilidad espermática (Ritar et al. 1991). Distintas concentraciones han sido usadas en la congelación de semen de oso: 10 y 20% en oso pardo (García-Macías et al. 2005; Anel et al. 2008), 15% en oso pardo de Hokkaido (Ishikawa et al. 2002) y 20% en oso negro japonés (Okano et al. 2006b). Estos autores no hacen estudios comparativos entre distintos porcentajes de yema y solamente describen su eficacia en sus condiciones de trabajo, así Ishikawa et al. (2002) observan una movilidad post-descongelación del 43% y un 67% de viabilidad. Okano et al. (2006b) obtienen una calidad inferior (movilidad individual: 16% y viabilidad: 54,3%) con un diluyente compuesto por TRIS-citrato-fructosa-glicerol y 20% de yema. En nuestro estudio, la concentración del 20% es la recomendada para la congelación de semen de oso pardo por proporcionar mejores resultados de movilidad, viabilidad y estatus acrosomal. No obstante, algunos autores han documentado un efecto negativo de concentraciones elevadas de yema de huevo tanto en la preservación de la movilidad como de la integridad acrosomal (gacela Mohor: Ritar et al. 1991). El efecto de la yema de huevo parece estar condicionado por la composición del diluyente (Watson et al. 1976). De este modo, Garde et al. (2008) usando un diluyente con rafinosa suplementado con dos

concentraciones de yema distintas (5% vs 20%) comprobó que la concentración más elevada de yema de huevo proporciona mejor preservación de la movilidad e integridad de membrana durante el proceso de refrigeración en espermatozoides de gacela Cuvier (Garde et al. 2008). Estos resultados concuerdan con los obtenidos en espermatozoides epididimarios de ciervo rojo usando diferentes concentraciones de yema (0; 5 y 20%), en que el efecto protector de la yema de huevo es más evidente al 20% (Fernández-Santos et al. 2006).

El glicerol es el crioprotector de elección para la criopreservación de semen de oso y su concentración en el medio varía entre 4-8% en las diferentes especies en las que se ha realizado congelación espermática (Seager et al. 1987; Anel et al. 1999; Ishikawa et al. 2002; Okano et al. 2004; Spindler et al. 2004; Zhang et al. 2005). Nuestros resultados concuerdan con las concentraciones de glicerol utilizadas en otros úrsidos. En algunas especies la movilidad espermática de las muestras descongeladas se ve influida por diferentes concentraciones de glicerol (caballo: Cochran et al. 1984; cerdo: Buhr et al. 2001; ovino: Fiser and Fairfull. 1984 y macaco Rhesus: Si et al. 2004). No obstante, en oso negro japonés la movilidad espermática no está afectada por distintas concentraciones de glicerol (4-12%) pero el empleo del 4 y 6% de glicerol proporcionó un porcentaje de viabilidad y de acrosomas intactos más elevado (Okano et al. 2006b).

En nuestro trabajo no se encontraron diferencias significativas en los parámetros estudiados (movilidad espermática, viabilidad, integridad acrosomal y actividad mitocondrial) para las dos concentraciones de glicerol analizadas (4 y 8%). Esto puede indicar que la concentración óptima de glicerol para la congelación de semen de oso pardo se sitúa en este rango. Trabajos posteriores, planteados por nuestro grupo, se

han centrado en determinar la concentración más adecuada de glicerol (de Paz et al. 2012) y el mejor momento de adición del mismo (Rodriguez-Alvarez et al. 2011). De Paz et al. (2012) evaluó diferentes concentraciones de glicerol (2-10%) y distintas rampas de congelación (-10 ºC/min, -20 ºC/min y -40 ºC/min) y definió la concentración del 6% de glicerol en combinación con la rampa de congelación media (-20 ºC/min) como la más indicada para la criopreservación de eyaculados de oso pardo. Con relación al momento de adición del glicerol, no se encontraron diferencias significativas en la movilidad y viabilidad de los espermatozoides en los tres modelos estudiados (adición total de glicerol a temperatura ambiente; adición fraccionada en dos pasos y adición después de la refrigeración). La concentración óptima de glicerol del 6%, definida por de Paz et al. (2012), está de acuerdo con los resultados obtenidos en este trabajo ya que se sitúa entre los dos porcentajes de glicerol ensayados que no marcaron diferencias entre sí. En cuanto al modelo de adición de glicerol se ha comprobado que permite un amplio margen de flexibilidad para la adaptación de los procedimientos del protocolo de congelación.

La incorporación de aditivos (componentes no habituales en los diluyentes patrón) en los medios de congelación espermática, responde al objetivo de aportar soluciones específicas a cuestiones particulares de los eyaculados de cada especie y de su congelabilidad. En nuestro caso, se han empleado dos sustancias (1% Equex STM paste y 2% EDTA) al objeto de controlar la espermioaglutinación, una de las problemáticas específicas que se plantean en el manejo de eyaculados de oso pardo obtenidos mediante electroeyaculación.

Aunque el uso del Equex STM paste y el EDTA no está documentado en la congelación de espermatozoides de oso, nuestros resultados indican que su

incorporación en el diluyente supone una mejora significativa en la calidad seminal. La adicción de Equex STM paste en el diluyente de congelación incrementa la movilidad post-descongelación de semen de perro (Alhaider and Watson. 2009) y disminuye el daño acrosomal post-descongelación en espermatozoides epididimarios de gato (Axner et al. 2004). El Equex STM paste es un agente surfactante que actúa como un detergente cuyo componente activo es el SDS (sulfato dodecil sódico) y que podría ejercer su efecto doblemente: modificando la estructura de las lipoproteínas de la yema de huevo en el medio extracelular (Arriola and Foote. 1987) y mejorando la estabilidad de los lípidos de la membrana plasmática (Peña et al. 2000; Gil et al. 2013). En este sentido, su uso como agente surfactante (principalmente de la yema de huevo) mejora la supervivencia de los espermatozoides tras la descongelación y se piensa que su función está relacionada con la estabilización de las membranas celulares, particularmente de la membrana acrosomal, protegiendo a los espermatozoides contra los efectos nocivos del glicerol durante el proceso de congelación-descongelación (Arriola and Foote. 1987; Anakkul et al. 2011). En la especie canina, se ha descrito que su efecto beneficioso en la congelación de espermática podría estar relacionado con una disminución de los cambios celulares similares a los que ocurren en la capacitación de los espermatozoides (implícitos al proceso de congelación), como son, cambios en la permeabilidad de la membrana espermática al Ca^{++} y la disminución de la hiperactivación espermática (Peña et al. 2003). No obstante, poco se conoce del mecanismo protector de esta sustancia pero su efecto solo ocurre en presencia de la yema de huevo, esto puede sugerir que ejerce su efecto positivo mediante la alteración de las lipoproteínas de baja densidad de la yema de huevo antes que directamente sobre las membranas celulares (Pursel et al. 1978).

El EDTA es un agente quelante que bloquea la acción del calcio como mediador de la capacitación espermática y reacción acrosomal (Aisen et al. 2000). La interacción del calcio (Ca^{++}) con los lípidos de la membrana influye considerablemente en la carga superficial neta, en la orientación de las cabezas polares, en el grado de solubilización y en la fase de transición con relación a los cambios de temperatura (Bakas and Disalvo. 1991).

Estudios en verraco, demuestran que tanto el calcio extracelular como la albumina sérica promueven la aglutinación (cabeza-cabeza) de los espermatozoides (Harayama et al. 1998; Harayama et al. 2000). También se observa que el calcio (Ca^{2+}) libre en el citoplasma está implicado en la aglutinación (cabeza-cabeza) inducida por un análogo del cAMP (Harayama et al. 2003). Además, la adicción de otro agente quelante del calcio, similar al EDTA pero más selectivo (EGTA), al medio de incubación atenúa el nivel de aglutinación espermática inducido por la concentración extracelular de calcio en eyaculados de verraco (Harayama et al. 1998). En toro, la adición de EGTA al diluyente seminal reduce el porcentaje de espermatozoides aglutinados tras un período de incubación de 72 horas (Yang et al. 2012).

Estudios previos en diferentes especies, indican los efectos beneficiosos del suplemento de los diluyentes con estos aditivos en la calidad seminal post-descongelación: perro (Rota et al. 1997; Peña et al. 2003; Ponglowhapan and Chatdarong. 2008; Alhaider and Watson. 2009), gato (Axner et al. 2004), ovino (Aisen et al. 2000), cerdo (Pursel et al. 1978), toro (Arriola and Foote. 1987) y ciervo (Cheng et al. 2004).

Una vez definida la mejor opción para los factores estudiados anteriormente (yema de huevo-20%, glicerol-8% y suplemento con aditivos Equex STM paste y EDTA)

se probaron dos diluyentes ajustados a 300 ó 320 mOsm/kg (E300 y E320, respectivamente). El E300 mostró mejores resultados para algunos de los parámetros cinéticos analizados (VAP, VCL, VSL y ALH). En este contexto, se confirma la hipótesis de que el uso de un diluyente con una osmolalidad (E300) ligeramente hipoosmótica respecto de la esperada para la muestra seminal (308 ± 38 mOsm/kg) sería la mejor opción para la criopreservación de semen de oso pardo.

Nuestros resultados estarían en desacuerdo con estudios realizados en otras especies (toro: Liu et al. 1998b; ovino: Aisen et al. 2000 y ciervo: Martinez-Pastor et al. 2006) en los que diluyentes ligeramente hiperosmóticos proporcionan mejor protección a los espermatozoides que los isosmóticos. En este sentido, la sensibilidad de los espermatozoides a los cambios en la osmolalidad del medio varía según la especie y se presenta como un factor decisivo en la eficacia del proceso de congelación (Liu et al. 1998b). Diversos estudios en ovino, demuestran los efectos osmóticos asociados a la adición y retirada de glicerol en la calidad seminal post-descongelación (Gao et al. 1995).

Considerando la escasa información sobre la criobiología del espermatozoide de oso pardo, los resultados de este estudio aportan una contribución importante para el desarrollo de un protocolo de criopreservación eficiente para esta especie. La mejor combinación de las testadas para la congelación de eyaculados de oso pardo sería una base de TES-Tris-Fructosa ajustada a 300 mOsm/kg y suplementada con 20% de yema de huevo, 4-8% de glicerol y aditivos (2% EDTA y 1% Equex STM paste).

A pesar de que el diluyente específicamente desarrollado para la congelación de eyaculados de oso pardo (Anel et al. 2010 modificado por de Paz et al. 2012) presenta buenos resultados en la evaluación in vitro post-descongelación, la posibilidad de

emplear diluyentes comerciales de fácil adquisición puede suponer una solución muy útil en situaciones de emergencia. Por ello, resulta interesante evaluar el nivel de especificidad de los espermatozoides de oso pardo, de cara al proceso de criopreservación, ensayando la tolerancia mostrada cuando se emplean medios de congelación formulados en función de las particularidades de otras especies. En nuestro caso, hemos empleado diluyentes comerciales desarrollados para especie bovina (Andromed® y Bioxcell®) y canina (Triladyl canine®, CaniPro® y Extender 2).

El Andromed® y el Bioxcell® son diluyentes comerciales que tienen en su constitución soja como componente sustitutivo de la yema de huevo. Los diluyentes sin componentes de origen animal (yema de huevo o leche) son cada vez más recomendables con el fin de evitar el riesgo de transmisión de enfermedades (de Ruigh et al. 2006). Estudios de nuestro grupo, usando el diluyente específico de oso TTF-ULE/bear suplementado con 5% de lecitina de soja tipo A (Alvarez-Rodriguez et al. 2013a), demuestran que la movilidad espermática es similar a la de las muestras congeladas con el mismo diluyente suplementado con yema de huevo, pero la viabilidad espermática y la integridad acrosomal son inferiores en las muestras congeladas con soja respecto al control con yema. En el presente trabajo, las muestras criopreservadas con Andromed® presentaron resultados similares a los obtenidos con TTF/ULE-bear en todos los parámetros estudiados. El Andromed® ha sido empleado en diversas especies con resultados muy variables. Estudios en ovino (Fukui et al. 2008), ciervo (Martinez-Pastor et al. 2006) y gacela (Saragusty et al. 2006) referencian el uso del Andromed® con resultados similares a los obtenidos con diluyentes basados en la yema de huevo. En cambio, en otras especies como el búfalo (Herold et al. 2004; Herold et al. 2006), el perro (Nothling et al. 2007) y el gato (Jimenez et al. 2013), la

utilización de Andromed® tiene un efecto deletéreo sobre la calidad de los espermatozoides. Los resultados contradictorios encontrados podrían ser explicados, tanto por el origen de los espermatozoides (epididimario o eyaculado), el tipo de lipoproteína empleado, así como por la respuesta específica al efecto protector de la lecitina de soja.

El Bioxcell® ha sido empleado en la criopreservación de semen de toro (Stradaioli et al. 2007), ovino (Gil et al. 2003a; Gil et al. 2003b) y caprino (Roof et al. 2012) con resultados similares a los obtenidos usando diluyentes basados en yema de huevo o leche. Además, el Bioxcell® se manifestó más efectivo que el Andromed® en la preservación del potencial fertilizante del semen de carnero tanto refrigerado como congelado (Khalifa et al. 2013); no obstante, el Bioxcell® demostró peores resultados de movilidad tras la congelación de semen de ciervo rojo (Martinez-Pastor et al. 2009). En este estudio, el Bioxcell® es menos efectivo en la protección de los espermatozoides de oso pardo durante la congelación que el TTF-ULE/bear y el Andromed®. Estos resultados indican que el Bioxcell® estará más específicamente indicado para rumiantes y sugieren que los componentes desconocidos presentes en este diluyente comercial no confieren un efecto protector eficiente sobre las estructuras celulares de los espermatozoides de oso pardo durante el proceso de criopreservación.

El Triladyl canine®, CaniPro® y Extender 2 son diluyentes utilizados en la criopreservación de semen de perro y tienen en su composición yema de huevo. Los resultados de este estudio indican que estos diluyentes adaptados a la especie canina, no son tan eficientes como el TTF-ULE/bear para la congelación de espermatozoides de oso pardo. A pesar de que la fracción de CaniPro® y Extender 2 que contiene el glicerol se añade normalmente solo después de la refrigeración (en dos pasos), en nuestro

trabajo se emplean en un solo paso -glicerol parcial desde la dilución inicial- para uniformizar con el resto de protocolos); no obstante, esta diferencia de procedimiento respecto a lo propuesto por la marca que manufactura los diluyentes comerciales no tendría que ser responsable de los pobres resultados obtenidos en la congelación espermática de oso pardo dado que en un estudio previo de nuestro grupo de trabajo, no se encontraron diferencias en la movilidad y viabilidad espermática post-descongelación, cuando el glicerol se añadió a temperatura ambiente ó a 5º C usando el TTF-ULE/bear (Alvarez-Rodriguez et al. 2011). Estos resultados evidencian una buena tolerancia del semen de oso pardo a la adición de glicerol a temperatura ambiente. El hecho de que los diluyentes basados en yema de huevo utilizados en este estudio, tengan la misma concentración de yema y una concentración de glicerol muy similar, sugiere que las diferencias encontradas entre ellos sean debidas a otros componentes, que para el caso de los diluyentes comerciales son de difícil identificación, debido a las normas de protección de patentes.

Los azúcares son otro de los componentes básicos de los diluyentes seminales que juegan un papel muy importante en el proceso de conservación de las células espermáticas. Okano et al. (2006a) refieren algunos estudios que indican que el suplemento efectivo de los azúcares varía de acuerdo con la especie; en la congelación de semen de oso negro japonés obtienen resultados similares (viabilidad post-descongelación mayor del 50%) con la utilización de tres diluyentes que contienen diferentes azucares (glucosa, fructosa y fructosa-lactosa-rafinosa). Dos de los diluyentes empleados en nuestro estudio contienen fructosa (TTF-ULE/bear y Andromed®) y otros dos contienen glucosa (CaniPro® y Extender 2); en los demás diluyentes se desconoce el azúcar presente. Del análisis de las experiencias realizadas

en la presente tesis doctoral, nuestros resultados parecen sugerir que la fructosa sería mejor opción que la glucosa en los diluyentes de criopreservación espermática adaptados al semen de oso pardo. En conclusión, aunque el diluyente específico, TTF-ULE/bear es el más adecuado para la congelación de semen de oso pardo, el Andromed® es una alternativa válida por presentar resultados similares; la disponibilidad comercial de este último, hace que pueda ser de gran utilidad en situaciones de emergencia o en aquellos ámbitos en los que no se disponga de la infraestructura necesaria para la elaboración de un diluyente específico.

Retomando de nuevo la problemática de la aglutinación espermática en esta especie y con el fin de establecer un control eficaz y temprano de la misma, en este trabajo hemos observado que la recogida de semen directamente sobre un medio TTF-H reduce significativamente el grado de aglutinación en las muestras frescas sin interferir en la calidad seminal de las muestras precongeladas y descongeladas. La aglutinación espermática puede ocurrir entre los espermatozoides (cabeza-cabeza o cabeza-cola) o con otras partículas presentes en la muestra. Monga y Roberts. (1994) refieren que las bacterias fimbriadas se pueden adherir a las glicoproteínas de la superficie de los espermatozoides induciendo la aglutinación de espermatozoides móviles. Además, algunas proteínas específicas de la superficie espermática están implicadas en la aglutinación espermática (Flaherty et al. 1993; Fornes and Burgos. 1994). También se ha descrito que una alta densidad espermática puede implicar una mayor frecuencia de colisiones entre regiones acrosomales y en estas zonas existen mayor número de proteínas que favorecen las asociaciones celulares. Por lo tanto, probablemente la densidad celular sea un factor más, que influye en la tasa de aglutinación espermática (Lindahl and Sjoblom. 1981). La disminución de la

aglutinación observada en nuestro estudio tras la dilución inicial, puede estar relacionada con la reducción de la actividad bacteriana, con el enmascaramiento de las proteínas de la superficie espermática y/o con la disminución de la densidad espermática.

La contaminación de los eyaculados con orina es un problema que ocurre con bastante frecuencia en las muestras de oso pardo obtenidas por electroeyaculación, afectando negativamente la calidad del semen y a su resistencia a la congelación. En otras especies, las muestras de semen contaminadas con orina normalmente son eliminadas, a no ser que la contaminación sea mínima o presenten una movilidad aceptable en la evaluación inicial. En oso pardo, teniendo en cuenta el valor de las muestras espermáticas, es importante investigar estrategias que permitan recuperar (aunque sea parcialmente) los eyaculados urospérmicos (Anel et al. 2008), pues la eliminación de los mismos en base a un criterio de selección inicial por calidad no parece admisible dadas las especiales condiciones que residen en dichas muestras y en la especie donante (especie con una población local amenazada).

En este contexto se evalúan la efectividad de un lavado pre-congelación con diluyentes de diferentes osmolalidades para revertir los efectos nocivos de la urospermia en la calidad del semen de oso pardo tras la descongelación, así como el uso de un gradiente de selección espermática (Puresperm®) para mejorar la calidad de los eyaculados urospérmicos de oso pardo destinados a ser criopreservados previamente a su almacenamiento en el banco de recursos genéticos de la especie.

La sensibilidad de los espermatozoides a los cambios osmóticos varía entre especies y determina la capacidad de cada una de ellas para restaurar la movilidad tras un estrés osmótico (Songsasen et al. 2002), que es el responsable directo de los daños

observados en las muestras urospérmicas. En el presente estudio se constató que la movilidad es el parámetro más afectado en la exposición de los espermatozoides de oso pardo a condiciones no isosmóticas. No obstante, también en nuestro trabajo, se observó que dicha movilidad se podría recuperar parcialmente con un lavado pre-congelación. Del mismo modo, estudios en otras especies documentan que la adición de un medio de lavado recupera la movilidad de las muestras de semen contaminadas con orina (antílope bongo: Wirtu et al. 2008; caballo: Griggers et al. 2001). En humana, se obtienen buenos resultados de fertilidad con semen urospérmico, aplicando un tratamiento de lavado y sometiéndole a centrifugación (reconcentración) previamente a la inseminación artificial (Crich and Jequier. 1978). Sin embargo, en caballo, la centrifugación no aporta ninguna mejora en la movilidad espermática con respecto a la simple adición de diluyente (Griggers et al. 2001). En oso pardo, la centrifugación es un procedimiento necesario para reconcentrar las muestras de semen, especialmente en los eyaculados urospérmicos que normalmente tienen una concentración de células menor a la deseable para abordar el proceso de criopreservación. Estudios previos de nuestro grupo indican que los espermatozoides de oso pardo toleran el proceso de centrifugación dentro de un amplio margen de variables (tiempo y velocidad) (Nicolas et al. 2011; Nicolas et al. 2012a). En nuestro trabajo, las muestras del grupo control sometidas solamente a centrifugación están entre las que presentan mejores resultados de movilidad, viabilidad y estatus acrosomal tanto en pre-congelación como en post-descongelación.

En especie humana, se observa que las variaciones de pH y osmolalidad tienen un efecto negativo en la movilidad espermática (Makler et al. 1981). En el presente estudio, el pH de las muestras urospérmicas presenta pequeñas variaciones sobre la

normalidad y no se encontraron diferencias significativas entre los niveles de urea de las tres clases de semen fresco contaminado con orina. Estos datos indican que el factor crítico responsable de la pérdida de movilidad espermática es el cambio osmótico.

El grado de recuperación de la movilidad espermática en los eyaculados urospérmicos con baja osmolalidad (clase A: osmolalidad inicial < 120 mOsm/kg) tras la centrifugación o lavado, sugiere que los espermatozoides de oso pardo son más tolerantes al estrés hiposmótico que los de otras especies en las que los efectos de la hiposmolalidad sobre las células espermáticas son más nocivos que los de la hiperosmolalidad (humana, ovino y toro: Blackshaw and Emmens. 1951; caballo: Pommer et al. 2002; Garcia et al. 2012). El porcentaje de espermatozoides con algún grado de endósmosis, también es claramente más elevado en la clase A que en las demás clases y parece estar correlacionado con una disminución más acentuada de la movilidad.

Uno de los resultados más destacables de este estudio indica que la movilidad espermática del semen fresco contaminado es el parámetro más afectado por las condiciones hiposmóticas, mientras que la viabilidad y el estatus acrosomal no se encuentran afectados. De esta forma, la movilidad espermática es el factor clave para evaluar el grado de recuperación de la calidad de las muestras seminales urospérmicas, ya que esta característica se ha demostrado que tiene cierta reversibilidad.

En el presente trabajo, la viabilidad espermática de las muestras urospérmicas frescas no difiere entre las distintas clases, lo que indica que la integridad de la membrana plasmática no está tan afectada como la movilidad por las condiciones hiposmóticas de la contaminación con orina. Del mismo modo, en toro, los mecanismos

fisiológicos responsables de la movilidad espermática están más afectados que la membrana plasmática tras la exposición a condiciones extremas de osmolalidad (Liu and Foote. 1998a). Hay diversos estudios que refieren que los cambios osmóticos provocan daños irreversibles en la membrana plasmática y mitocondrias de los espermatozoides de caballo (Pommer et al. 2002; Gonzalez-Fernandez et al. 2012), demostrándose una mayor sensibilidad de las mitocondrias al estrés osmótico (Garcia et al. 2012). De igual modo, se observó un descenso brusco de la actividad mitocondrial de los espermatozoides de ratón sometidos a variaciones osmóticas (Willoughby et al. 1996). Esta mayor sensibilidad de las mitocondrias al estrés osmótico podría explicar la acentuada perdida de movilidad observada en nuestro estudio.

Los espermatozoides son menos tolerantes a excursiones osmóticas (de condiciones hiposmóticas o hiperosmóticas a condiciones isosmóticas) que a la simple exposición a osmolalidades no fisiológicas (humana: Gao et al. 1995; caballo: Garcia et al. 2012). A pesar de que no hay estudios previos sobre la tolerancia a los cambios osmóticos de los espermatozoides de oso pardo, nuestros resultados muestran que los espermatozoides sufren cambios osmóticos bruscos (ej. clase A de 96.1 a 499 mOsm/kg) como evidencia el alto porcentaje de células que presentaron endosmosis en algún grado, pero en la evaluación pre-congelación (después de estas excursiones osmóticas), la movilidad y la viabilidad espermática no mostraron diferencias significativas salvo en casos muy puntuales (que afectan solo a la viabilidad). El tratamiento de las muestras seminales de las clases B y C con el medio de 700 mOsm/kg y con una osmolalidad final mayor de 520 tiene un efecto prejudicial sobre la viabilidad espermática en pre-congelación. Estos resultados concuerdan, en parte, con los obtenidos por Songsasen et al. (2002) en espermatozoides caninos, lo que indica

que la tolerancia de la membrana plasmática de los espermatozoides de oso pardo al estrés osmótico sería similar a la de los espermatozoides caninos.

Por otro lado y respecto a la estrategia de selección espermática, señalar que si partimos de una muestra, afectada en mayor o menor medida por el estrés osmótico -urospermia-, seria de indudable interés de cara a la congelación espermática eficiente, poder separar las células resistentes a la agresión osmótica, para proceder de esa forma congelar una muestra de mejor calidad inicial. Los gradientes de selección han sido empleados en diferentes especies para mejorar la calidad de las muestras seminales, mediante la selección de células espermáticas con mejor patrón de motilidad: humano (Claassens et al. 1998; Esteves et al. 2000); toro (Rodriguez-Martinez et al. 1997); ovino (Garcia-Alvarez et al. 2010); mono Marmoset (Hernandez-Lopez et al. 2005); caballo (Morrell et al. 2009) y antílope Bongo (Wirtu et al. 2008). En estudios previos de nuestro grupo en oso pardo, se ha demostrado que el uso del gradiente Puresperm® tanto en semen fresco (Nicolas et al. 2012b) como en semen descongelado sometido a dos ciclos de congelación (Alvarez-Rodriguez et al. 2013b) mejora la calidad de la población espermática seleccionada. En el presente estudio, el empleo del gradiente Puresperm® en eyaculados urospérmicos de oso pardo mejora la movilidad y viabilidad de los espermatozoides seleccionados, además los espermatozoides seleccionados presentan mejor congelabilidad. Estos resultados concuerdan con los estudios realizados en semen de perro (Dorado et al. 2011) y en humana (Mousset-Simeon et al. 2004). En antílope Bongo (Wirtu et al. 2008), se observó también una mejora en la integridad de la membrana y movilidad de los espermatozoides descongelados seleccionados con el gradiente de selección, sin embargo comparando la movilidad pre y post-descongelación, proporcionalmente, la

población espermática seleccionada manifestó una pérdida de movilidad más acentuada que los espermatozoides congelados sin tratamiento. Esto sugiere que, al contrario que en nuestro estudio, los espermatozoides seleccionados son más sensibles a los daños de la congelación. La mejora de la congelabilidad en las muestras separadas mediante un gradiente de centrifugación (Puresperm®), ya fue observada por nuestro grupo en espermatozoides de oso que posteriormente fueron sometidos a un segundo ciclo de congelación (Alvarez-Rodriguez et al. 2013b); sin embargo, Nicolás et al. (2012b) no comprueban esta mejora de congelabilidad en eyaculados "normales" de esta especie sometidos a un proceso estándar de congelación. Esta aparente discrepancia, estaría justificada por el hecho de que las subpoblaciones espermáticas afectadas por diferentes procesos deletéreos (urospermia, recongelación) seguramente son coincidentes con las criolábiles en una muestra "normal", lo que aparentemente determinaría una mejor congelabilidad en muestras problemáticas.

A pesar de que nuestros resultados indican que el Puresperm® es una alternativa útil en la mejora de la calidad de los eyaculados de oso pardo contaminados con orina, el principal problema de su uso es la baja tasa de recuperación de espermatozoides (rendimiento cuantitativo). En el presente estudio, el porcentaje de espermatozoides recuperados en el PS-Pellet (31.5%) fue más bajo que el obtenido en nuestros estudios previos no usando gradientes de densidad (60%: Nicolas et al. 2012a; 58.3%: Alvarez-Rodriguez et al. 2013b). Maxwell et al., (2007) usando el gradiente Puresperm® en eyaculados de toro, obtuvo una tasa de recuperación de espermatozoides en semen descongelado más elevada (35.8%) que la obtenida por nosotros en estudios previos en semen descongelado de oso pardo (12.5%: Nicolas et al. 2012b), pero similar a la de este estudio. Estos resultados indican que la eficiencia de los gradientes de selección

podría variar entre las diferentes especies, pero sobre todo y dentro de ellas en función de diversos factores como la muestra tratada. En este sentido, en futuros estudios sería importante desarrollar estrategias que permitan optimizar las técnicas de selección adaptadas a los eyaculados de oso pardo, minimizando la pérdida de espermatozoides durante el procedimiento, pero manteniendo una capacidad selectiva aceptable del gradiente.

En resumen, la contaminación de los eyaculados de oso pardo con orina reduce considerablemente la movilidad espermática pero no interfiere con la viabilidad espermática. Cuando los eyaculados urospérmicos son sometidos al lavado pre-congelación con los medios de diferentes osmolalidades, los mejores resultados tanto de movilidad como de viabilidad espermática se obtuvieron con el diluyente de 200 mOsm/kg, siendo similares a los obtenidos con la dilución empleada en el protocolo de criopreservación estándar (sin lavado pre-congelación). De este modo, la osmolalidad inicial del semen parece ser el factor clave en la determinación del medio de lavado más adecuado. El gradiente de selección Puresperm® demuestra ser un método eficaz en la mejora de calidad de los eyaculados contaminados con orina y además, los espermatozoides seleccionados son más resistentes a la congelación.

BIBLIOGRAFÍA

Aisen EG, Alvarez HL, Venturino A, Garde JJ, 2000: Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. Theriogenology *53* 1053-1061.

Alhaider AK, Watson PF, 2009: Cryopreservation of dog semen: the effects of Equex STM paste on plasma membrane fluidity and the control of intracellular free calcium. Anim.Reprod.Sci. *110* 147-161.

Althouse GC, Seager SW, Varner DD, Webb GW, 1989: Diagnostic aids for the detection of urine in the equine ejaculate. Theriogenology *31* 1141-1148.

Alvarez-Rodriguez M, Alvarez M, Anel-Lopez L, Martinez-Rodriguez C, Martinez-Pastor F, Borragan S, Anel L, de Paz P, 2013a: The antioxidant effects of soybean lecithin- or low-density lipoprotein-based extenders for the cryopreservation of brown-bear (Ursus arctos) spermatozoa. Reprod.Fertil.Dev. *25* 1185-1193.

Alvarez-Rodriguez M, Alvarez M, Lopez-Uruena E, Martinez-Rodriguez C, Borragan S, Anel-Lopez L, de Paz P, Anel L, 2013b: Brown bear sperm double freezing: Effect of elapsed time and use of PureSperm® gradient between freeze-thaw cycles. Cryobiology *67* 339-346.

Alvarez-Rodriguez M, Alvarez M, Gomes-Alves S, Borragan S, Martinez-Pastor F, de Paz P, Anel L, 2011: Quality of frozen-thawed semen in brown bear is not affected by timing of glycerol addition. Theriogenology *75* 1561-1565.

Anakkul N, Suwimonteerabutr J, Singlor J, Phutikanit N, Tharasanit T, Techakumphu M, 2011: Effect of Equex STM Paste on the quality and motility characteristics of post thawed cryopreserved goat semen. Thai J. Vet. Med *41* (3) 345-351.

Anel L, Gomes-Alves S, Alvarez M, Borragan S, Anel E, Nicolas M, Martinez-Pastor F, de Paz P, 2010: Effect of basic factors of extender composition on post-thawing quality of brown bear electroejaculated spermatozoa. Theriogenology *74* 643-651.

Anel L, Alvarez M, Martinez-Pastor F, Gomes S, Nicolas M, Mata M, Martinez AF, Borragan S, Anel E, de Paz P, 2008: Sperm cryopreservation in brown bear (Ursus arctos): preliminary aspects. Reprod.Domest.Anim. *43 Suppl 4* 9-17.

Anel L, de Paz P, Alvarez M, Chamorro CA, Boixo JC, Manso A, Gonzalez M, Kaabi M, Anel E, 2003: Field and in vitro assay of three methods for freezing ram semen. Theriogenology *60* 1293-1308.

Anel L, Martinez F, Alvarez M, Anel E, Boixo JC, Kaabi M, de Paz P, Chamorro C, Herraez P, 1999: Post-mortem spermatozoa recovery and freezing in Cantabric brown bear (*Ursus arctos*): a preliminary report. Theriogenology *51* 277.

Arriola J, Foote RH, 1987: Glycerolation and thawing effects on bull spermatozoa frozen in detergent-treated egg yolk and whole egg extenders. J.Dairy Sci. *70* 1664-1670.

Axner E, Hermansson U, Linde-Forsberg C, 2004: The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. Anim.Reprod.Sci. *84* 179-191.

Bakas LS, Disalvo EA, 1991: Effect of Ca2+ on the cryoprotective action of trehalose. Cryobiology *28* 347-353.

Blackshaw AW, Emmens CW, 1951: The interaction of pH, osmotic pressure and electrolyte concentration on the motility of ram, bull and human spermatozoa. J.Physiol. *114* 16-26.

Bollwein H, Petschow K, Weber F, Leiding C, Stolla R, 2004: The incidence of agglutination and its influence on sperm quality and fertility of boar semen. Berl.Munch.Tierarztl.Wochenschr. *117* 327-333.

Buhr MM, Fiser P, Bailey JL, Curtis EF, 2001: Cryopreservation in different concentrations of glycerol alters boar sperm and their membranes. J.Androl. *22* 961-969.

Chen D, Scobey MJ, Jeyendran RS, 1995: Effects of urine on the functional quality of human spermatozoa. Fertil.Steril. *64* 1216-1217.

Chen LM, Hou R, Zhang ZH, Wang JS, An XR, Chen YF, Zheng HP, Xia GL, Zhang MJ, 2007: Electroejaculation and semen characteristics of Asiatic black bears (Ursus thibetanus). Anim.Reprod.Sci. *101* 358-364.

Cheng FP, Wu JT, Chan JP, Wang JS, Fung HP, Colenbrander B, Tung KC, 2004: The effect of different extenders on post-thaw sperm survival, acrosomal integrity and

longevity in cryopreserved semen of Formosan Sika deer and Formosan Sambar deer. Theriogenology *61* 1605-1616.

Claassens OE, Menkveld R, Harrison KL, 1998: Evaluation of three substitutes for Percoll in sperm isolation by density gradient centrifugation. Hum.Reprod. *13* 3139-3143.

Cochran JD, Amann RP, Froman DP, Pickett BW, 1984: Effects of centrifugation, glycerol level, cooling to 5 degrees C, freezing rate and thawing rate on the post-thaw motility of equine sperm. Theriogenology *22* 25-38.

Crich JP, Jequier AM, 1978: Infertility in men with retrograde ejaculation: the action of urine on sperm motility, and a simple method for achieving antegrade ejaculation. Fertil.Steril. *30* 572-576.

de Paz P, Alvarez-Rodriguez M, Nicolas M, Alvarez M, Chamorro C, Borragan S, Martinez-Pastor F, Anel L, 2012: Optimization of glycerol concentration and freezing rate in the cryopreservation of ejaculate from brown bear (Ursus arctos). Reprod.Domest.Anim. *47* 105-112.

de Ruigh L, Bosch JC, Brus MC, Landman B, Merton JS, 2006: Ways to improve the biosecurity of bovine semen. Reprod.Domest.Anim. *41* 268-274.

Dorado J, Alcaraz L, Duarte N, Portero JM, Acha D, Demyda S, Munoz-Serrano A, Hidalgo M, 2011: Centrifugation on PureSperm((R)) density-gradient improved quality of spermatozoa from frozen-thawed dog semen. Theriogenology *76* 381-385.

Esteves SC, Sharma RK, Thomas AJ,Jr, Agarwal A, 2000: Improvement in motion characteristics and acrosome status in cryopreserved human spermatozoa by swim-up processing before freezing. Hum.Reprod. *15* 2173-2179.

Fahy GM, 1986: The relevance of cryoprotectant "toxicity" to cryobiology. Cryobiology *23* 1-13.

Fernandez-Santos MR, Esteso MC, Soler AJ, Montoro V, Garde JJ, 2006: Effects of egg yolk and cooling rate on the survival of refrigerated red deer (Cervus elaphus hispanicus) epididymal spermatozoa. Reprod.Domest.Anim. *41* 114-118.

Fiser PS, Fairfull RW, 1984: The effect of glycerol concentration and cooling velocity on cryosurvival of ram spermatozoa frozen in straws. Cryobiology *21* 542-551.

Flaherty SP, Swann NJ, Primakoff P, Myles DG, 1993: A role for the WH-30 protein in sperm-sperm adhesion during rouleaux formation in the guinea pig. Dev.Biol. *156* 243-252.

Fornes MW, Burgos MH, 1994: Epididymal glycoprotein involved in rat sperm association. Mol.Reprod.Dev. *38* 43-47.

Fukui Y, Kohno H, Togari T, Hiwasa M, Okabe K, 2008: Fertility after artificial insemination using a soybean-based semen extender in sheep. J.Reprod.Dev. *54* 286-289.

Gao DY, Liu J, Liu C, McGann LE, Watson PF, Kleinhans FW, Mazur P, Critser ES, Critser JK, 1995: Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. Hum.Reprod. *10* 1109-1122.

Garcia BM, Moran AM, Fernandez LG, Ferrusola CO, Rodriguez AM, Bolanos JM, da Silva CM, Martinez HR, Tapia JA, Pena FJ, 2012: The mitochondria of stallion spermatozoa are more sensitive than the plasmalemma to osmotic-induced stress: role of c-Jun N-terminal kinase (JNK) pathway. J.Androl. *33* 105-113.

Garcia-Alvarez O, Maroto-Morales A, Ramon M, del Olmo E, Montoro V, Dominguez-Rebolledo AE, Bisbal A, Jimenez-Rabadan P, Perez-Guzman MD, Soler AJ, 2010: Analysis of selected sperm by density gradient centrifugation might aid in the estimation of in vivo fertility of thawed ram spermatozoa. Theriogenology *74* 979-988.

García-Macías V, Martínez-Pastor F, Martínez F, González N, Álvarez M, Anel E, de Paz P, Borragán S, Celada M, Anel L, 2005: Problems using JC-1 to assess mitochondrial status in brown bear (*Ursus arctos*) semen. Reprod. Fertil. Dev. *17* (1-2) 246.

Garde JJ, Del Olmo A, Soler AJ, Espeso G, Gomendio M, Roldan ERS, 2008: Effect of egg yolk, cryoprotectant, and various sugars on semen cryopreservation in endangered Cuvier's gazelle (Gazella cuvieri). Anim. Reprod. Sci. *108* (3‑4) 384-401

Gil J, Rodriguez-Irazoqui M, Lundeheim N, Soderquist L, Rodriguez-Martinez H, 2003b: Fertility of ram semen frozen in Bioexcell and used for cervical artificial insemination. Theriogenology *59* 1157-1170.

Gil J, Fierro S, Bentancur O, Olivera-Muzante J, 2011: Chilled storage of ram semen improves with the addition of egg yolk and glycerol to milk-based extenders. Reprod Domest Anim.*46* 503-507.

Gil L, Galindo-Cardiel I, Malo C, González N, and Álvarez C, 2013: Effect of Cholesterol and Equex-STM Addition to an Egg Yolk Extender on Pure Spanish Stallion Cryopreserved Sperm ISRN veterinary science 01/2013; 2013:280143. DOI: 10.1155/2013/28014

Gil J, Lundeheim N, Soderquist L, Rodriguez-Martinez H, 2003a: Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. Theriogenology *59* 1241-1255.

Gilmore JA, Liu J, Peter AT, Critser JK, 1998: Determination of plasma membrane characteristics of boar spermatozoa and their relevance to cryopreservation. Biol.Reprod. *58* 28-36.

Gonzalez-Fernandez L, Morrell JM, Pena FJ, Macias-Garcia B, 2012: Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. Theriogenology *78* 415-422.

Griggers S, Paccamonti DL, Thompson RA, Eilts BE, 2001: The effects of ph, osmolarity and urine contamination on equine spermatozoal motility. Theriogenology *56* 613-622.

Guthrie HD, Liu J, Critser JK, 2002: Osmotic tolerance limits and effects of cryoprotectants on motility of bovine spermatozoa. Biol.Reprod. *67* 1811-1816.

Harayama H, Okada K, Miyake M, 2003: Involvement of cytoplasmic free calcium in boar sperm: head-to-head agglutination induced by a cell-permeable cyclic adenosine monophosphate analog. J.Androl. *24* 91-99.

Harayama H, Miyake M, Kato S, 2000: Role of cyclic adenosine 3',5'-monophosphate and serum albumin in head-to-head agglutination of boar spermatozoa. Reprod.Fertil.Dev. *12* 307-318.

Harayama H, Miyake M, Shidara O, Iwamoto E, Kato S, 1998: Effects of calcium and bicarbonate on head-to-head agglutination in ejaculated boar spermatozoa. Reprod.Fertil.Dev. *10* 445-450.

Hernandez-Lopez L, Umland N, Mondragon-Ceballos R, Nayudu PL, 2005: Comparison of the effects of Percoll and PureSperm on the common marmoset (Callithrix jacchus) semen. J.Med.Primatol. *34* 86-90.

Herold FC, de Haas K, Colenbrander B, Gerber D, 2006: Comparison of equilibration times when freezing epididymal sperm from African buffalo (Syncerus caffer) using Triladyl or AndroMed. Theriogenology *66* 1123-1130.

Herold FC, De Haas K, Cooper D, Colenbrander B, Nothling JO, Theunisen W, Spillings B, Gerber D, 2004: Comparison of three different media for freezing of epididymal sperm from the African buffalo (Syncerus caffer) and influence of equilibration time on the post-thaw sperm quality. Onderstepoort J.Vet.Res. *71* 203-210.

Holt WV, Pickard AR, 1999: Role of reproductive technologies and genetic resource banks in animal conservation. Rev.Reprod. *4* 143-150.

Holt WV, Abaigar T, Jabbour HN, 1996: Oestrous synchronization, semen preservation and artificial insemination in the Mohor gazelle (Gazella dama mhorr) for the establishment of a genome resource bank programme. Reprod.Fertil.Dev. *8* 1215-1222.

Ishikawa A, Matsu M, Sakamoto H, Katagiri S, Takahashi Y, 2002: Cryopreservation of the semen collected by electroejaculation from the Hokkaido brown bear (Ursus arctos yesoensis). J.Vet.Med.Sci. *64* 373-376.

Jimenez E, Perez-Marin CC, Vizuete G, Millan Y, Aguera EI, 2013: Effect of different extenders on in vitro characteristics of feline epididymal sperm during cryopreservation. Reprod.Domest.Anim. *48* 665-672.

Khalifa T, Lymberopoulos A, Theodosiadou E, 2013: Association of soybean-based extenders with field fertility of stored ram (Ovis aries) semen: a randomized double-blind parallel group design. Theriogenology *79* 517-527.

Kim SC, Kim HW, 1998: Effects of nitrogenous components of urine on sperm motility: an in vitro study. Int.J.Androl. *21* 29-33.

Kojima E, Tsuruga H, Komatsu T, Murase T, Tsubota T, Kita I, 2001: Characterization of semen collected from beagles and captive Japanese black bears (Ursus thibetanus japonicus). Theriogenology *55* 717-731.

Leibo SP, Songsasen N, 2002: Cryopreservation of gametes and embryos of non-domestic species. Theriogenology *57* 303-326.

Lindahl PE, Sjoblom P, 1981: On mechanisms of head-to-head association in bovine spermatozoa. Biol.Reprod. *25* 29-43.

Liu Z, Foote RH, 1998a: Bull sperm motility and membrane integrity in media varying in osmolality. J.Dairy Sci. *81* 1868-1873.

Liu Z, Foote RH, Brockett CC, 1998b: Survival of bull sperm frozen at different rates in media varying in osmolarity. Cryobiology *37* 219-230.

Makler A, David R, Blumenfeld Z, Better OS, 1981: Factors affecting sperm motility. VII. Sperm viability as affected by change of pH and osmolarity of semen and urine specimens. Fertil.Steril. *36* 507-511.

Martinez-Pastor F, Martinez F, Alvarez M, Maroto-Morales A, Garcia-Alvarez O, Soler AJ, Garde JJ, de Paz P, Anel L, 2009: Cryopreservation of Iberian red deer (Cervus elaphus hispanicus) spermatozoa obtained by electroejaculation. Theriogenology *71* 628-638.

Martinez-Pastor F, Martinez F, Garcia-Macias V, Esteso MC, Anel E, Fernandez-Santos MR, Soler AJ, de Paz P, Garde J, Anel L, 2006: A pilot study on post-thawing quality of Iberian red deer spermatozoa (epididymal and electroejaculated) depending on glycerol concentration and extender osmolality. Theriogenology *66* 1165-1172.

Maxwell WM, Parrilla I, Caballero I, Garcia E, Roca J, Martinez EA, Vazquez JM, Rath D, 2007: Retained functional integrity of bull spermatozoa after double freezing and

thawing using PureSperm density gradient centrifugation. Reprod.Domest.Anim. *42* 489-494.

Monga M, Roberts JA, 1994: Spermagglutination by bacteria: receptor-specific interactions. J.Androl. *15* 151-156.

Morrell JM, Dalin AM, Rodriguez-Martinez H, 2009: Comparison of density gradient and single layer centrifugation of stallion spermatozoa: yield, motility and survival. Equine Vet.J. *41* 53-58.

Mousset-Simeon N, Rives N, Masse L, Chevallier F, Mace B, 2004: Comparison of six density gradient media for selection of cryopreserved donor spermatozoa. J.Androl. *25* 881-884.

Nicolas M, Alvarez M, Anel E, Martinez F, Borragan S, Martinez-Pastor F, de Paz P, Anel L, 2012a: Spermatozoa recovery and post-thawing quality of brown bear ejaculates is affected for centrifugation regimes. Eur J Wild Res *58* 77-84.

Nicolas M, Alvarez M, Gomes-Alves S, Mata-Campuzano M, Borragan S, Martinez-Pastor F, de Paz P, Anel L, 2011: Effects on brown bear (Ursus arctos) spermatozoa freezability of different extender and dilution ratios used for prefreezing centrifugation. Eur J Wild Res *57* 259-266.

Nicolas M, Alvarez M, Borragan S, Martinez-Pastor F, Chamorro CA, Alvarez-Rodriguez M, de Paz P, Anel L, 2012b: Evaluation of the qualitative and quantitative effectiveness of three media of centrifugation (Maxifreeze, Cushion Fluid Equine, and PureSperm 100) in preparation of fresh or frozen-thawed brown bear spermatozoa. Theriogenology *77* 1119-1128.

Nothling JO, Gerber D, Colenbrander B, Dijkstra M, Bakker T, De Cramer K, 2007: The effect of homologous prostatic fluid on motility and morphology of dog epididymal spermatozoa extended and frozen in Biladyl with Equex STM paste or Andromed. Theriogenology *67* 264-275.

Okano T, Murase T, Yayota C, Komatsu T, Miyazawa K, Asano M, Tsubota T, 2006a: Characteristics of captive Japanese black bears (Ursus thibetanus japonicus) semen collected by electroejaculation with different voltages for stimulation and frozen-thawed under different conditions. Anim.Reprod.Sci. *95* 134-143.

Okano T, Nakamura S, Komatsu T, Murase T, Miyazawa K, Asano M, Tsubota T, 2006b: Characteristics of frozen-thawed spermatozoa cryopreserved with different concentrations of glycerol in captive Japanese black bears (Ursus thibetanus japonicus). J.Vet.Med.Sci. *68* 1101-1104.

Okano T, Murase T, Tsubota T, 2004: Electroejaculation and semen cryopreservation of free-ranging Japanese black bears (Ursus thibetanus japonicus). J.Vet.Med.Sci. *66* 1371-1376.

Peña AI, Lugilde LL, Barrio M, Herradon PG, Quintela LA, 2003: Effects of Equex from different sources on post-thaw survival, longevity and intracellular Ca2+ concentration of dog spermatozoa. Theriogenology *59* 1725-1739.

Peña A and Linde-Forsberg C, 2000: Effects of equex, one- or two step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. Theriogenology *54* (6) 859–875.

Pommer AC, Rutllant J, Meyers SA, 2002: The role of osmotic resistance on equine spermatozoal function. Theriogenology *58* 1373-1384.

Ponglowhapan S, Chatdarong K, 2008: Effects of Equex STM Paste on the quality of frozen-thawed epididymal dog spermatozoa. Theriogenology *69* 666-672.

Pukazhenthi B, Noiles E, Pelican K, Donoghue A, Wildt D, Howard J, 2000: Osmotic effects on feline spermatozoa from normospermic versus teratospermic donors. Cryobiology *40* 139-150.

Pursel VG, Schulman LL, Johnson LA, 1978: Effect of Orvus ES Paste on acrosome morphology, motility and fertilizing capacity of frozen-thawed boar sperm. J.Anim.Sci. *47* 198-202.

Purroy FJ, 2008: Oso Pardo: Ursus arctos. En: Enciclopedia Virtual de los Vertebrados Españoles. Carrascal, L. M., Salvador A. (Eds). Museo Nacional de Ciencias Naturales, Madrid. http:/www.vertebradosibericos.org/

Ritar AJ, Salamon S, 1991: Effects of month of collection, method of processing, concentration of egg yolk and duration of frozen storage on viability of Angora goat spermatozoa. Small Rumin Res. *4* 29-37.

Rodriguez-Martinez H, Larsson B, Pertoft H, 1997: Evaluation of sperm damage and techniques for sperm clean-up. Reprod.Fertil.Dev. *9* 297-308.

Roof DJ, Bowley S, Price LL, Matsas DJ, 2012: Comparison of two commercial extenders for cryopreservation of goat semen without sperm washing. Theriogenology *77* 412-420.

Rota A, Strom B, Linde-Forsberg C, Rodriguez-Martinez H, 1997: Effects of equex STM paste on viability of frozen-thawed dog spermatozoa during in vitro incubation at 38 degrees C. Theriogenology *47* 1093-1101.

Salamon S, Maxwell WM, 2000: Storage of ram semen. Anim.Reprod.Sci. *62* 77-111.

Santiago-Moreno J, Toledano-Diaz A, Sookhthezary A, Gomez-Guillamon F, Salas de la Vega R, Pulido-Pastor A, Lopez-Sebastian A, 2011: Effects of anesthetic protocols on electroejaculation variables of Iberian ibex (Capra pyrenaica). Res.Vet.Sci. *90* 150-155.

Santos IP, Cunha ICN, Melo EJT, 2011: Effects of urine and NaCl solutions of different osmolarities on canine sperm. Anim.Reprod. *8* 73-79.

Saragusty J, Gacitua H, King R, Arav A, 2006: Post-mortem semen cryopreservation and characterization in two different endangered gazelle species (Gazella gazella and Gazella dorcas) and one subspecies (Gazella gazelle acaiae). Theriogenology *66* 775-784.

Seager SWJ, Dulensek EP, He G, Schaller GB, 1987: Giant panda (Ailuropoda melanoleuca) semen collection, evaluation and freezing in Southwest China. In: Proceedings of the 15th Annual Meeting of the Society for Cryobiology. Tokyo, Japan, 123-126.

Si W, Zheng P, Li Y, Dinnyes A, Ji W, 2004: Effect of glycerol and dimethyl sulfoxide on cryopreservation of rhesus monkey (Macaca mulatta) sperm. Am.J.Primatol. *62* 301-306.

Songsasen N, Yu I, Murton S, Paccamonti DL, Eilts BE, Godke RA, Leibo SP, 2002: Osmotic sensitivity of canine spermatozoa. Cryobiology *44* 79-90.

Spindler RE, Huang Y, Howard JG, Wang P, Zhang H, Zhang G, Wildt DE, 2004: Acrosomal integrity and capacitation are not influenced by sperm cryopreservation in the giant panda. Reproduction *127* 547-556.

Stradaioli G, Noro T, Sylla L, Monaci M, 2007: Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: comparison between two extenders. Theriogenology *67* 1249-1255.

White IG, 1993: Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. Reprod.Fertil.Dev. *5* 639-658.

Willoughby CE, Mazur P, Peter AT, Critser JK, 1996: Osmotic tolerance limits and properties of murine spermatozoa. Biol.Reprod. *55* 715-727.

Wirtu G, Pope CE, MacLean RA, Godke RA, Paccamonti DL, Dresser BL, 2008: Reversal of motility loss in bongo antelope (Tragelaphus eurycerus isaaci) spermatozoa contaminated with hyposmotic urine during electroejaculation. Anim.Reprod.Sci. *103* 392-397.

Yang DH, McMillan AG, Standley NT, Shannon P, Xu ZZ, 2012: Extracellular calcium is involved in egg yolk-induced head-to-head agglutination of bull sperm. Theriogenology *78* 1476-1486.

Yoshida M, 2000: Conservation of sperms: current status and new trends. Anim.Reprod.Sci. *60-61* 349-355.

Zhang M, Tang H, Shen G, Zhou B, Wu Z, Peng Z, Zhang J, Yan J, Xia G, 2005: Atrial natriuretic peptide induces an acrosome reaction in giant panda spermatozoa and enhances their penetration of salt-stored porcine oocytes. Theriogenology *64* 1297-1308.

CONCLUSIONES

El diluyente más adecuado para la criopreservación de eyaculados de oso pardo es el medio específico TTF-ULE/bear compuesto por TES-Tris-Fructosa ajustado a 300 mOsm/Kg y suplementado con 20% de yema de huevo, 4-8% de glicerol, 2% de EDTA y 1% de Equex paste.

1. El diluyente comercial Andromed® puede ser una alternativa válida al uso del diluyente específico de oso (TTF-ULE/bear) en la congelación de semen de oso pardo.

2. La recogida de los eyaculados de oso pardo sobre medio TES-Tris-Fructosa ajustado a 300 mOsm/Kg y suplementado con 1% de yema de huevo (TTF-H) disminuye el grado de aglutinación espermática en las muestras frescas sin interferir con la calidad de las muestras seminales en pre-congelación y post-descongelación.

3. El lavado pre-congelación de muestras seminales urospérmicas de oso pardo con medios de distinta osmolalidad, no aporta beneficios a la calidad de las muestras tratadas respecto a la dilución sin lavado pre-congelación empleada en el protocolo estándar de criopreservación.

4. El gradiente de selección Puresperm® es un método útil para mejorar la calidad de los eyaculados urospérmicos de oso pardo; los espermatozoides seleccionados mediante esta técnica son más resistentes a la congelación.

RESUMEN

El desarrollo de protocolos de criopreservación espermática en el oso pardo *(Ursus arctos)*, es un paso fundamental para el establecimiento de un banco de recursos genéticos que constituye una herramienta básica en la conservación de esta especie, seriamente amenazada en España.

En el presente trabajo se pretende estudiar diferentes aspectos de la congelabilidad de los espermatozoides de oso pardo que permitan desarrollar estrategias para la mejora de la calidad post-descongelación. Para ello se desarrolló un diluyente específico analizando diferentes parámetros relativos a la composición del diluyente seminal, y se evaluaron diferentes medios comerciales como alternativa al diluyente específico. Por otro lado se estudiaron diferentes estrategias para mejorar la calidad de los eyaculados problemáticos en esta especie (urospermia y espermioglutinación).

Para el desarrollo de un diluyente específico, se evaluaron dos concentraciones de glicerol (4 y 8%) y de yema de huevo (10 y 20%), un suplemento con dos aditivos (Equex paste -surfactante- y EDTA -quelante-) y dos ajustes de la osmolalidad del diluyente (300 y 320 mOsm/Kg). No se encontraron diferencias entre las dos concentraciones de glicerol estudiadas, lo que podría indicar una cierta tolerancia de los espermatozoides de oso pardo al agente crioprotector.

Con relación a la concentración de yema se obtuvieron mejores resultados de movilidad, viabilidad y porcentaje de espermatozoides viables con acrosomas intactos para el diluyente con yema al 20%.

La incorporación de aditivos al diluyente mostró una mejoría significativa en todos los parámetros estudiados. Por último, la osmolalidad de 300 mOsm/kg parece tener un efecto positivo sobre algunos parámetros cinéticos (valores más altos de

velocidad media (VAP), velocidad curvilínea (VCL), velocidad linear progresiva (VSL) y amplitud lateral del desplazamiento de la cabeza (ALH). El análisis global de los datos indica por tanto que el diluyente más adecuado para la congelación de espermatozoides de oso pardo sería TES-TRIS-Fructosa ajustado a 300 mOsm/Kg, suplementado con 20% de yema de huevo, 4-8% de glicerol y aditivos (1% Equex paste y 2% EDTA).

Como segundo paso en la estrategia de validar protocolos eficaces en la congelación de espermatozoides de oso pardo, se evaluó el uso de diluyentes comerciales diseñados para otras especies (bovino -Andromed®, Bioxcell®- y canino -Triladyl Canine®, CaniPro® y Extender 2-). El diluyente TTF-ULE/bear (Tes-Tris-fructosa-yema de huevo-glicerol) específicamente desarrollado para oso pardo se utilizó como control en la comparación con los demás diluyentes. Tras la descongelación, TTF-ULE/bear y Andromed® presentaron valores, significativamente, más elevados para la movilidad individual y progresiva y viabilidad espermática que el Bioxcell®. TTF-ULE/bear también mostró mejores resultados cuando comparado con Triladyl Canine®, CaniPro® y Extender 2. El TTF-ULE/bear se presenta como el diluyente más adecuado para la criopreservación de semen de oso pardo, sin embargo resultados similares pueden ser obtenidos empleando el Andromed®.

Finalmente, se evaluaron estrategias para la mejora de la congelación espermática de eyaculados problemáticos de oso pardo. En la recogida de semen directamente sobre un diluyente de manejo espermático -TTF-H-, se observó una disminución significativa en la incidencia de la espermioaglutinación en las muestras frescas, no afectando esta manipulación a la calidad seminal pre y post-descongelación.

Por otro lado, se evaluaron alternativas para mejorar la calidad post-descongelación de espermatozoides procedentes de eyaculados urospérmicos basadas en la reversión de los efectos deletéreos de la urospermia mediante la aplicación de un lavado pre-congelación con diluyentes de diferente osmolalidad, o en el empleo en pre-congelación, de un gradiente de selección espermática.

Con respecto a la primera propuesta, se evaluaron diferentes tratamientos de lavado pre-congelación (medios con diferentes presiones osmóticas: 200, 300, 400, 500 y 700 mOsm/kg). Los eyaculados con osmolalidad inicial menor de 120mOsm/kg tanto sometidos al lavado pre-congelación (independientemente de la presión osmótica del medio) o no (solo centrifugación-Control) presentaron mejores resultados en la movilidad pre-congelación que el semen fresco, sin embargo la viabilidad espermática no se vio alterada. En pre-congelación, las muestras tratadas con la solución de 700 mOsm/Kg mostraron la viabilidad espermática más baja. Tras la descongelación, los tratamientos de lavado pre-congelación no aportaron ninguna mejoría en comparación con el grupo Control (solo centrifugación sin lavado) y el tratamiento con el medio de 700 mOsm/Kg tuvo un efecto perjudicial en todas las muestras urospérmicas. Como resumen de esta experiencia, el protocolo estándar de congelación ofrece resultados post-descongelación similares a los de los tratamientos más positivos de lavado espermático pre-congelación. Por otro lado, el gradiente de separación espermática Puresperm® aplicado a las muestras de semen fresco contaminadas con orina, mostró un buen rendimiento cualitativo en la selección celular, ya que los espermatozoides seleccionados tras el proceso muestran un aumento en la movilidad y viabilidad tanto en pre-congelación como en post-descongelación respecto a las muestras no seleccionadas. El problema de esta técnica reside en el bajo rendimiento cuantitativo

sobre las células inicialmente tratadas, cuestión que es de mucha importancia si tenemos en cuenta el alto valor de las muestras.

SUMMARY

Development of sperm cryopreservation protocols from brown bear *(Ursus arctos)*, is a necessary step on the establishment of a genetic resource bank that appear as a valuable tool on the conservation of this species, seriously endangered in Spain.

The aim of the present work was to study different aspects of brown bear sperm freezability that allow to develop strategies for improving post- thawing semen quality.

To develop a specific extender we analyzed different parameters related to the composition of seminal extender and different commercial media were evaluated, as an alternative to the specific extender. Furthermore different strategies were studied to improve the quality of the problematic ejaculates in this species (urospermia and sperm agglutination).

For the development of the specific extender, we evaluated two glycerol concentrations (4 and 8%) and two egg yolk concentrations (10 and 20%), supplement with two additives (Equex paste –surfactant agent- and EDTA –chelating agent-) and two osmolality adjustments of the extender (300 and 320 mOsm/Kg). No differences were found among the two glycerol concentrations studied. This could suggest a certain brown bear sperm tolerance to the cryoprotectant.

With regard to egg yolk concentration, 20% egg yolk showed the highest motility results, percentages of viable spermatozoa and viable spermatozoa with intact acrosome.

Extender supplementation with additives rendered significantly higher results for all studied parameters. For extender osmolality, 300 mOsm/kg showed higher values of average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL) and amplitude of lateral head displacement (ALH). We conclude that the most suitable extender to cryopreserve brown bear spermatozoa was Tes-Tris-Fructose adjusted to

300 mOsm/Kg, supplemented with 20% egg yolk, 4-8% glycerol and additives (1% Equex paste and 2% EDTA).

As a second step in the strategy of validating effective protocols on brown bear sperm freezing, we evaluated the use of commercial extenders designed to other species (bovine -Andromed®, Bioxcell®- and canine -Triladyl Canine®, CaniPro® and Extender 2-). The TTF-ULE/bear extender (Tes-Tris-fructose-egg yolk-glycerol), specifically developed for brown bear served as control in both experiments. After thawing, total and progressive sperm motility and sperm viability were, significantly, greater for TTF-ULE/bear and Andromed® than for Bioxcell®. TTF-ULE/bear also showed better results than Triladyl Canine®, CaniPro® y Extender 2. TTF-ULE/bear is the most suitable extender for the brown bear semen cryopreservation, but comparable results can be obtained with Andromed®.

Finally, strategies for improving cryopreservation of problematic ejaculates from brown bear were tested. Collection of semen directly into a handling extender -TTF-H-, resulted in a significant decrease of sperm agglutination in fresh samples, but it had no effect on pre-freeze and post-thawing semen quality.

Furthermore, other alternatives were evaluated in order to improve post-thawing quality of spermatozoa from urospermic ejaculates based on reversing the deleterious effects of urine contamination by applying a pre-freezing washing with extenders with different osmolality, or use of a sperm centrifugation gradient in pre-freezing.

With regarding to the first proposal, different pre-freezing washing treatments (extenders of different osmolality: 200, 300, 400, 500 y 700 mOsm/kg) were tested.

Ejaculates with an initial osmolality of less than 120mOsm/kg treated with pre-freezing washing (independently of extender osmolality), and the Control sample

showed higher pre-freezing sperm motility than the raw ejaculate, but sperm viability did not changed.

The samples washed with 700 mOsm/kg solutions showed the lowest pre-freezing viability. In the post-thawing evaluation, pre-freezing washing treatments did not provide any improvement in comparison with the Control sample (only centrifugation without washing), and treatment with 700 mOsm/kg extender had deleterious effects in all urospermic samples. In summary, the standard freezing protocol provides pot-thawing results similar to those obtained with the most positive pre-freezing washing treatments.

Puresperm® density gradient centrifugation applied to urospermic raw semen showed good performance in the cell selection, since selected spermatozoa show an improvement of sperm motility and viability of pre and post-thawing samples.

The problem of this technique resides in the low quantitative yield on the initially treated cells, issue that is very important considering the high value of the samples.




APÉNDICE I: ANEXO GRÁFICO

Figura 1. a) Ejemplar de un macho adulto de oso pardo *(Ursus arctos).* b) Osa con crías interaccionando con otra hembra. c) Vista panorámica del recinto de osos en el Parque de la Naturaleza de Cabárceno, Cantabria, España. d) Pelea entre dos machos adultos en época reproductiva. E) Cópula en la época reproductiva.

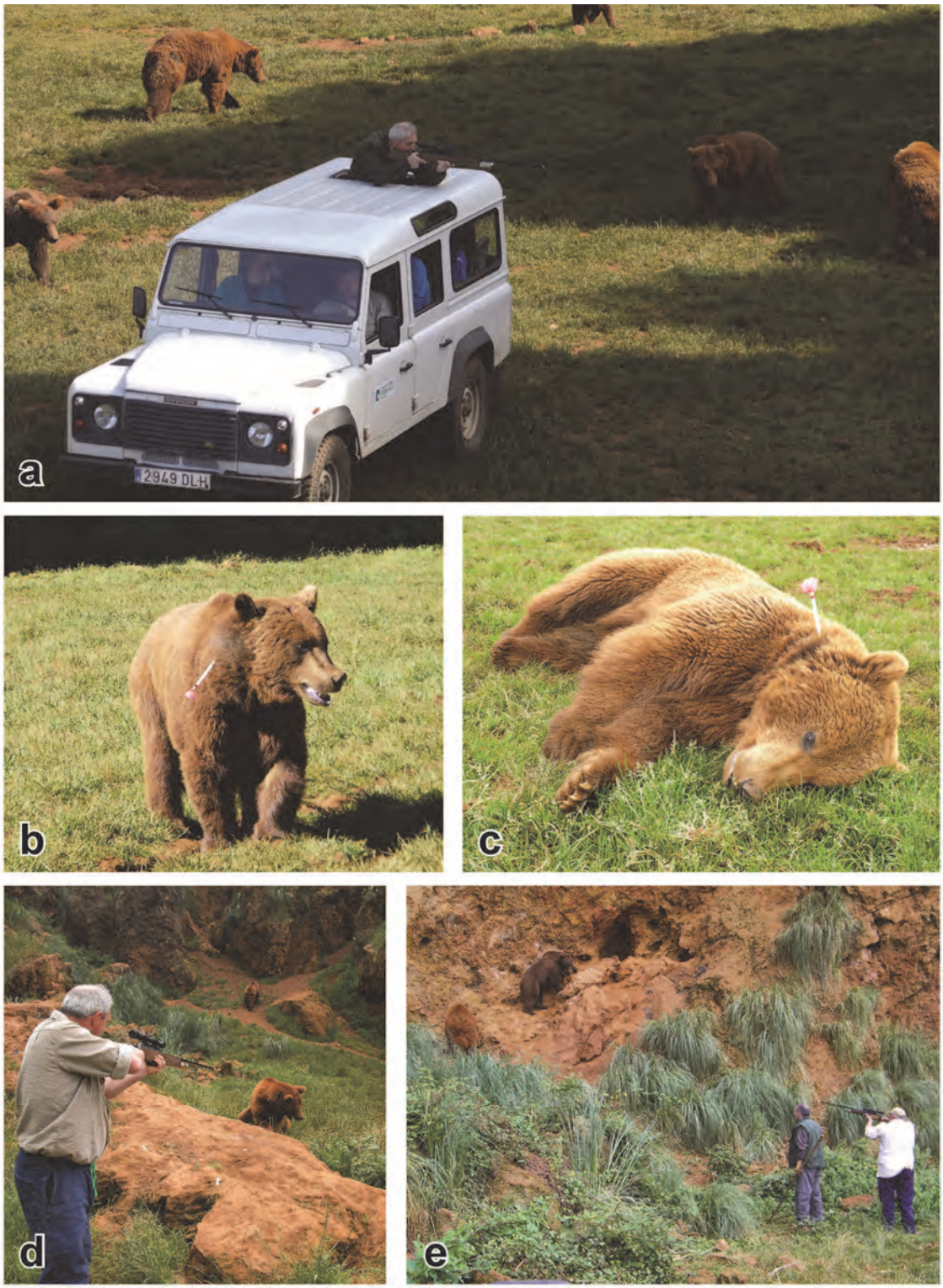

Figura 2. Captura de los animales en el recinto de osos del Parque de Naturaleza de Cabárceno mediante teleanestesia. a) Disparo del dardo anestésico desde el coche de vigilancia. b) Animal huyendo después del disparo con el dardo anestésico. c) Animal anestesiado. d) y e) Elección del macho y disparo del dardo anestésico.

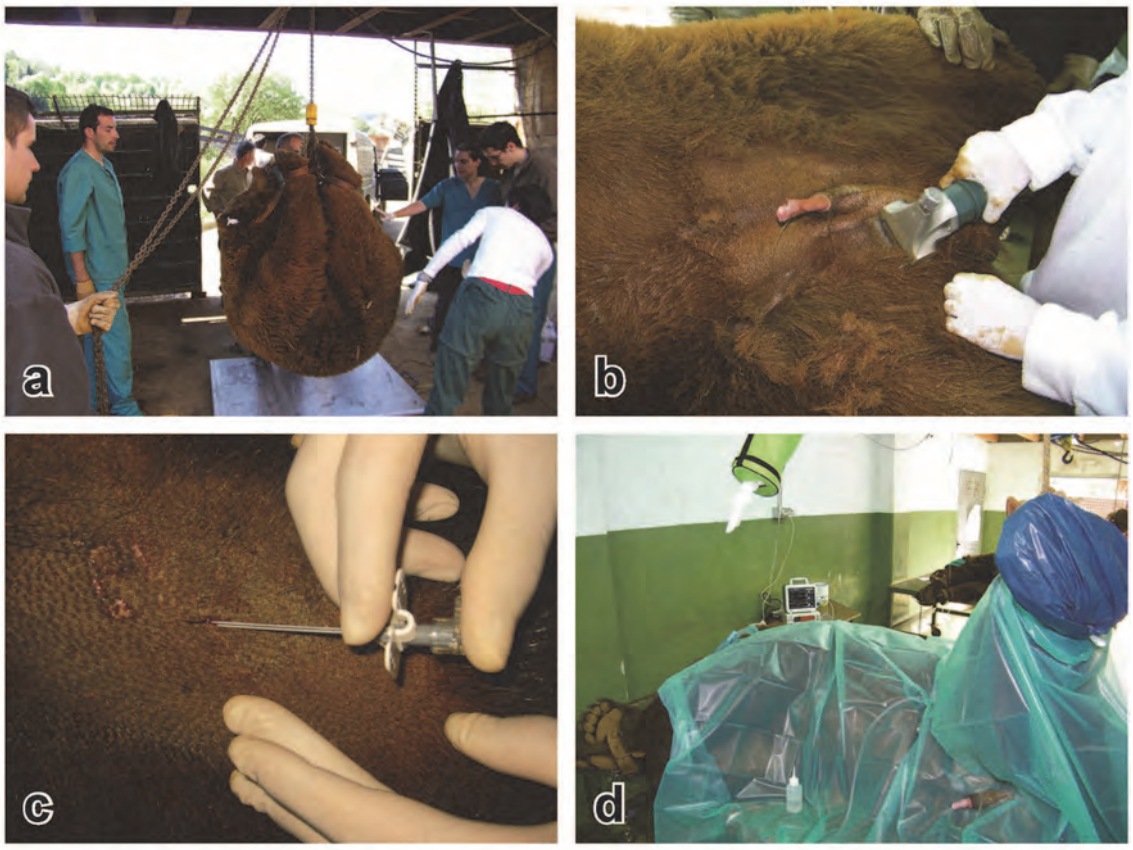

Figura 3A. Preparación de los animales y proceso de electroeyaculación. a) Determinación del peso del animal. b) Rasurado del área prepucial. c) Colocación de un catéter endovenoso en la extremidad anterior (vena cefálica). d) Animal anestesiado e inmovilizado en la mesa de trabajo con cadenas y trabones. Monitorización de las constantes vitales y saturación de O_2.

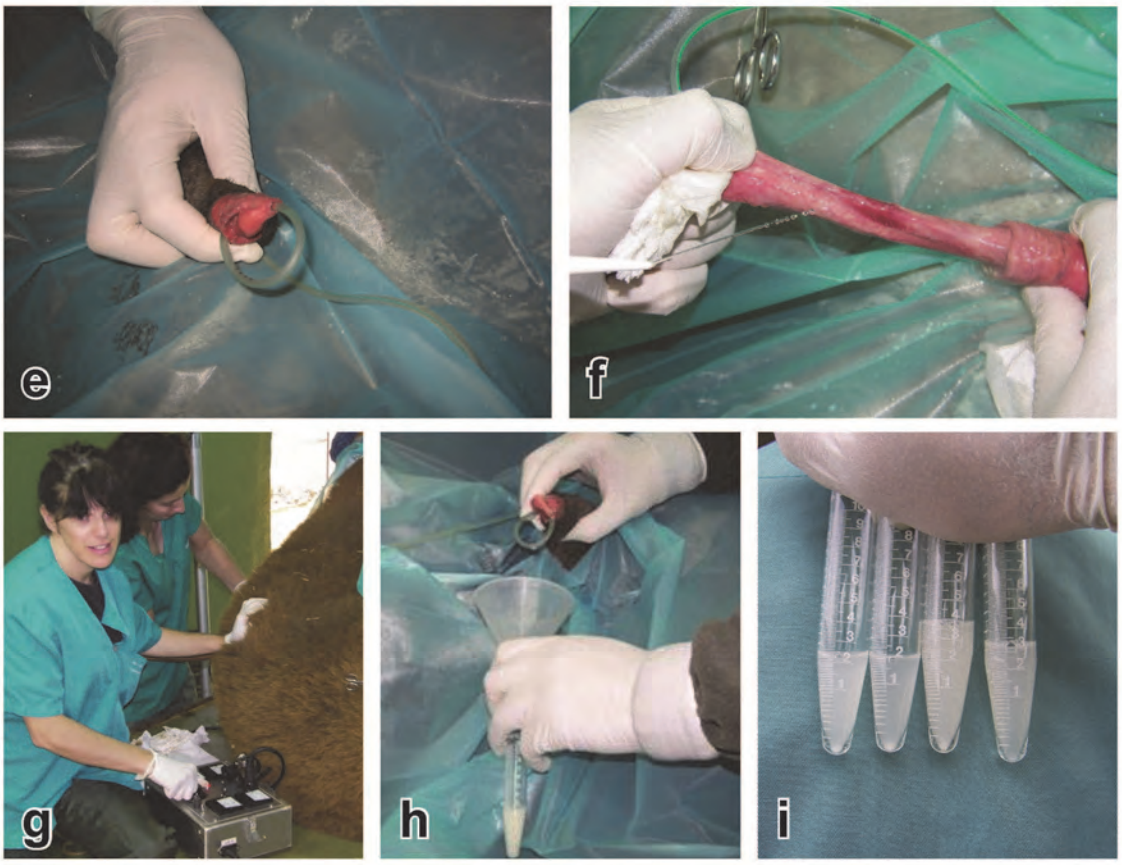

Figura 3B. Preparación de los animales y proceso de electroeyaculación. e) Sondaje de la vejiga urinaria. f) Limpieza del pene con suero fisiológico. g) Electroeyaculación: aplicación de estímulos eléctricos. h) Recogida de las muestras seminales usando un sistema de embudo conectado a un tubo de vidrio graduado. i) Muestras seminales obtenidas durante el proceso de recogida fraccionado.

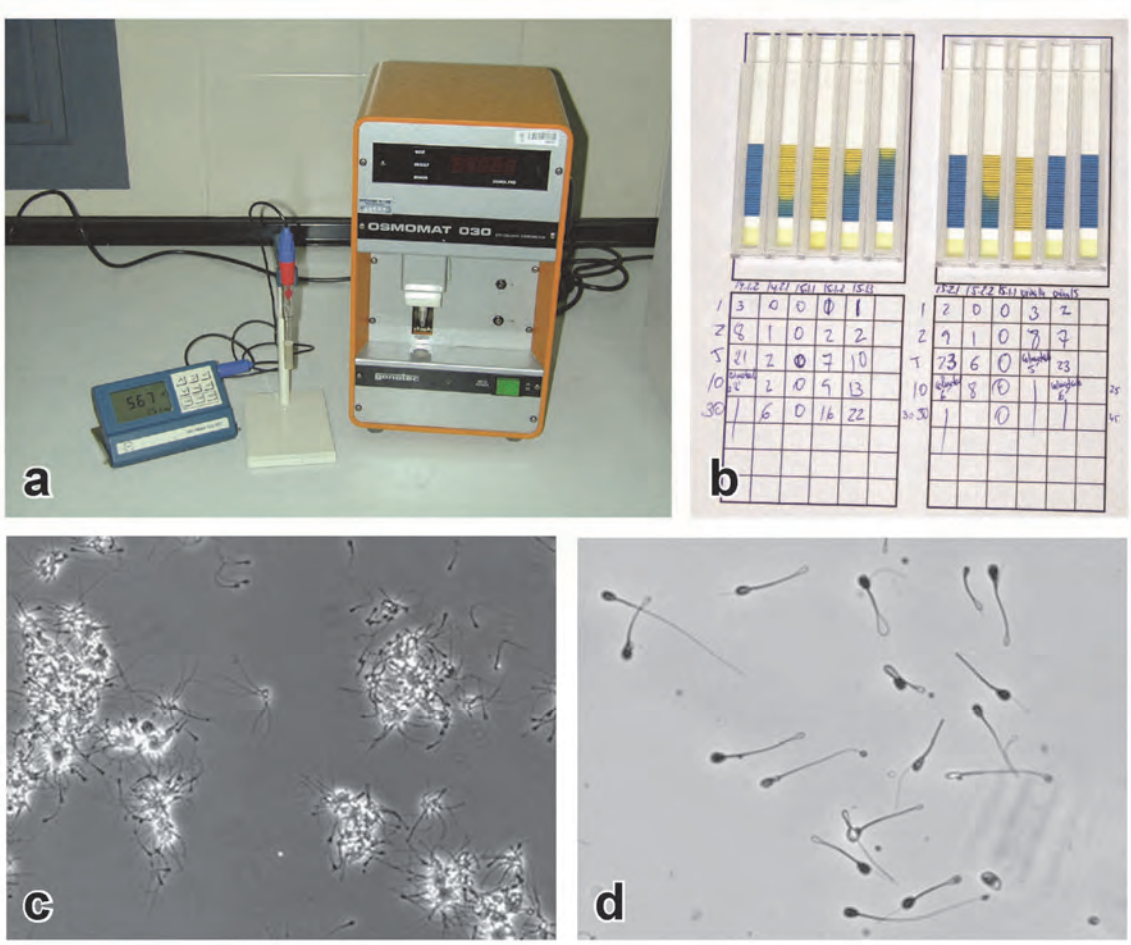

Figura 4. Evaluación de la calidad de las muestras espermáticas. a) Determinación de la presión osmótica (Osmomat-030 Gonotec ™; Berlin) y pH (CG 837-Schott; Germany). b) Test rápido para determinación de urea (Merckgnost Urea Rapid Screening test, Merck,Barcelona, Spain). c) Muestra seminal de oso pardo con alto nivel de aglutinación espermática. d) Espermatozoides de oso pardo contaminados com orina hiposmótica (nótese el grado de endosmósis).

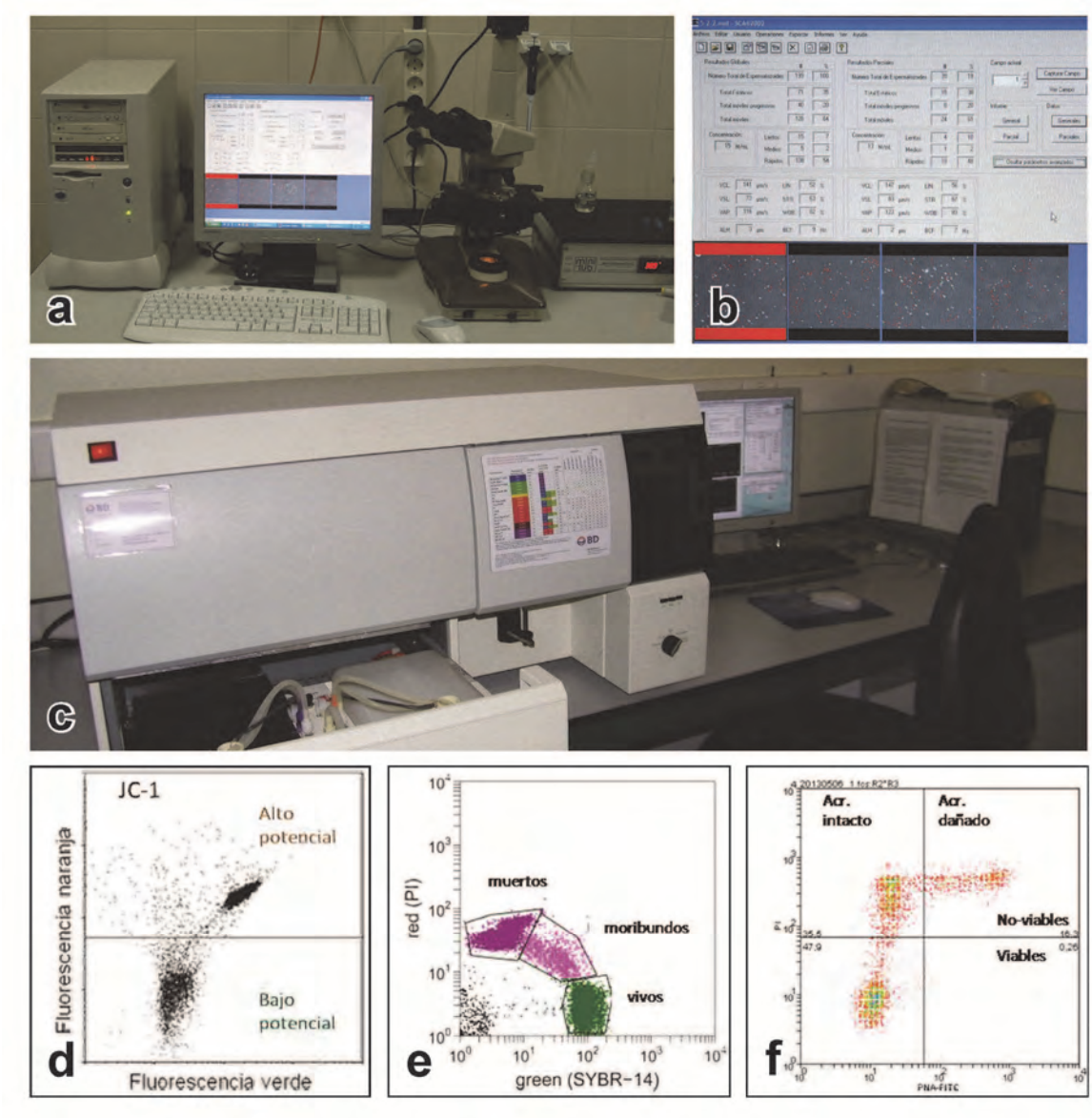

Figura 5. Evaluación de la calidad de las muestras espermáticas. a) Sistema de análisis computerizado de la movilidad espermática (CASA: Proiser, Valencia). b) Captura de campos de espermatozoides mediante el sistema CASA. c) Citómetro de flujo FACScalibur (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA). d) Análisis JC-1. Identifica dos poblaciones, alto y bajo potencial mitocondrial. e) Análisis SYBR-14/PI. Se diferencian tres poblaciones espermáticas. SYBR-14+/PI-: vivos; SYBR-14+/PI+: moribundos; SYBR-14-/PI+: muertos. El dotplot G procede de Reprod Dom Ani 45 (2), 67-78 (2010). f) Análisis PNA-FITC/PI. Se distinguen cuatro poblaciones espermáticas, PNA-FITC-/PI-: viables con el acrosoma intacto; PNA-FITC+/PI-: viables con el acrosoma dañado; PNA-FITC-/PI+: no viables con el acrosoma intacto; PNA-FITC+/PI+: no viables con el acrosoma dañado.

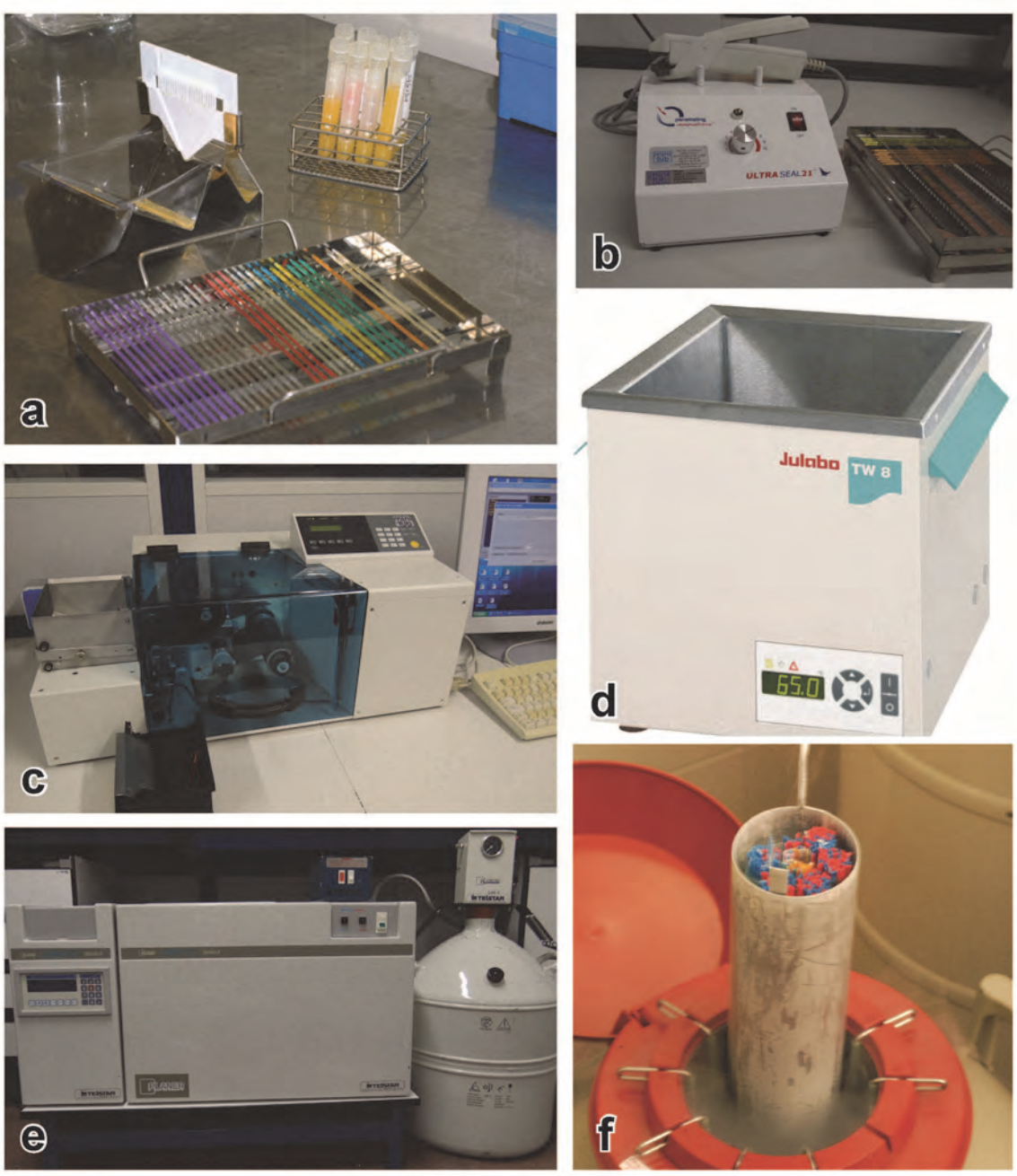

Figura 6. Equipo laboratorial utilizado en la congelación y descongelación de semen. a) Soporte con peine para la creación de cámara de aire previa al sellado y rack de congelación con pajuelas. b) Impresora de pajuelas (Minitub, Tiefenbach, Germany). c) Biocongelador (Planner Krio II). d) Selladora de pajuelas (ULTRASEAL 21, Minitub, Tiefenbach, Germany). e) Baño de agua para descongelación de pajuelas (Julabo TW8). f) Tanque de almacenamiento de pajuelas en nitrógeno líquido.

APÉNDICE II: FACTOR DE IMPACTO DE LAS PUBLICACIONES

ISI Web of Knowledge℠

Journal Citation Reports®

WELCOME | HELP

2013 JCR Science Edition

Journal Summary List

Journal Title Changes

Journals from: subject categories VETERINARY SCIENCES | VIEW CATEGORY SUMMARY LIST

Sorted by: Impact Factor | SORT AGAIN

Mark	Rank	Abbreviated Journal Title (linked to journal information)	ISSN	JCR Data						Eigenfactor® Metrics	
				Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life	Eigenfactor® Score	Article Influence® Score
	1	VET RES	0928-4249	3521	3.383	3.849	0.339	124	6.7	0.00728	1.080
	2	TRANSBOUND EMERG DIS	1865-1674	866	3.116	2.622	0.783	106	2.5	0.00324	0.655
	3	FISH SHELLFISH IMMUN	1050-4648	8000	3.034	3.452	0.466	440	5.0	0.01228	0.558
	4	VET MICROBIOL	0378-1135	12896	2.726	3.123	0.637	488	5.8	0.02778	0.823
	5	VET PARASITOL	0304-4017	14204	2.545	2.719	0.608	498	6.6	0.02169	0.578
	6	PREV VET MED	0167-5877	4966	2.506	2.530	0.408	218	7.2	0.00805	0.600
	7	EQUINE VET J	0425-1644	6124	2.369	2.267	0.993	138	>10.0	0.00698	0.527
	8	MED VET ENTOMOL	0269-283X	2890	2.333	2.512	0.740	50	>10.0	0.00379	0.753
	9	MED MYCOL	1369-3786	3132	2.261	2.205	0.542	120	5.3	0.00832	0.667
	10	J VET INTERN MED	0891-6640	5410	2.224	2.378	0.293	208	6.9	0.00925	0.590
	11	VET J	1090-0233	5621	2.165	2.480	0.630	413	5.0	0.01431	0.646
	12	COMP IMMUNOL MICROB	0147-9571	1307	2.107	2.367	0.726	62	6.2	0.00267	0.635
	13	ZOONOSES PUBLIC HLTH	1863-1959	1155	2.065	2.352	0.848	66	3.7	0.00447	0.649
	14	AVIAN PATHOL	0307-9457	2866	2.041	2.139	0.237	76	>10.0	0.00352	0.572
	15	VET PATHOL	0300-9858	4473	2.038	1.893	0.420	138	>10.0	0.00601	0.548
	16	VET DERMATOL	0959-4493	1575	1.993	2.292	0.402	102	5.3	0.00290	0.473
	17	THERIOGENOLOGY	0093-691X	12505	1.845	2.146	0.369	309	8.5	0.01577	0.532
	18	J MED ENTOMOL	0022-2585	7352	1.815	2.255	0.420	169	9.9	0.00948	0.645
	19	ANIMAL	1751-7311	2506	1.784	1.922	0.270	282	3.7	0.00992	0.548
	20	VET ANAESTH ANALG	1467-2987	1210	1.776	1.986	0.281	89	5.3	0.00265	0.450

MARK ALL | UPDATE MARKED LIST

Journals 1 - 20 (of 129) | [1 | 2 | 3 | 4 | 5 | 6 | 7] | Page 1 of 7

ISI Web of Knowledge℠

Journal Citation Reports®

WELCOME | HELP

2013 JCR Science Edition

Journal Summary List

Journal Title Changes

Journals from: subject categories AGRICULTURE, DAIRY & ANIMAL SCIENCE | VIEW CATEGORY SUMMARY LIST

Sorted by: Impact Factor | SORT AGAIN

Journals 1 - 20 (of 51) | [1 | 2 | 3] | Page 1 of 3

MARK ALL | UPDATE MARKED LIST

Ranking is based on your journal and sort selections.

Mark	Rank	Abbreviated Journal Title (linked to journal information)	ISSN	JCR Data						Eigenfactor® Metrics	
				Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life	Eigenfactor® Score	Article Influence® Score
	1	GENET SEL EVOL	0999-193X	1717	3.747	3.365	0.205	44	8.1	0.00391	1.067
	2	J DAIRY SCI	0022-0302	31754	2.550	3.080	0.505	782	9.4	0.03273	0.622
	3	ANIM GENET	0268-9146	3329	2.210	2.682	0.532	109	6.2	0.00611	0.631
	4	ANIM FEED SCI TECH	0377-8401	6437	2.086	2.066	0.217	143	8.1	0.00934	0.539
	5	J ANIM BREED GENET	0931-2668	1168	2.059	1.706	0.388	49	6.7	0.00215	0.485
	6	J ANIM SCI	0021-8812	25061	1.920	2.551	0.439	602	>10.0	0.02286	0.564
	7	ANIMAL	1751-7311	2506	1.784	1.922	0.270	282	3.7	0.00992	0.548
	8	DOMEST ANIM ENDOCRIN	0739-7240	1865	1.783	2.127	0.358	53	8.3	0.00311	0.621
	9	J REPROD DEVELOP	0916-8818	1652	1.635	1.693	0.191	89	5.5	0.00401	0.467
	10	APPL ANIM BEHAV SCI	0168-1591	6323	1.626	2.016	0.206	141	9.7	0.00720	0.497
	11	ANIM REPROD SCI	0378-4320	5717	1.581	1.854	0.152	217	7.7	0.00956	0.483
	12	POULTRY SCI	0032-5791	13522	1.544	2.001	0.264	394	>10.0	0.01332	0.442
	13	J DAIRY RES	0022-0299	2632	1.394	1.574	0.297	64	>10.0	0.00258	0.446
	14	J ANIM PHYSIOL AN N	0931-2439	1283	1.317	1.263	0.261	138	5.4	0.00306	0.339
	15	REPROD DOMEST ANIM	0936-6768	2828	1.177	1.442	0.202	178	4.9	0.00825	0.393
	16	WORLD POULTRY SCI J	0043-9339	1342	1.158	1.489	0.155	58	9.7	0.00152	0.399
	17	LIVEST SCI	1871-1413	3193	1.100	1.501	0.193	295	4.6	0.01053	0.425
	18	SMALL RUMINANT RES	0921-4488	4004	1.099	1.342	0.189	265	7.3	0.00547	0.317
	19	ANIM SCI J	1344-3941	851	1.044	1.006	0.171	111	4.5	0.00225	0.256
	20	CAN J ANIM SCI	0008-3984	2019	0.983	1.130	0.186	59	>10.0	0.00175	0.323

MARK ALL | UPDATE MARKED LIST

APÉNDICE III: CV SUSANA GOMES-ALVES

FORMACIÓN ACADÉMICA

DIPLOMA DE ESTUDIOS AVANZADOS (D.E.A.) - "SANIDAD ANIMAL Y REPRODUCCIÓN"
Universidad de León - Facultad de Veterinaria; Octubre de 2006.
Titulo: "Estudio de la contaminación de semen de caballo"

LICENCIATURA EN MEDICINA VETERINARIA
Universidad de Trás-Os-Montes e Alto Douro - Vila Real (Portugal); Julio de 2004.

EXPERIENCIA PROFESIONAL

VETERINARIA CLINICA
Clinica Veterinaria de Vale d´Álvaro – Braganza, (Portugal). Marzo 2014-continua.

PROFESORA ASISTENTE INVITADA
Instituto Politécnico de Braganza - Escuela Superior Agraria, Departamento de Ciencia Animal, Braganza (Portugal).
Asignatura: Introducción a Farmacología e Anestesiología. Titulación: Licenciatura en Enfermería Veterinaria. Octubre 2014-Febrero 2015.

PROFESORA ASISTENTE INVITADA
Instituto Politécnico de Braganza - Escuela Superior Agraria, Departamento de Ciencia Animal, Braganza (Portugal).
Asignatura: Enfermería Veterinaria. Titulación: Licenciatura en Enfermería Veterinaria. Febrero 2014-Julio 2014.

BECARIA DE INVESTIGACIÓN-FCT (Fundación Ciencia y Tecnología-Portugal).
Universidad de León, Departamento de Medicina, Cirugía y Anatomía Veterinaria-Unidad de Reproducción y Obstetricia. Tesis de doctorado titulada: "Estudio de la congelabilidad de eyaculados de oso pardo". Julio 2009-Diciembre 2013.

PROFESORA ASISTENTE INVITADA
Instituto Politécnico de Braganza - Escuela Superior Agraria, Departamento de Ciencia Animal, Braganza (Portugal).
Asignatura: Enfermería Veterinaria. Titulación: Licenciatura en Enfermería Veterinaria. Marzo 2012-Mayo 2012.

PROFESORA ASISTENTE INVITADA
Instituto Politécnico de Braganza - Escuela Superior Agraria, Departamento de Ciencia Animal, Braganza (Portugal).
Asignatura: Enfermería Veterinaria. Titulación: Licenciatura en Enfermería Veterinaria. Abril 2011-Junio 2011.

BECARIA ASIMILADA
Universidad de León, Departamento de Medicina, Cirugía y Anatomía Veterinaria-Unidad de Reproducción y Obstetricia.

Proyecto de investigación: “Gestión reproductiva de un centro de reproducción para la raza ovina Assaf”. Enero 2006-Junio 2009.

BECARIA ASIMILADA
Universidad de León, Departamento de Medicina, Cirugía y Anatomía Veterinaria-Unidad de Reproducción y Obstetricia.
Proyecto de investigación: “Transplante de embriones en la raza Assaf”. Julio 2005-Diciembre 2005.

BECARIA DE INVESTIGACIÓN
Universidad de León, Departamento de Medicina, Cirugía y Anatomía Veterinaria-Unidad de Reproducción y Obstetricia.
Proyecto de investigación: “Desarrollo del programa de mejora genética ovina para la raza Assaf en la Provincia de León”. Noviembre 2004-Febrero 2005.

ARTÍCULOS INDEXADOS (EN REVISTAS SCI)

GARCÍA-MACÍAS V, PAZ P, MARTÍNEZ-PASTOR F, ÁLVAREZ M, **GOMES S,** BERNARDO J, ANEL E, ANEL L (2007). DNA fragmentation assessment by flow cytometry (SCSA®) and Sperm-Bos-Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm. *Int J Androl*, 30 (2): 88-98.

ANEL L, ÁLVAREZ M, MARTÍNEZ-PASTOR F, **GOMES S**, NICOLÁS M, MATA M, MARTINEZ AF, BORRAGÁN S, ANEL E, PAZ P (2008). Sperm cryopreservation in brown bear (*Ursus arctos*): preliminary aspects. *Reproduction in Domestic Animals* 43: 9-17.

ANEL L, **GOMES S**, ÁLVAREZ M, BORRAGÁN S, ANEL E, NICOLÁS M, MARTÍNEZ-PASTOR F, PAZ P (2010). Effect of basic factors of extender composition on post-thawing quality of brown bear electroejaculated spermatozoa. *Theriogenology* 74: 643–651.

NICOLÁS M, ÁLVAREZ M, **GOMES-ALVES S**, MATA-CAMPUZANO M, BORRAGÁN S, MARTÍNEZ-PASTOR F, DE PAZ P, ANEL L (2011). Effects on brown bear (*Ursus arctos*) spermatozoa freezability of different extender and dilution ratios used for prefreezing centrifugation. *European Journal of Wildlife Research* 57(2): 259-266.

ÁLVAREZ-RODRÍGUEZ M, ÁLVAREZ M, **GOMES-ALVES S,** BORRAGÁN S, MARTÍNEZ-PASTOR F, DE PAZ P, ANEL L (2011). Quality of frozen-thawed semen in Brown bear is not affected by timing of glycerol addition. *Theriogenology* 75(8): 1561-5.

ÁLVAREZ M, TAMAYO-CANUL J, MARTÍNEZ-RODRÍGUEZ C, LÓPEZ-URUEÑA E, **GOMES-ALVES S**, ANEL L, MARTÍNEZ-PASTOR F, DE PAZ P (2012). Specificity of the extender used for freezing ram sperm depends of the spermatozoa source (ejaculate, electroejaculate or epididymis). *Anim Reprod Sci* 132(3-4): 145-54.

LÓPEZ-URUEÑA E, ÁLVAREZ M, **GOMES-ALVES S**, MARTÍNEZ-RODRÍGUEZ C, BORRAGÁN S, ANEL-LÓPEZ L, DE PAZ P, ANEL L (2014). Tolerance of brown bear spermatozoa to conditions of pre-freezing cooling rate and equilibration time. *Theriogenology* 81(9): 1229-38.

MARTÍNEZ-RODRÍGUEZ C, ÁLVAREZ M, LÓPEZ-URUEÑA E, **GOMES-ALVES S**, ANEL-LÓPEZ L, CHAMORRO C, ANEL L, DE PAZ P (2014). Ram spermatozoa migrating through artificial mucus in vitro reduce the membrane potential of their mitochondria but retaining their viability. *Reproduction, Fertility and Development*. doi.org/10.1071/RD13377.

GOMES-ALVES S, ÁLVAREZ M, NICOLÁS M, LÓPEZ-URUEÑA E, MARTÍNEZ-RODRÍGUEZ C, BORRAGÁN S, DE PAZ P, ANEL L (2014). Use of commercial extenders and alternatives to prevent sperm agglutination for cryopreservation of brown bear semen. *Theriogenology* 82(3): 469-74.

GOMES-ALVES S, ÁLVAREZ M, NICOLÁS M, MARTÍNEZ-RODRÍGUEZ C, BORRAGÁN S, CHAMORRO CA, ANEL L, DE PAZ P (2014). Salvaging urospermic ejaculates from brown bear (Ursus arctos). *Anim Reprod Sci* 2014 Sep 22 doi: 101016/janireprosci201409007 [Epub ahead of print].

LÓPEZ-URUEÑA E, ÁLVAREZ M, **GOMES-ALVES S**, MANRIQUE P, ANEL-LÓPEZ L, CHAMORRO CA, BORRAGÁN S, DE PAZ P, Anel L (2014). Alternatives procedures for the cryopreservation of brown bear ejaculates depending on the flexibility of the "in cooling" period (5 °C). *Cryobiology*. Aceptado: Octubre 2014.

ABSTRACTS INDEXADOS (EN REVISTAS SCI)

ÁLVAREZ M, MARTÍNEZ-PASTOR F, GARCÍA-MACÍAS V, BORRAGÁN S, CELADA M, BERNARDO J, GONZÁLEZ N, **GOMES S**, ANEL, L (2006). Epididymal sperm cryopreservation of one somalia wild ass (*Equus africanus somaliensis*) using six different extenders. *Reproduction Fertility and Development,* 18(1-2):215.

MURO J, ÁLVAREZ M, GARCÍA-MACÍAS V, MARTÍNEZ-PASTOR F, GONZÁLEZ N, BERNARDO J, **GOMES S**, ANEL E, ANEL L (2006). Effect of five extenders on the fertility of refrigerated ram semen. *Reproduction Domestic Animals*, 41 (4): 327.

GONZÁLEZ M, ANEL E, ÁLVAREZ M, GARCÍA-MACÍAS V, MARTÍNEZ-PASTOR F, **GOMES S,** ANEL L (2006). Depth of thawed semen deposition (vaginal AI) affects fertility in alpine goats. *Reproduction Domestic Animals*, 41 (4): 328.

ÁLVAREZ M, BOIXO JC, GARCÍA-MACÍAS V, MARTÍNEZ-PASTOR F, MURO J, BERNARDO J, **GOMES S,** ANEL E, ANEL L (2006). Effect of two anesthesic protocols in blood cortisol levels of electroejaculated bulls. *Reproduction Domestic Animals*, 41 (Suplemento 2): 118.

ANEL L, GARCÍA-MACÍAS V, MARTÍNEZ-PASTOR F, ÁLVAREZ M, BORRAGÁN S, BERNARDO J, **ALVES S,** CHAMORRO C, PAZ P (2007). Effect of spermatozoa head morphometric dimensions on freezability in brown bear (*Ursus arctos*). *Reproduction Fertility and Development,* 19(1):237.

NICOLÁS M, ÁLVAREZ M, **ALVES S,** MATA M, PAZ P, BORRAGÁN S, MARTÍNEZ-PASTOR F, ANEL L (2008). Immediate dilution decreases agglutination in brown bear electroejaculates. Reprod Domest Anim 43 Suppl 4:77.

ANEL E, BORRAGÁN S, ÁLVAREZ M, NICOLÁS M, **GOMES S,** MATA M, BOIXO JC, ANEL L (2008). Sperm charateristics in ejaculated and epididymal Blue wildebeest (*Connochaetes taurinus*). *Reproduction in Domestic Animals,* 43(3):209.

GOMES S, ANEL E, ÁLVAREZ M, BORRAGÁN S, NICOLÁS M, MARTÍNEZ-PASTOR F, PAZ P, ANEL L (2008). Effects of dilution before centrifugation on spermatozoa quality from brown bear (*Ursus arctos*). *Reproduction in Domestic Animals,* 43(3):210.

ANEL L, BORRAGÁN S, ÁLVAREZ M, MARTÍNEZ-PASTOR F, MATA M, **GOMES S,** ANEL E, PAZ P (2009). Study of three consecutive electroejaculations in brown bear (*Ursus arctos*). *Reproduction Fertility and Development,* 21(1): 175.

ÁLVAREZ-RODRIGUEZ M, ÁLVAREZ M, MATA-CAMPUZANO M, NICOLÁS M, **GOMES-ALVES S**, MARTÍNEZ-PASTOR F, BOIXO JC, DE PAZ P, ANEL L (2009).
Comparison of Three Extenders in the Cryopreservation of Electroejaculated Semen of Fighting Bulls. *Reproduction in Domestic Animals* 44 (3): 93.

ANEL L, ÁLVAREZ M, NICOLÁS M, **GOMES-ALVES S**, ANEL E, MATA-CAMPUZANO M, BORRAGÁN S, MARTÍNEZ-PASTOR F, DE PAZ P (2009). Preliminary Approach to Salvage Urospermic Ejaculates of Brown Bear (*Ursus arctos*). *Reproduction in Domestic Animals* 44 (3): 93.

NICOLÁS M, ÁLVAREZ M, **GOMES S**, MATA M, PAZ P, BORRAGÁN S, MARTÍNEZ-PASTOR F, ANEL L (2010). Comparison of two cryopreservation extenders for dog semen based on TES-Tris-Fructose or the Tris-Citric acid-Glucose. *Reproduction in Domestic Animals,* 45 (2): 88.

GOMES S, NICOLÁS M, ÁLVAREZ M, ÁLVAREZ-RODRIGUEZ M, MATA M, PAZ P, BORRAGÁN S, ANEL E,MARTÍNEZ-PASTOR F, ANEL, L (2010). Descriptive study of electroejaculated and epididymal sperm from a llama (*Lama glama*). *Reproduction in Domestic Animals,* 45 (2): 100.

GOMES-ALVES S, NICOLÁS M, BORRAGÁN S, MARTINEZ F, DE PAZ P, ANEL L, ÁLVAREZ M (2011). Seasonal variation in testicular volume and serum testosterone concentrations in the brown bear (*Ursus Arctos*). *Reproduction in Domestic Animals,* 46 (3): 108-109.

LÓPEZ-URUEÑA E, ÁLVAREZ M, BORRAGÁN S, MANRIQUE P, ANEL-LÓPEZ L, **GOMES-ALVES S,** MARTÍNEZ-PASTOR F, DE PAZ P, ANEL L (2012). Preliminary approach of cooling protocol for brown bear sperm cryopreservation. *Reproduction in Domestic Animals,* 47 (3): 94.

ÁLVAREZ-RODRIGUEZ M, ÁLVAREZ M, BORRAGÁN S, LÓPEZ-URUEÑA E, **GOMES-ALVES S**, MARTÍNEZ-PASTOR F, ANEL L, DE PAZ P (2012). Effect of 37 ºC incubation test after thawing of brown bear (*Ursus arctos*) semen cryopreserved with soybean extenders. *Reproduction in Domestic Animals,* 47 (5): 73.

MANRIQUE P, LÓPEZ-URUEÑA E, ÁLVAREZ M, BORRAGÁN S, **GOMES-ALVES S**, NICOLÁS M, DE PAZ P, MARTÍNEZ-PASTOR F, ANEL L (2012). Effects of season and male's age on brown bear (*Ursus arctos*) mating length. *Reproduction in Domestic Animals,* 47 (5): 95-96.

GOMES-ALVES S, LÓPEZ-URUEÑA E; ÁLVAREZ M, ANEL-LÓPEZ L, MARTÍNEZ-RODRÍGUEZ C, DE PAZ P, ANEL L (2013). Effect of anaerobic environment on motility from ram sperm long-term storage at 5 °C. *Reproduction in Domestic Animals*, 48 (1): 78.

LÓPEZ-URUEÑA E, **GOMES-ALVES S**, ÁLVAREZ M, NICOLÁS M, ÁLVAREZ-RODRÍGUEZ M, MANRIQUE P, BORRAGÁN S, DE PAZ P, ANEL L (2013). Descriptive study of epididymal spermatozoa from tiger (*Panthera tigris tigris*). *Reproduction in Domestic Animals,* 48 (1): 82.

LÓPEZ-URUEÑA E, ÁLVAREZ M, **GOMES-ALVES S**, BORRAGÁN S, ANEL-LÓPEZ L, MARTÍNEZ-RODRÍGUEZ C, DE PAZ P, ANEL L (2014). Beneficial effects of glycerol in long-term storage (5 °C) of brown bear (*Ursus arctos*) ejaculates. *Reproduction in Domestic Animals,* 49(3): 80.

LÓPEZ-URUEÑA E, ÁLVAREZ M, **GOMES-ALVES S**, BORRAGÁN S, MARTÍNEZ-RODRÍGUEZ C, ANEL-LÓPEZ L, MANRIQUE P, DE PAZ P, ANEL L (2014). Cryopreservation of brown bear (Ursus arctos) electroejaculates: rapid cooling rate to 5°C affects spermatozoa viability but not apoptosis. *Reproduction in Domestic Animals*, 49 (4): 98.

ÁLVAREZ M, LÓPEZ-URUEÑA E, **GOMES-ALVES S**, BOIXO JC, ANEL-LÓPEZ L, MARTÍNEZ-RODRÍGUEZ C, DE PAZ P, ANEL L (2014). Preliminary results of superovulatory response after two treatments (Pluset® vs Folltropin®) in Assaf sheep for embryo transfer. *Reproduction in Domestic Animals*, 49 (4): 122-3.

LÓPEZ-URUEÑA E, ÁLVAREZ M, **GOMES-ALVES S,** BORRAGÁN S, MANRIQUE P, ANEL-LÓPEZ L, MARTÍNEZ-RODRÍGUEZ C, DE PAZ P, ANEL L (2014). Glycerol presence during prefreezing storage up to 48h (5°C) is recommended to enhance post-thawing quality of brown bear semen. *Reproduction in Domestic Animals*, 49 (4):123-4.

APORTACIONES A CONGRESOS

ÁLVAREZ M, MARTÍNEZ-PASTOR F, GARCÍA-MACÍAS V, BORRAGÁN S, CELADA M, BERNARDO J, GONZÁLEZ N, **GOMES S**, ANEL, L. Epididymal sperm cryopreservation of one somalia wild ass (*Equus africanus somaliensis*) using six different extenders. *Annual Conference IETS.* Orlando, Florida, USA, Enero, 2006.

GARCÍA-MACÍAS V, ÁLVAREZ M, PAZ P, MARTÍNEZ-PASTOR F, **GOMES S,** BORRAGÁN S, ANEL L. Annexin V-FITC/PI assay in fresh and cryopreserved brown bear (*Ursus arctos*) sperm. *10th International Symposium on Spermatology*. Madrid, España, Septiembre, 2006.

GONZÁLEZ M, ANEL E, ÁLVAREZ M, GARCÍA-MACÍAS V, MARTÍNEZ-PASTOR F, **GOMES S**, ANEL L. Depth of thawed semen deposition (vaginal AI) affects fertility in alpine goats. *10th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).* Portorož, Eslovenia, Septiembre, 2006.

MURO J, ÁLVAREZ M, GARCÍA-MACÍAS V, MARTÍNEZ-PASTOR F, GONZÁLEZ N, BERNARDO J, **GOMES S**, ANEL E, ANEL L. Effect of five extenders on the fertility of refrigerated ram semen. *10th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).* Portorož, Eslovenia, Septiembre, 2006.

ÁLVAREZ M, BOIXO JC, GARCÍA-MACÍAS V, MARTÍNEZ-PASTOR F, MURO J, BERNARDO J, **GOMES S**, ANEL E, ANEL L. Effect of two anesthesic protocols in blood cortisol levels of electroejaculated bulls. *8º Congreso Internacional de AERA*. Murcia, España, Octubre, 2006.

ANEL L, GARCÍA-MACÍAS V, MARTÍNEZ-PASTOR F, ÁLVAREZ M, BORRAGÁN S, BERNARDO J, **ALVES S,** CHAMORRO C, PAZ P. Effect of spermatozoa head morphometric dimensions on freezability in brown bear (*Ursus arctos*). *Annual Conference IETS*. Kyoto, Japon, Enero, 2007.

ANEL E, BORRAGÁN S, ÁLVAREZ M, NICOLÁS M, **GOMES S**, MATA M, BOIXO JC, ANEL L. Sperm charateristics in ejaculated and epididymal Blue wildebeest (*Connochaetes taurinus*). *16th International Congress on Animal Reproduction (Icar 2008).* Budapest, Hungria, Julio, 2008.

GOMES S, ANEL E, ÁLVAREZ M, BORRAGÁN S, NICOLÁS M, MARTÍNEZ-PASTOR F, PAZ P, ANEL L. Effects of dilution before centrifugation on spermatozoa quality from brown bear (*Ursus arctos*). *16th International Congress on Animal Reproduction (Icar 2008)*. Budapest, Hungria, Julio, 2008.

NICOLÁS M, **GOMES-ALVES S**, MATA-CAMPUZANO M, GARCÍA-MACIAS V, ÁLVAREZ M, TAMAYO J, DE PAZ P, ANEL L. Centrifugation and dilution effects on sperm quality and freezability from dog. *16th International Congress on Animal Reproduction (Icar 2008).* Budapest, Hungria, Julio, 2008.

ÁLVAREZ M, BERNARDO J, BOIXO JC, **GOMES S**, MATA M, ANEL E, DE PAZ P, ANEL L. Evaluation of different lipid sources in frozen-thawed ram semen. *16th International Congress on Animal Reproduction (Icar 2008).* Budapest, Hungria, Julio, 2008.

NICOLÁS M, ÁLVAREZ M, **GOMES S,** MATA M, PAZ P, BORRAGÁN S, MARTÍNEZ-PASTOR F, ANEL L. Immediate dilution decreases agglutination in Brown bear electroejaculates. *1st Joint International Meeting of the Spanish-Association-for-Animal-Reproduction/British-Andrology-Society.* Gijón, España, Octubre, 2008.

DE LIMA H, ÁLVAREZ M, **GOMES S**, NICOLÁS M, MATA M, ANEL E, BOIXO JC, PAZ P, ANEL L. Mechanical effect of speculum on fertility in ovine vaginal artificial insemination. *1st Joint International Meeting of the Spanish-Association-for-Animal-Reproduction/British-Andrology-Society.* Gijón, España, Octubre, 2008.

ANEL L, ÁLVAREZ M, MARTÍNEZ-PASTOR F, **GOMES S,** NICOLÁS M, MATA M, MARTÍNEZ AF, BORRAGÁN S, ANEL E, PAZ P. Sperm cryopreservation in brown bear (Ursus arctos): Preliminary aspects. *1st Joint International Meeting of the Spanish-Association-for-Animal-Reproduction/British-Andrology-Society.* Gijón, España, Octubre, 2008.

ÁLVAREZ M, MARTÍNEZ-PASTOR F, PAZ P, **GOMES-ALVES S**, LÓPEZ-URUEÑA E, PÉREZ JJ, APARICIO N, NICOLÁS M, MATA-CAMPUZANO M, ANEL, L. Evolución de un programa de inseminación en la raza assaf en la provincia de León. *XIII Jornadas sobre producción animal, Asociación*. Interprofesional para el Desarrollo Agrario, Zaragoza, Spain, 2009.

ANEL L, ÁLVAREZ M, NICOLÁS M, **GOMES-ALVES S**, ANEL E, MATA-CAMPUZANO, BORRAGÁN S, MARTÍNEZ-PASTOR F, de PAZ P. Preliminary approach to salvage urospermic ejaculates of brown bear (Ursus arctos). *13th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).* Gante, Belgica, Septiembre, 2009.

ÁLVAREZ -RODRIGUEZ M, ÁLVAREZ M, MATA M, NICOLÁS M, **GOMES S**, MARTÍNEZ-PASTOR F, BOIXO JC, PAZ P, ANEL L. Comparison of three extenders in the cryopreservation of electroejaculated semen of fighting bulls. *13th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).* Gante, Belgica, Septiembre, 2009.

ANEL L, BORRAGÁN S, ÁLVAREZ M, MARTÍNEZ-PASTOR F, MATA M, **GOMES S,** ANEL E, PAZ P. Study of three consecutive electroejaculations in brown bear (*Ursus arctos*). *Annual Conference IETS.* San Diego, USA, Enero, 2009.

NICOLÁS M, ÁLVAREZ M, **GOMES S,** MATA M, PAZ P, BORRAGÁN S, MARTÍNEZ-PASTOR F, ANEL L. Comparison of two cryopreservation extenders for dog semen based on TES-Tris-Fructose or the Tris-Citric acid-Glucose. *10º Congreso de la Asociación Española de Reproducción Animal.* Cáceres, España, Junio, 2010.

GOMES S, NICOLÁS M, ÁLVAREZ M, ÁLVAREZ-RODRIGUEZ M, MATA M, PAZ P, BORRAGÁN S, ANEL E,MARTÍNEZ-PASTOR F, ANEL L. Descriptive study of electroejaculated and epididymal sperm from a llama (*Lama glama*). *10º Congreso de la Asociación Española de Reproducción Animal.* Cáceres, España, Junio, 2010.

ÁLVAREZ-RODRIGUEZ M, NICOLÁS M, **GOMES-ALVES S,** MATA-CAMPUZANO M, MARTÍNEZ-PASTOR F, ÁLVAREZ M, PAZ P, ANEL L. Storage method affects the antioxidant capacity of non-commercial extenders used for semen cryopreservation. *Annual Meeting of British Andrology Society.* Cheshire, Inglaterra, Noviembre, 2010.

GOMES-ALVES S, NICOLÁS M, BORRAGÁN S, MARTINEZ F, DE PAZ P, ANEL L, ÁLVAREZ M**.** Seasonal variation in testicular volume and serum testosterone concentrations in the brown bear (*Ursus Arctos*). *15th Annual Conference of the European Society of Domestic Animals Reproduction.* Antalya, Turquía, Septiembre, 2011.

LÓPEZ-URUEÑA E, ÁLVAREZ M, BORRAGÁN S, MANRIQUE P, ANEL-LÓPEZ L, **GOMES-ALVES S,** MARTÍNEZ-PASTOR F, DE PAZ P, ANEL, L. Preliminary approach of cooling protocol for brown bear sperm cryopreservation. *11º Congreso de la Asociación Española de Reproducción Animal (AERA.)* Córdoba, España, Junio, 2012.

ÁLVAREZ-RODRIGUEZ M, ÁLVAREZ M, BORRAGÁN S, LÓPEZ-URUEÑA E, **GOMES-ALVES S,** MARTÍNEZ-PASTOR F, ANEL L, DE PAZ P. Effect of 37 ºC incubation test after thawing of brown bear (*Ursus arctos*) semen cryopreserved with soybean extenders. *16th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).* Dublin, Ireland, 29 Agosto -1 Septiembre, 2012.

MANRIQUE P, LÓPEZ-URUEÑA E, ÁLVAREZ M, BORRAGÁN S, GOMES-ALVES S, NICOLÁS M, DE PAZ P, MARTÍNEZ-PASTOR F, ANEL L. Effects of season and male's age on brown bear (*Ursus arctos*) mating length. *16th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).* Dublin, Ireland, 29 Agosto -1 Septiembre, 2012.

ÁLVAREZ-RODRÍGUEZ M, ÁLVAREZ M, BORRAGÁN S, LÓPEZ-URUEÑA E, **GOMES-ALVES S**, HOLT WV, FAZELI A, DE PAZ P, ANE L. Effect of the HSPA8 addition to cryopreservation media on the quality of brown bear (*Ursus arctos*) sperm submitted to deferred freezing up to 48 h. *3rd Inter. Congress on Controversies in Cryopreservation of Stem Cells,Reproductive Cells,Tissue and Organs (Cryo)*. Berlin, Germany, Marzo, 2013.

GOMES-ALVES S, LÓPEZ-URUEÑA E; ÁLVAREZ M, ANEL-LÓPEZ L, MARTÍNEZ-RODRÍGUEZ C, DE PAZ P, ANEL L (2013). Effect of anaerobic environment on motility from ram sperm long-term storage at 5 °C. *17th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).* Bologna, Italia, Septiembre, 2013.

LÓPEZ-URUEÑA E, **GOMES-ALVES S**, ÁLVAREZ M, NICOLÁS M, ÁLVAREZ-RODRÍGUEZ M, MANRIQUE P, BORRAGÁN S, DE PAZ P, ANEL L. Descriptive study of epididymal spermatozoa from tiger (*Panthera tigris tigris*). *17th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).* Bologna, Italia, Septiembre, 2013.

LÓPEZ-URUEÑA E, ÁLVAREZ M, **GOMES-ALVES S**, BORRAGÁN S, ANEL-LÓPEZ L, MARTÍNEZ-RODRÍGUEZ C, DE PAZ P, ANEL L (2014). Beneficial effects of glycerol in long-term storage (5 °C) of brown bear (*Ursus arctos*) ejaculates. *18th Annual Conference of the European Society for Domestic Animal Reproduction (***ESDAR***).* Helsinki, Finland, Sepetiembre, 2014.

LÓPEZ-URUEÑA E, ÁLVAREZ M, **GOMES-ALVES S**, BORRAGÁN S, MARTÍNEZ-RODRÍGUEZ C, ANEL-LÓPEZ L, MANRIQUE P, DE PAZ P, ANEL L (2014). Cryopreservation of brown bear (Ursus arctos) electroejaculates: rapid cooling rate to 5°C affects spermatozoa viability but not apoptosis. *12º Congreso Internacional* AERA 2014. Alicante, España, Octubre, 2014.

ÁLVAREZ M, LÓPEZ-URUEÑA E, **GOMES-ALVES S**, BOIXO JC, ANEL-LÓPEZ L, MARTÍNEZ-RODRÍGUEZ C, DE PAZ P, ANEL L (2014). Preliminary results of superovulatory response after two treatments (Pluset® vs Folltropin®) in Assaf sheep for embryo transfer. *12º Congreso Internacional* AERA2014. *12º Congreso Internacional* AERA 2014. Alicante, España, Octubre, 2014.

LÓPEZ-URUEÑA E, ÁLVAREZ M, **GOMES-ALVES S,** BORRAGÁN S, MANRIQUE P, ANEL-LÓPEZ L, MARTÍNEZ-RODRÍGUEZ C, DE PAZ P, ANEL L (2014). Glycerol presence during prefreezing storage up to 48h (5°C) is recommended to enhance post-thawing quality of brown bear semen. *12º Congreso Internacional* AERA2014. Alicante, España, Octubre, 2014.

PROYECTOS Y CONTRATOS DE INVESTIGACIÓN

"Estudio sobre congelabilidad de semen de caballos de raza Hispano-Bretón". Asociación Nacional de Ganaderos Criadores de ganado equino de raza Hispano-Bretón: 2002.

"Convenio específico de colaboración entre la Excma Diputación Provincial de León y la Universidad de León para el desarrollo de un programa de mejora genética ovina para la raza Assaf en la provincia de León". Diputación Provincial de León: 2002-2005.

"Convenio marco entre CANTUR SA (Parque de la Naturaleza de Cabarceno) y la Universidad de León": 2003.

"Convenio para la realización de servicios técnicos (Programa de transplante de embriones en la raza assaf)". Diputación Provincial de León: 2005.

"Caracterización y mejora de eyaculados de oso pardo (Ursus arctos) obtenidos mediante electroeyaculación". CICYT (CLG2004-02178/BOS): 2005-2007.

“Convenio de colaboración entre la Consejería de Agricultura y Ganadería de la Junta de Castilla y León y la Universidad de León para el desarrollo de un marco de colaboración en materia de reproducción ovina y caprina”. Junta de Castilla y León: 2006.
“Contrato para la realización de servicios técnicos y asesoramiento (Asesoría técnica al programa de inseminación artificial ovina en raza Assaf)”. Assaf. E.: 2006-2007.

“Mejora de resultados de la inseminación artificial ovina”. Ovigen: 2006-2007.

“Mejora de la eficacia del banco de recursos genéticos (semen congelado) en las razas ovinas Castellana Negra y Ojalada”. INIA (RZ2007-00011-00-00): 2007-2009.

“Criopreservación de eyaculados de oso pardo (Ursus arctos)”. CICYT (CLG2007-63748/BOS): 2007-2010.

“Estrategias para la conservación de eyaculados de oso pardo adaptadas al tratamiento diferido con técnicas de mejora espermática”. MICINN (CGL2010-19213/BOS): 2010-2013.

“Inseminación Artificial en el oso pardo”. CANTUR S.A.: 25/03/2004 – 31/12/2013.