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FREEZABILITY MARKERS FOR BOAR SPERM: NEW PROTEOMIC APPROACHES

Ingrid Vilagran Martí

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Universitat de Girona

Freezability markers for boar sperm: new proteomic approaches

Marcadors de congelació per l'esperma de porcí: noves aproximacions proteòmiques

Ingrid Vilagran Martí

2015

Cover images: 3D structure of Triosephosphate isomerase (TPI), Acrosin-binding protein (ACRBP), Fibronectin 1 (FN1) and Voltage-dependent anion channel 2 (VDAC2). Source: (Berman *et al.*, 2000)

Doctoral thesis

Freezability markers for boar sperm: new proteomic approaches

Ingrid Vilagran Martí

2015

Doctoral programme in Technology

Supervised by:

Dr. Sergi Bonet Marull

Dra. Sílvia Sancho Badell

Dr. Marc Yeste Oliveras

A thesis dissertation submitted to obtain the degree of Doctor of Philosophy at the University of Girona

This work was supported by a predoctoral BR Grant from Girona University (BR2012).

Sergi Bonet Marull, Catedràtic de l'Àrea de Biologia Cel·lular del Departament de Biologia de la Universitat de Girona i Rector de la Universitat de Girona.

CERTIFICA:

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Dr. Sergi Bonet Marull

Sílvia Sancho Badell, Doctora de l'Àrea de Biologia Cel·lular del Departament de Biologia de la Universitat de Girona.

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Dra. Sílvia Sancho Badell

Marc Yeste Oliveras, Marie Curie Senior Research Fellow at Nuffield Department of Obstetrics and Gynaecology, University of Oxford.

CERTIFIES:

That the Doctoral Thesis entitled "*Freezability markers for boar sperm: new proteomic approaches*", by Ingrid Vilagran Martí to obtain the degree of Doctor of Philosophy at the University of Girona has been conducted under his supervision and it is ready to be submitted for being evaluated by the corresponding Commitee.

Oxford, 9th September of 2015.

Dr. Marc Yeste Oliveras

Als meus pares Josep i Paquita

Tot el que tinc i el que puc aconseguir és només possible gràcies al seu amor i suport

A l'Oriol

Pel seu amor incondicional i per dur-me sempre a la llum quan he caminat a les fosques

Acknowledgements/Agraïments

El procés de doctorat que ha culminat amb l'escriptura de la present tesi, per mi ha sigut un procés de creixement. La llavor que contenia tot el meu amor per la ciència, expectativa per nous reptes i avidesa de nous coneixements ha anat creixent al llarg d'aquests anys, dels quals no canviaria ni un sol instant. Tan tots els moments alegres com tots els obstacles han fet que aquella llavor esdevingués una espectacular planta. Tot aquest procés de germinació i desenvolupament no hagués estat possible sense els adobs conformats per totes les persones que he anat trobant al llarg dels anys de doctorat.

Primer, voldria donar les gràcies al meu director de tesi i director de TechnoSperm, a en Sergi Bonet, per dipositar la seva confiança en mi i permetrem formar part del grup TechnoSperm. Gràcies per posar a la nostra disposició les fantàstiques instal·lacions de les que he pogut gaudir i també per estar sempre disponible quan calia en tot aquest procés de creixement. També voldria agrair a la meva co-directora de tesi, la Sílvia Sancho, el seu suport.
M'agradaria fer una especial menció al meu co-director Dr. Marc Yeste, per esdevenir no només un director, un mentor i un guia en tota aquest procés, sinó també un amic. Gràcies Marc per estar en tot moment (tan aquí a Girona, com a Barcelona, com a Oxford) ben a prop. Gràcies per meravellar-me i per aportar-me tant amb tot el teu bagatge i coneixements i per ser aquella figura admirada i respectada que ha constituït un adob imprescindible per aquella llavor. Aquesta tesi no hauria mai continuat ni sigut possible sense tu. De tot cor, moltes gràcies.

Gràcies també als membres, tan actuals com antics, del grup TechnoSperm: Mailo, Eva B., Eva T., Eva F., Anna, Isabel i Sara per ser un suport i resoldre sempre els meus dubtes quan ho he necessitat. Voldria agrair especialment, per compartir el dia a dia al laboratori: A l'Estela per la seva ajuda i per ser una mentora quan ha fet falta, a la Míriam per representar per mi un model d'eficiència i perseverança i per sempre ser-hi a l'hora de compartir coneixements, a la Lili pel seu bon humor i ajuda i a l'Albert per regalar-me tots els moments de bromes, riures, safareig i sobretot pel "bon rotllo" amb que inundaves tot el laboratori. Per últim, gràcies proteolítiques, nenes sabeu que heu esdevingut no només unes companyes sinó unes amigues molt estimades. Noe gràcies per la teva vitalitat, alegria i genuïnitat; ja saps que sempre podràs comptar amb mi. Dra. Roser Morató, Xarito voldria que sabessis que no només representes el meu ideal com a persona sinó també com a científica. Gràcies per meravellar-me amb el teu treball i manera de ser: Roser, Xarito, Rosales, però sobretot amiga.

Cal tenir en compte però, que aquesta llavor no hagués germinat i crescut només amb els adobs endògens, sinó també als exògens. Per això, voldria agrair a Rafael Oliva per acceptar-me al seu laboratori de l'Hospital Clínic de Barcelona, deixant que fos un

membre més del seu magnífic grup. També, voldria agrair la seva implicació, els coneixements que m'ha aportat com a "sensei" i tots els moments que hem passat juntes a la Judit Castillo. També gràcies, Rubén, Meri, Xana, Carla, Toni i Claudio per deixar-me formar part de la vostra família i per esdevenir sempre un suport quan ha fet falta.

No em voldria descuidar dels membre de la Universitat Autònoma de Barcelona, per això voldria agraïr a l'Efrén i la Elissa els coneixements, rialles i bons moments compartits. Aquest agraïment el voldria fer extensiu a l'equip del Departament de Reproducció de l'Hospital Clínic i Veterinari de la UAB el qual ha resolt sempre els meus dubtes i problemes sempre que ho he necessitat, com la Montse Rivera.

Cal tenir en compte que per suposat, aquest creixement no hagués estat mai possible per tots els que esteu sempre ben a prop. Gràcies als meus amics: Tània R., Amàlia, Jordi, Tània G., Imma i Roser per interessar-vos per la tesi encara que això del porc no sigui l'ho vostre i pels vostres "quan acabes?". També gràcies a les meves nenes: Patry, Irene, Paula i Marina per ser-hi sempre, per compartir primer el nostre amor per la biologia i després la nostra gran amistat.

Per acabar, gràcies a tota la meva família per sempre fer-me costat: avis, tiets, cosins.. com també a la meva família política que ja considero pròpia. Als meus pares gràcies per deixar-me somiar, gràcies per deixar que aconseguís tot el que em proposés, gràcies per la vostra ajuda i ànims en tot moment, gràcies per donar-m'ho tot i sobretot per ensenyar-me que amb perseverança tot es pot superar. No puc fer més que dedicar-vos aquesta tesi i tot el meu amor.
A l'Oriol, gràcies per ser l'aigua de la meva llavor, el ingredient imprescindible pel seu creixement. Sense tu, el teu suport i el teu amor això no hagués sigut possible. Gràcies per no només ser la persona estimada per compartir la vida, sinó també per ser amb qui compartir els meus reptes i somnis. T'estimo.

Finalment, gràcies a tu per llegir encara que sigui una pàgina de la meva tesi, la qual representa tot aquest esforç, il·lusió i perseverança pel creixement tan personal com científic del qual no només jo en sóc la responsable sinó totes aquestes persones que d'alguna manera n'han format part.

Tot el que descobrim és bell i agrest,
fosc en l'origen, però clar en la forma,
perquè en la forma creix i s'interroga.

Dels ulls estant, tots els secrets són dòcils,
dòcil l'espai en que els somnis s'exalten
i fecunden el goig i l'esperança,
i dòcil també el riu d'aigües profundes
que ens vincla joncs al fons de la mirada.

Visible molts miralls, la vida, lenta,
s'escriu a si mateixa en els propòsits
i s'esborra en la por i les defallences.
Només pel foc del desig i l'espera
l'esclat de tot es justifica.

Només pel foc, només amb el foc,
cremant-nos,
podem mirar la vida cara a cara.

Miquel Martí i Pol (Poema 1, 1982)

List of papers

The thesis is presented as a compendium of three papers:

PAPER 1

Vilagran I, Castillo J, Bonet S, Sancho S, Yeste M, Estanyol J M, Oliva R. **Acrosin-binding protein (ACRBP) and triosephosphate isomerise (TPI) are good markers to predict boar sperm freezing capacity**. Theriogenology, 2013. 80:443-450

Theriogenology has an Impact Factor of 1.845 and it is situated in the first quartile (Q1) in Veterinary Sciences category (© 2015 Journal Citation Reports Science Edition, published by Thomson Reuters).

PAPER 2

Vilagran I, Yeste M, Sancho S, Casas I, Rivera del Álamo M M, Bonet S. **Relationship of sperm small heat-shock protein 10 and voltage-dependent anion channel 2 with semen freezability in boars**. Theriogenology, 2014. 82:418-426

Theriogenology has an Impact Factor of 1.845 and it is situated in the first quartile (Q1) in Veterinary Sciences category (© 2015 Journal Citation Reports Science Edition, published by Thomson Reuters).

PAPER 3

Vilagran I, Yeste M, Sancho S, Castillo J, Oliva R, Bonet S. **Comparative analysis of boar seminal plasma proteome from different freezability ejaculates and identification of Fibronectin 1 as sperm freezability marker**. Andrology, 2015. 3(2):345-56

Andrology has not yet an Impact Factor since it became the union in 2013 between Journal of Andrology with an Impact Factor of 1.694 and it was situated in the third quartile (Q3) in Andrology category and International Journal of Andrology with an Impact Factor of 3.206 and it was situated in the first quartile (Q1) in Andrology category (© 2015 Journal Citation Reports Science Edition, published by Thomson Reuters).

Abbreviations

µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
2D	Two dimensional
2D-DIGE	Two-dimensional fluorescence difference gel electrophoresis
ACRBP	Acrosin-binding protein
AFLPs	Amplified fragment length polymorphisms
AI	Artificial insemination
ALH	Amplitude of lateral head displacement
arcsin	Arcsine
ATP	Adenosin triphosphate
B.C.	Before Christ
BCF	Beating frequency
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium ion
CASA	Computer Assisted Sperm Analysis
cFN	Cellular fibronectin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Cl^{-}	Clor ion
cm	Centimeters
CPAs	Cryoprotective agents
Cu/Zn SOD	Copper/Zinc containing Superoxide dismutase
Cy2	Cyanine dye 2
Cy3	Cyanine dye 3
Cy5	Cyanine dye 5
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EEUU	United States
et al.	*et alii* (and others)
etc.	*et cetera* (and other things)
EU	European Union
FAO	Food and Agricultural Organization
Fig.	Figure

FITC	Fluorescein isothiocyanate
FN1	Fibronectin 1
g	Gram
GFE	Good freezability ejaculates
GLUT 3	Glucose transporter protein 3
GPX5	Glutathione peroxidase 5
h	Hours
HCl	Hydrogen chloride
HCO^{3-}	Bicarbonate
HRP	Horseradish peroxidase
HSP90AA1	Heat-shock protein 90 kDa alpha (cytosolic), class A member 1
HSPB10/ODF1	Small heat-shock protein 10 / Outer-dense fiber protein 1
Hz	Hertz
i.e.	*id est* (that is)
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
iTRAQ	Isobaric tags for relative and absolute quantitation
K^+	Potassium ion
kDa	Kilodalton
Kg	Kilogram
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LEY	Lactose egg-yolk
LEYGO	Lactose egg-yolk, glycerol and Orvus-ES®/EQUEZ STM® Paste
LIN	Linearity index (VSL/VCL)
M	Molar (mol/liter)
mA	Milliamper
Mg^{2+}	Magnesium ion
Min	Minutes
mL	Mililiter
mm	Milimeter
mM	Milimolar (nmol/liter)
mm^2	Square milimeters
mOsm	Miliosmoles
MS/MS	Tandem mass spectrometry
MW	Molecular weight
Na^+	Sodium ion
NaCl	Sodium chloride
nL	Nanoliter
°C	Degree Centigrade
OEP	Orvus-ES® Paste

ORT	Osmotic resistance test
Orvus-ES®	Commercial name of a synthetic detergent
P32	Acrosin-binding protein
PBS	Phosphate buffered saline
PCA	Principal component analysis
PFE	Poor freezability ejaculates
pFN	Plasma fibronectin
pH	Power of Hydrogen
PI	Propidium iodide
pmol	Picomol
PMOT	Progressive motility
PNA	Peanut agglutinin
PPi	Inorganic pyrophosphate
ROS	Reactive oxygen species
RT	Room temperature
s	Seconds
S.A.	Anonymous Society
SCA5	Sperm class analyzer software version 5
SDS-PAGE	Sodium dodecyl sulphate
SEM	Standard error of the mean
sHSPs	Small heat-shock proteins
SP32	Acrosin-binding protein
STR	Straightness index (VSL/VAP)
SYBR14	Commercial name of nucleic acids synthetic dyes
TBS	Tris-buffered saline
TMOT	Total motility
TPI	Triosephosphate isomerase
Tris	Tyrosine amino acids
Tween20	Polysorbate 20
UK	United Kingdom
v	Volume
V	Volts
VAP	Average velocity
VCL	Crvilinear velocity
VDAC2	Voltage-dependent anion channel 2
vol	Volume
VSL	Straghtlinear velocity
w	Weight
WOB	Oscillation index (VAP/VCL)
wt	Weight
x g	Centrifuge force (g-force units)
Zn^{+}	Zinc ion

α-tub	Alpha tubuline

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Abstract

Sperm cryopreservation is currently the most suitable technique to store boar sperm samples for a long period of time. This method has many advantages, including preservation of specific genetic material from breeds and lineages, planning of reproductive trials in breeding farms and sanitary control of samples. However, this technique also has limitations that heavily rely upon the cell sensitivity to cold shock, very high in the case of boar sperm. In addition, not all boar ejaculations present the same freezability (i.e. resilience to withstand cryopreservation), but rather a great variation between and within boars exists. This is related to the sperm resistance to cold shock and allows the classification of boars and their ejaculations into good (GFE) and poor (PFE) freezability boars/ejaculates. In this context, the present Thesis aims to gain new insights into the evaluation and prediction of sperm freezability, thereby preventing PFE cryopreservation. With this purpose, three different objectives were set and addressed by separate, but complementary studies. The first aim was to determine, through Western Blot, whether small heat shock protein 10 (ODF1/HSPB10) and voltage-dependent anion channel 2 (VDAC2) were reliable freezability markers. The second objective was to find potential freezability markers from comparing the sperm proteome of GFE and PFE through two-dimensional difference gel electrophoresis (2D-DIGE). The third aim was to compare the fresh seminal plasma proteome of GFE and PFE through 2D-DIGE, again seeking potential markers for boar sperm freezability. Results from 2D-DIGE obtained were in all cases validated by Western Blot. For the first two objectives, 26 and 34 boar ejaculations, respectively, were split into two fractions, one for protein determination and the other for cryopreservation purposes. Ejaculations were subsequently classified into two groups (GFE and PFE) using progressive motility and sperm viability at 30 and 240 minutes post-thawing. In the first study, VDAC2 amounts in fresh sperm were found to be significantly higher in GFE than in PFE. In contrast, ODF1/HSPB10 did not significantly differ between groups. Furthermore, principal component and multiple regression analyses showed the component explaining the 78.41% of the variance in ejaculate freezability at 240 min post-thawing was significantly correlated with VDAC2 amounts, demonstrating that this protein may be used to predict boar sperm freezability before cryopreservation procedures take place. As far as the second objective is concerned, 2D-DIGE allow to identify two potential freezability markers in boar fresh sperm: acrosin binding protein (ACRBP) and triosephosphate isomerase (TPI). After validation through Western Blot, ACRBP content was significantly higher in GFE than in PFE, whereas TPI amounts were significantly lower in the former that in the latter. Pearson's linear correlations were used to confirm the association of these two proteins with post-thaw sperm viability and motility For the third objective, 18 boar ejaculations were classified as GFE and PFE (as in the other studies) and fresh seminal plasma proteomes were compared. Following 2D-DIGE, two different proteins (fibronectin-1, FN1, and glutathione peroxidase 5, GPX5) were found to significantly

differ between GFE and PFE. However, after Western Blot validation and correlation analysis FN1 but not GPX5 was found to be a reliable marker of boar sperm freezability. Therefore, the main conclusion of this Dissertation is that VDAC2, ACRBP and TPI in fresh sperm and FN1 is fresh seminal plasma may be used to predict the sperm resilience to withstand cryopreservation procedures. Taking together, these findings provide new insights into molecules involved in boar sperm freezability shedding light on the mechanisms involved in boar sperm cryotolerance. Furthermore, they may help to develop new tests to detect sperm freezability capacity at molecular level avoiding the costs and time on cryopreserving PFE. This could ultimately lead the swine industry to reconsider its interest for cryopreserved sperm doses.

Resum

La criopreservació espermàtica és actualment la tècnica més eficient per emmagatzemar les mostres d'esperma de porc durant un llarg període de temps. Aquest mètode presenta nombrosos avantatges que inclouen la preservació de material genètic de races i llinatges específics, la organització dels assaigs reproductius a les granges de producció i el control sanitari de les mostres. Malgrat això, també presenta limitacions que en gran part depenen de la sensibilitat de la cèl·lula al xoc per fred, molt elevada en el cas de l'espermatozoide porcí. A més, no totes les ejaculacions presenten la mateixa congelabilitat, definida com a resistència a la criopreservació, sinó que existeix una gran variació inter- i intra-individual. Aquest fet, que està relacionat amb la resistència de l'esperma al xoc per fred, permet classificar tant els porcs com les seves ejaculacions entre els/les de bona (GFE) i mala (PFE) congelabilitat. Dins d'aquest context, aquesta Tesi pretén adquirir noves aproximacions quant a l'avaluació i predicció de la congelabilitat espermàtica, a fi d'evitar la criopreservació de les PFE. Amb aquest propòsit, es van establir tres objectius diferents que es van dur a terme mitjançant sengles estudis complementaris. El primer objectiu va ser determinar, mitjançant Western Blot, si la proteïna petita de xoc de calor 10 (ODF1/HSPB10) i el canal d'anions dependent de voltatge 2 (VDAC2) eren marcadors de congelabilitat fiables. El segon objectiu va ser buscar potencials marcadors de congelabilitat comparant el proteoma espermàtic de les GFE i les PFE mitjançant gels d'electroforesi diferencials i bidimensionals (2D-DIGE). El tercer objectiu va ser comparar el proteoma del plasma seminal fresc de porcí entre les GFE i les PFE mitjançant 2D-DIGE, per tal de cercar marcadors potencials de la congelabilitat espermàtica del porc. Els resultats obtinguts emprant la 2D-DIGE van ser validats, en tots els casos, mitjançant Western Blot. Per assolir els dos primers objectius, es van dividir 26 i 34 ejaculacions procedents de diferents mascles reproductors porcins en dues fraccions, una va ser destinada a les determinacions proteiques i l'altra a la criopreservació. Seguidament, les ejaculacions es van classificar en dos grups (GFE i PFE) segons la seva motilitat progressiva i viabilitat espermàtiques als 30 i 240 min post-descongelació. En el primer estudi, la quantitat de VDAC2 a l'esperma fresc va resultar ser significativament major a les GFE que a les PFE. En contraposició, la quantitat de ODF1/HSPB10 no va diferir significativament entre els dos grups. Endemés, les anàlisis de components principals i de regressió lineal múltiple van mostrar que la component principal que explicava el 78.41% de la variància de la congelabilitat dels ejaculats a 240 min post-descongelació presentava una correlació significativa amb la quantitat de VDAC2. Aquest resultat va corroborar que aquesta proteïna pot ser emprada com a predictor de la congelabilitat d'una ejaculació de porcí abans que aquesta sigui criopreservada. Pel que fa al segon objectiu, l'anàlisi per 2D-DIGE va permetre la identificació de dos marcadors potencials de la congelabilitat de l'esperma fresc de porcí: la proteïna d'unió a la acrosina (ACRBP) i la trifosfat isomerasa (TPI). Després de la seva validació per

Western Blot, la quantitat de la ACRBP va resultar significativament major a les GFE que a les PFE. Per contra, els nivells de TPI van ser inferiors a les GFE que a les PFE. Es van utilitzar correlacions lineals de Pearson per confirmar la associació d'aquestes dues proteïnes amb la viabilitat i motilitat espermàtiques a la post-descongelació. Pel tercer objectiu, es van classificar 18 ejaculacions porcines com a GFE o PFE (com en els altres estudis) i es va comparar el proteoma del plasma seminal fresc entre ambdós grups. L'anàlisi 2D-DIGE va determinar que les quantitats de dues proteïnes diferents, la fibronectina-1 (FN1) i la glutatió peroxidasa 5, GPX5) diferien entre les GFE i les PFE. Malgrat això, després de la validació per Western Blot i les anàlisis de correlació es va demostrar que només la FN1 era un marcador fiable de la congelabilitat de les ejaculacions de porcí. D'aquesta manera, les principals conclusions d'aquesta Dissertació són que la VDAC2, la ACRBP i la TPI en l'esperma fresc i la FN1 en el plasma seminal poden ser emprades a fi de predir la resistència de les ejaculacions porcines a la criopreservació. Amb tot, aquestes troballes representen noves aproximacions per l'estudi de les molècules relacionades amb la congelabilitat de les ejaculacions porcines i contribueixen a la comprensió dels mecanismes involucrats en la criotolerància de l'espermatozoide d'aquesta espècie. D'altra banda, els resultats obtinguts poden ajudar al desenvolupament de nous testos que detectin la congelabilitat espermàtica a nivell molecular, evitant, així, els costos i el temps esmerçats en la criopreservació de les PFE. Aquest fet pot, en definitiva, conduir la indústria a reconsiderar el seu interès per les dosis seminals criopreservades.

Resumen

La criopreservación espermática es actualmente la técnica más eficiente para almacenar el semen verraco durante un largo periodo de tiempo. Este método presenta múltiples ventajas que incluyen la preservación de material genético de razas y linajes específicos, la organización de ensayos reproductivos en granjas de producción y el control sanitario de las muestras. Sin embargo, también presenta limitaciones que en gran medida dependen de la sensibilidad de la célula al choque por frío, el cual tiene una gran incidencia en la espermatozoide de verraco. Asimismo, no todas las eyaculaciones presentan la misma congelabilidad, esto es, la resistencia a la criopreservación, sino que existe una gran variación inter- e intra-individual. Este hecho está relacionado con la resistencia del espermatozoide al choque por frío y permite la clasificación de los verracos y sus eyaculaciones entre los de buena (GFE) y mala (PFE) congelabilidad. En este contexto, la presente Tesis pretende adquirir nuevas aproximaciones para la evaluación y predicción de la congelabilidad espermática, ahorrando así las pérdidas económicas que supone la criopreservación de PFE. Con este propósito, se establecieron tres objetivos distintos que se llevaron a cabo mediante sendos estudios complementarios. El primer objetivo fue determinar, mediante Western Blot, si la proteína pequeña de choque de calor 10 (ODF1/HSPB10) y el canal aniónico dependiente de voltage 2 (VDAC2) eran marcadores de congelabilidad fiables. El segundo objetivo fue buscar marcadores de congelabilidad potenciales comparando el proteoma espermático de GFE y PFE mediante geles de electroforesis diferenciales y bidimensionales (2D-DIGE). El tercer objetivo fue comparar el proteoma del plasma seminal fresco entre GFE y PFE mediante 2D-DIGE, con el último fin de encontrar marcadores potenciales de congelabilidad en el espermatozoide de verraco. En todos los casos, los resultados de 2D-DIGE obtenidos fueron validados mediante Western Blot. Para los dos primeros objetivos, 26 and 34 eyaculaciones de verraco, respectivamente, fueron divididas en dos fracciones, una destinada a las determinaciones proteicas y la otra a la criopreservación. Las eyaculaciones fueron posteriormente clasificadas en dos grupos (GFE y PFE) de acuerdo con su motilidad progresiva y viabilidad espermáticas a 30 y 240 minutos post-descongelación. En el primer estudio, la cantidad de VDAC2 en esperma fresco resultó ser significativamente mayor en GFE que en PFE. Por el contrario, la cantidad de ODF1/HSPB10 no difirió significativamente entre ambos grupos. Además, los análisis de componentes principales y de regresión lineal múltiple mostraron que el componente que explicaba el 78.41% de la varianza de la congelabilidad de los eyaculados a 240 min post-descongelación presentaba una correlación significativa con la cantidad de VDAC2, demostrando así que dicha proteína puede ser usada para predecir la congelabilidad espermática de verraco antes de que los procedimientos de criopreservación tengan lugar. En cuanto al segundo objetivo, la 2D-DIGE permitió la identificación de dos potenciales marcadores de la congelabilidad en esperma fresco de verraco: la proteína de unión a la acrosina (ACRBP) y la triosafosfato isomerasa (TPI). Después de su

validación con Western Blot, la cantidad de ACRBP resultó significativamente mayor en las GFE que en las PFE, mientras que la de TPI fue menor en los primeros que en los segundos. Se usaron correlaciones lineales de Pearson para confirmar la asociación de estas dos proteínas con la viabilidad y motilidad espermáticas a la post-descongelación. Para el tercer objetivo, se clasificaron 18 eyaculaciones en GFE y PFE (como en los otros estudios) y se compararon los proteomas del plasma seminal fresco entre ambos grupos. Según el análisis de 2D-DIGE, las cantidades de dos proteínas diferentes (Fibronectina-1, FN1, y glutatión peroxidasa 5, GPX5) resultaron ser significativamente diferentes entre GFE y PFE. Sin embargo, después de la validación con Western Blot y de los análisis de correlación, solo la FN1 fue confirmada como marcador de congelabilidad. De esta manera, las principales conclusiones de esta Disertación son que la VDAC2, la ACRBP y la TPI en esperma fresco y la FN1 en el plasma seminal pueden ser utilizadas para predecir la resistencia del espermatozoide de verraco a la criopreservación. Con todo, estos hallazgos representan nuevas aproximaciones a las moléculas relacionadas con la congelabilidad del espermatozoide de verraco y arrojan luz a los mecanismos involucrados en su criotolerancia. Además, dichos hallazagos pueden contribuir al desarrollo de nuevos tests para detectar la congelabilidad espermática a nivel molecular, evitando, así, los costes económicos y temporales que entraña la criopreservación de las PFE. Todo ello puede, en último término, hacer que la industria del porcino reconsidere su interés por las dosis seminales criopreservadas.

1

Introduction

Fifty years ago, Polge and colleagues discovered the cryoprotective effect of glycerol (Polge *et al.*, 1949) which led to the first successful attempt of cryopreserving mammalian cells: spermatozoa (Woelders, 1997). This fact marked the beginning of an era in which practical methods for freezing and banking of blood cells, semen, tissues and organs were focused upon fundamental and theoretical research in cryobiology. Since then, cattle sector has been using artificial insemination with frozen-thawed semen, thereby exploiting its advantages and improving cattle stocks (Curry, 2000; Sieme and Oldenhof, 2015).

Initially, the use of frozen-thawed boar semen was as promising as in other livestock species and was thus expected to present the same early success. However, difficulties on the application of the technique and discouraging fertility outcomes were reported from the first attempts (Watson, 1990a; Holt, 2000a; Johnson *et al.*, 2000; Gobfeld *et al.*, 2008; Knox, 2011). Despite the intensive efforts made in the last three decades, cryopreservation of boar sperm is infrequently used in commercial swine AI programs and its use is less than 1% of the total inseminations worldwide (Johnson *et al.*, 2000; Roca *et al.*, 2006; Yeste, 2015). Nevertheless, due to the advantages that boar sperm cryopreservation presents with respect to liquid-sorted semen, such as: long-term conservation of high-value samples, gene banking strategies, international export in safer conditions since the donor is quarantined at original location, better control for transmission of pathogens and storage of sex-sorted sperm (Bailey *et al.*, 2008; Riesenbeck, 2011; Men *et al.*, 2012), great efforts of the scientific community have been directed towards its improvement and widespread use. In this regard, one should take into account that the notoriously sensitive of boar sperm to cold shock affects the stability and integrity of their plasma membrane at low temperatures (Maxwell and Johnson, 1999; Holt, 2000b; Casas and Flores, 2013). For example, "cryocapacitation" or capacitation-like changes take place as a result of cold-shock, compromise lifespan of sperm and decrease their fertilizing ability (Green and Watson, 2001; Kumaresan *et al.*, 2011; Leahy and Gadella, 2011).

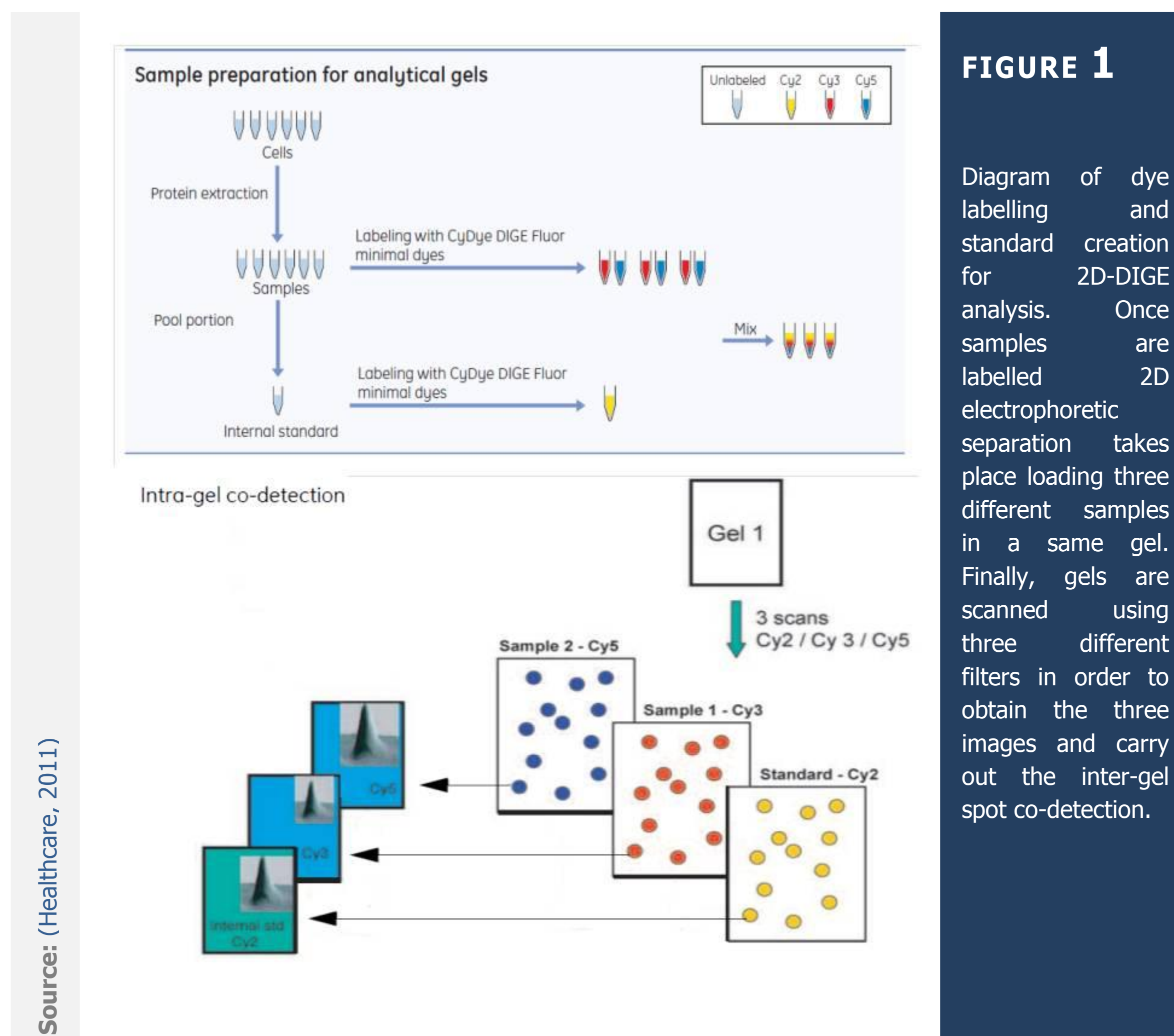
Apart from this handicap and the limited reproductive efficiency of frozen-thawed boar sperm, a high variability in the capability of sperm to survive to cryopreservation protocols (i.e. freezability) has been largely described (Thurston *et al.*, 2002; Holt *et al.*, 2005; Roca *et al.*, 2006; Hernández *et al.*, 2007a; Casas *et al.*, 2009; Benson *et al.*, 2012; Yeste *et al.*, 2013a; Yeste, 2015). It has been reported that about 70% of the variability observed in sperm quality parameters at post-thawing is attributed to the boar, i.e. to an individual factor (being only the rest percentage, about 30%, explained by initial quality sperm analyses) (Roca *et al.*, 2006). Thus, boars were traditionally classified into "good freezers" or "bad freezers" on the basis of the freezability of their ejaculates (Medrano *et al.*, 2002; Hernández *et al.*, 2007a). However, further studies in the field demonstrated that this variability not only existed between males, but also between breeds (Thurston *et al.*, 2002; Waterhouse *et al.*, 2006) and even between ejaculations from the same male. These findings led classifying ejaculates, rather than boars, into good freezability ejaculates (GFE) and poor freezability ejaculates (PFE) (Watson, 1995; Gil *et al.*, 2005; Casas *et al.*, 2009; Casas and Flores, 2013;

Yeste *et al.*, 2013a). The mechanisms underlying these differences in ejaculate freezability remain to be elucidated since this realm has mainly been focused upon improvements of cryopreservation procedures, extenders and artificial insemination (AI) practices rather than on identifying the molecular basis of sperm freezability differences (Casas *et al.*, 2010a; Yeste, 2015). Despite little progress being made to address this issue, genetic-based principles have been reported to underlie these differences (Thurston *et al.*, 2002). Indeed, Thurston and colleagues demonstrated that genetic variation, involving amplified fragment length polymorphism markers (AFLPs), existed between "good" and "bad" freezers (Thurston *et al.*, 2002). This was followed by the idea that individuals should not only be selected by their fertility outcomes, but also on the basis of their sperm freezability, since cryopreservation of PFE should be avoided. Towards this goal, and taking into account that conventional sperm parameters analyzed in fresh/extended ejaculations do not correlate with sperm quality at post-thawing (Casas *et al.*, 2009; Yeste, 2015), the efforts of research community were directed to identify markers predicting boar sperm freezability. Related to this, Flores and colleagues (2009) demonstrated that subpopulation patterns of motile sperm could be useful for predicting the potential freezability of a given ejaculation (Flores *et al.*, 2009). Sperm kinematic parameters such as percentages of linearity (LIN) and straightness (STR) evaluated at 5ºC, i.e. following the first cryopreservation step, were also found to match with this purpose (Casas *et al.*, 2009). Recently, acrosin activity has been shown to be a cryopreservation marker, since GFE present higher acrosin activity than PFE not only after but also before starting freeze-thawing procedures (Estrada *et al.*, 2015; Pinart *et al.*, 2015).

Another promising strategy in the prediction of boar sperm freezability is the research of molecular markers that can predict such freezability in refrigerated semen. This requires urgent attention since can promote further the use of frozen-thawed boar sperm in AI programs. With regard to this angle, one needs to mention the study by Casas and colleagues (2010) in which the expression of three freezability-related proteins (Cu/ZnSOD, HSP90AA1, GLUT3) in extended semen was compared between GFE and PFE. The researchers found encouraging results since heat shock protein 90 (HSP90AA1) amounts differed between GFE and PFE, thereby confirming this protein as a reliable freezability marker in boar sperm (Casas *et al.*, 2010a). As stated by the aforementioned authors, one could think that molecules involved in the pathways related to cryopreservation and its effects could become molecular markers of sperm freezability, hypothesis largely demonstrated by the identification of HSP90AA1 as boar freezability marker (Casas *et al.*, 2010a). Thus, heat-shock proteins, which play key roles in cold-induced stress on sperm cell and plasma membrane proteins involved in osmotic cell regulation, which is vital during cryopreservation, are firm candidates to be studied as freezability markers. Therefore, the aim of the first study (**PAPER I**) was to determine whether two molecules involved in the events described before could be able of predicting boar sperm freezability. Indeed, expression levels of small heat-shock protein 10 or outer-dense fibre protein 1 (HSPB10/ODF1) that could be related to cold-shock effects on boar sperm due to its role as chaperone (Fontaine *et al.*, 2003), and voltage-dependent anion channel 2 (VDAC2), which exerts a clue role in

mammalian sperm motility and membrane permeability (Shoshan-Barmatz *et al.*, 2010), were compared in extended semen of GFE and PFE. This required taking a protein aliquot prior to cryopreservation, since the identification of GFE and PFE only came later. Once the sperm samples were classified the HSPB10/ODF1 and VDAC2 amounts were assessed using Western Blot technique.

While the approach taken in **PAPER I** provided interesting results, another, optimized strategy comparing the entire proteome of GFE and PFE rather than freezability marker candidates one-by-one was taken in **PAPER II** and **PAPER III**. To achieve this objective, two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) technique was used to assess differences in protein spot profiles between 10 GFE and 10 PFE. Later on, results were confirmed through Western Blot assessment. In this context, it is worth mentioning that in order for proteins to be studied in such a large scale, the application of powerful proteomic techniques, such as protein arrays, 2D-DIGE or iTRAQ, are mandatory. While this does not avoid further determination through Western Blotting, it allows a wider screening that is not possible otherwise. In fact, and because of its high resolution and sensitivity for the analysis of complex mixtures, two-dimensional (2D) electrophoretic separation of proteins followed by liquid chromatography and mass spectroscopy have been widely used for the study of sperm from other mammalian species (Martínez-Heredia *et al.*, 2006; Paradowska *et al.*, 2006; de Mateo *et al.*, 2007; Cardozo *et al.*, 2008; Petit *et al.*, 2013). However, this approach implies an important handicap: the complexity of generating matched-sets from the same spots in different gels due to variations in spot profiles (Ünlü *et al.*, 1997; Oliva *et al.*, 2008, 2010). In this regard, 2D-DIGE technique improves the standard 2D since overcomes variation between gels (Tonge *et al.*, 2001; Baker *et al.*, 2005; Oliva *et al.*, 2010; Capaldi *et al.*, 2011). As first reported by Ünlü and colleagues (1997), 2D-DIGE allows covalent labelling of samples with three, structurally similar but spectrally distinct, cyanine dyes which do not have any effect on the protein migration in the 2D gel electrophoresis (Ünlü *et al.*, 1997; Tonge *et al.*, 2001). Through this way, equal protein amounts of the differentially labelled samples to be compared are mixed with an internal labelled standard. The inclusion of this standard in each gel, made by a pool of equal amounts of each experimental sample, allows the variation adjustment in all gel-system for gel to gel matches and comparison of protein-spot profiles. Subsequently, samples are subjected to 2D electrophoretic separation followed by fluorescence imaging/scanning and protein identification by tandem mass spectrometry (LC-MS/MS) (Timms and Cramer, 2008; Capaldi *et al.*, 2011) **(Fig. 1)**. Applications of 2D-DIGE in the study of sperm provide encouraging results (Baker *et al.*, 2005; Forné *et al.*, 2009; Liao *et al.*, 2009; D'Amours *et al.*, 2010). However, as aforementioned, the power and reliability of this approach is increased when combined with Western Blot technique (Baker *et al.* 2005; Tan *et al.* 2014).



Source: (Healthcare, 2011)

FIGURE 1

Diagram of dye labelling and standard creation for 2D-DIGE analysis. Once samples are labelled 2D electrophoretic separation takes place loading three different samples in a same gel. Finally, gels are scanned using three different filters in order to obtain the three images and carry out the inter-gel spot co-detection.

Finally, in addition to the approach based upon sperm (**PAPERS I and II**), the present dissertation also tackled the seminal plasma angle. In fact, despite its vital role for reproductive physiology, little attention has been paid thus far about its effects upon boar sperm freezability. In fact, it is widely understood that this complex mixture of secretions from male accessory sexual glands (Sancho and Vilagran, 2013) modulates sperm function (reviewed by (Maxwell and Johnson, 1999; Juyena and Stelletta, 2012). However, its role during sperm cryopreservation still remains controversial, since studies that examined its effect when added to freezing and thawing extenders have reported conflicting results (Okazaki *et al.*, 2009b; Mogielnicka-Brzozowska and Kordan, 2011; Caballero *et al.*, 2012; Fernández-Gago *et al.*, 2013). Such differences have been attributed to variations in its composition and, based on this, it has been demonstrated that seminal plasma from boars with good sperm freezability improves the sperm quality parameters at post-thawing (Hernández *et al.*, 2007a). Hence, against this background and taking into account that boar seminal plasma proteins are yet to be tested as freezability markers, the aim of the third study (**PAPER III**) was to compare the proteome of boar seminal plasma between GFE and PFE through 2D-DIGE. Western Blotting was further used for validation as it has been stated above.

To sum up, it is important to know that boar sperm cryopreservation is nowadays confined in a specific niche of the pig industry: breeding farms that select specific valuable genetic traits and store them for its dissemination to production farms (Knox, 2011; Rodríguez-Gil and Estrada, 2013). Since frozen-thawed boar sperm is not used worldwide in commercial AI programs due to its fertility limitations compared to extended semen, research on cryopreservation techniques is warranted. In this context, investigations for designing new freezability prediction tests are extremely required since they may represent an important forward step in the dissemination of this sperm preservation technique and its application in pig farms.

2

Bibliographic revision

Bibliographic revision
Bibliographic revision

2.1 Evolution of the domestic pig and its global production

2.1.1 The phylogenetics and origin of the Domestic Pig

According to the current classification (systematists do not yet agree with the exact number or how some orders and families are related and new information is always arisen based on molecular evidences and new fossils which change the understanding of many groups), pigs belong to the order Artiodactyla, an order phylogenetically closer to Primates than Rodentia, concretely the suborder Suiformes and they are members of the family Suidae (Chen *et al.*, 2007; Ruvinsky *et al.*, 2011). The Suidae family traces back to the upper Eocene or possibly later and it consists of 15 species grouped into 4 genera: Sus (domestic and wild pigs) from Eurasia; Porcula (pygmy hogs) from northern India; Babyrousa (babirusa) from Sulawesi island and its satellites and Potamochoerus (bush pig and red river hog) from Sub-Saharan Africa **(Fig. 2)**.

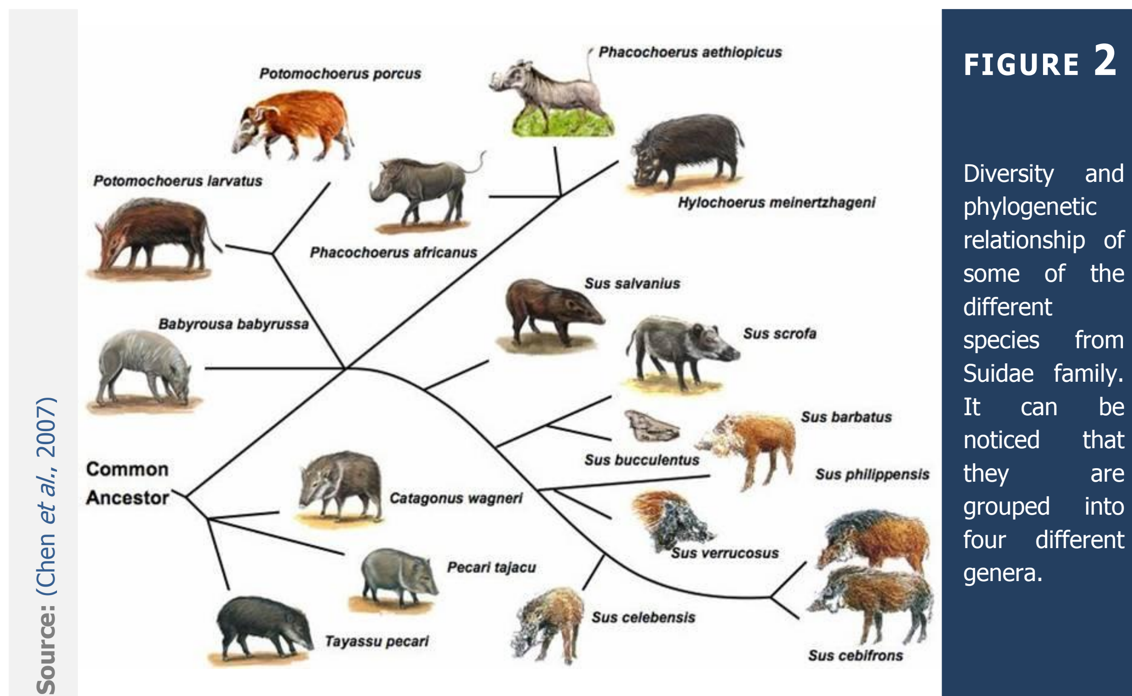


FIGURE 2

Diversity and phylogenetic relationship of some of the different species from Suidae family. It can be noticed that they are grouped into four different genera.

The domestic pig (*Sus scrofa domesticus*) originates from the Eurasian wild boar (*Sus scrofa*) (Giuffra *et al.*, 2000). Archaeological evidences suggest that pigs were domesticated in multiple centres by 10,500–9,500 year before Christ (B.C.) (Larson *et al.*, 2005; Zeder, 2009). According to a multiple, independent domestication hypothesis, pigs would have been domesticated separated by thousands of kilometres and also by thousands of years (Chen *et al.*, 2007). The time of its domestication coincides with their geographical expansion as domesticate livestock worldwide (Zeder, 2008). This highlights the dramatic capability of this animal to adapt to different food and climate conditions (Larson *et al.*, 2011). Domestication provided a rapid genetic variation

through human and novel environmental pressures which generated an inter-breed phenotypic evolution that ultimately resulted in the formation of many unique breeds (Chen *et al.*, 2007).

To date, there are likely over 730 pig breeds worldwide, and the most distributed are the following five international transboundary breeds: Large white (117 countries), Duroc (93 countries), Landrace (91 countries), Hampshire (54 countries) and Piétrain (35 countries) (Chen *et al.*, 2007).

2.1.2 Livestock and porcine market

Nowadays, livestock supports directly the livelihoods and food security of almost a billion of people, supplying around 12.9 percent of calories consumed across the world according to Food and Agriculture Organization of the United Nations (FAO) reports (FAO, 2009). During the past 40 decades meat production has grown strikingly, concretely, pig meat increased 294% of its production (FAO, 2011).

Pig presents good adaptability to diverse environments, an excellent conversion index and high reproductive efficiency, (which involves a short interval to reach sexual maturity, short gestation period, multiple offspring per pregnancy and quick tendency to rebreed). These features make this animal one of the most efficient livestock species for global food production (Knox, 2014). Indeed, pork together with poultry are the most important meats in world trade (USDA, 2014), the pork meat being the preferred in the European Union (EU) (EU, 2011).

In 2014, the main producer of pork worldwide was China with 56.5 million tons of pork, followed by the EU with 22.4 million (USDA, 2014). In 2013, Spain became the second EU producer with approximately 3.4 million tons of pork (EUROSTAT 2013). Figures for this country in the same year indicate that pig production accounted for approximately 39.3% of the final livestock production and 14.2% of the final agricultural production (i.e. percentage of pig production from the total dirty production of all productive units that constitute the agricultural production). The main producing region was Catalonia accounting for 43.7% of the total pig production in Spain, followed by Castile and Leon with 12.9% (MAGRAMA, 2014). All these data indicate that swine production occupies an important position in the global economy and its production has been playing an important role in many European countries and regions such as Spain and Catalonia.

2.2 The Piétrain breed

Since all semen samples used in the present Dissertation came from the Piétrain breed, the present section is intended to summarise its main features. First, the birthplace of Piétrain breed was in Belgium in the twenties (Buxadé, 1984), in a village that gave it the name (BPA, 2006). It was originated from Normand pigs with a genetic mutation that affects 80% of the breed and that was carried out by eight selection centres in Belgium (MAGRAMA, 2006; FAEGAS, 2010). Thereafter, it was imported into Germany in 1960-61 and into the United Kingdom (UK) in 1964 (Oklahoma University, 1995; BPA, 2006).

The Piétrain breed is very popular as a terminal sire in the two UE's largest pig producing countries: Germany and Spain (BPA, 2006). Being approximately 1,600 reproduction farms of this breed found in Spain (Sabor artesano, 2008; Sánchez, 2010); it is a breed widely produced in different regions mainly in Catalonia, Murcia, Castile La Mancha and Galicia (FAEGAS, 2010).

2.2.1 Interesting characteristics of Piétrain pigs

In morphologic terms, Piétrain breed is medium-size characterized with white skin with black spots irregularly distributed, carrying the ears erected (Briggs, 1983)**(Fig. 3)**.

Source: (Sabor artesano, 2008)

FIGURE 3

Piétrain female with piglets. It can be appreciate the white colour with black spots, characteristic of this breed. Around these spots are present white hair that confers a characteristic light pigmentation (Briggs, 1983).

As pure strain, Piétrain breed shows exceptional muscular conformation and high yield in meat with no fat in their flesh, thereby giving the highest ratio in terms of meat/total weight compared to all other strains (FAEGAS, 2010). For this reason, Piétrain boar is commonly used as terminal sire in crossing programs, since regardless of the mother's breed, the improvement in terms of quality and quantity of meat is impressive. This leads a better commercial ranking (BPA, 2006; FAEGAS, 2010; Sánchez, 2010), so its production is aimed to fresh products, the carcases giving 72-75% yield (FAEGAS, 2010).

However, Piétrain breed presents low growth with low conversion index and reproductive performance (requires a high level of feeding to increase its weight and reproduction) (Bencomo, 2010; FAEGAS, 2010). Moreover, females do not produce much milk, present relatively under-developed mammary glands and demonstrate aggressive behaviour to their piglets. This explains why they are not used as reproductive females, but rather other breeds are preferred (e.g. Landrace, Large-White) (Quiniou *et al.*, 2007; Bencomo, 2010; Chomwisarutkun *et al.*, 2012).

2.3 The boar reproductive system and ejaculation

2.3.1 Boar reproductive anatomy and functions

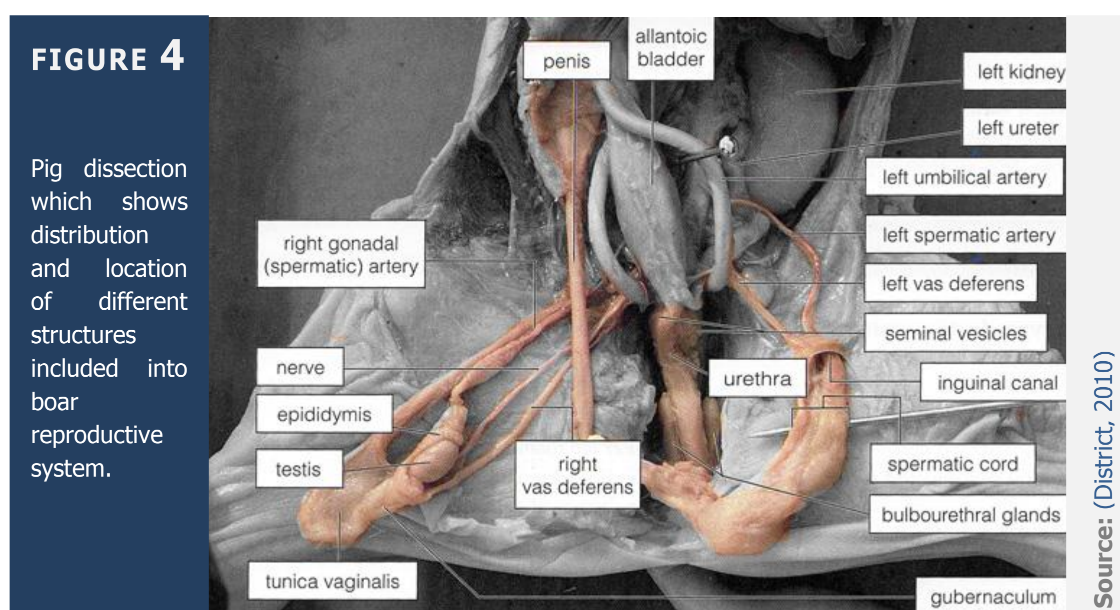


FIGURE 4 Pig dissection which shows distribution and location of different structures included into boar reproductive system.

Source: (District, 2010)

Boar reproductive system include six different structures consisting of two testes, two epididymis, two deferent ducts, the urethra, accessory sex glands (which comprise two seminal vesicles, the prostate and two bulbourethral glands or Cowper's glands), and a copulatory organ or penis (Bonet *et al.*, 2013) **(Fig. 4)**.

In the pig embryo, sex organs start their development about 20 days after oocyte fertilization. This development is complete around day 90 (McGlone and Pond, 2003). Correct development of boar reproduction system is of crucial importance for its proper functioning, which consists of: production, maturation and ejaculation of semen and contribution to sexual characters through secretion of hormones (Knobil and Neill, 1994; Bonet *et al.*, 2013).

2.3.2 The boar ejaculate

Ejaculation consists of the ejection of semen from the reproductive tract of post-pubertal and adult males (Bonet *et al.*, 2013). While boars are considered to be post-pubertal when they reach eight to twelve months of age, and adults from one year, first ejaculations may occur between five and six months of age (Córdova-Izquierdo *et al.*, 2004; Yeste, 2008; Sancho and Vilagran, 2013).

Semen is composed of a cell fraction made up of spermatozoa and a non-cellular fraction called seminal plasma (Bonet *et al.*, 2013). Boar ejaculation is characteristic for its abundant volume, between 150 and 600 mL which represents the highest volume among domestic livestock species (Yeste, 2008; Knox, 2010; Sancho and Vilagran, 2013). In all mammalian species, semen is ejaculated in fractions, but contents from the epididymis and accessory sex glands are only mixed during ejaculation. While the three fractions cannot be clearly distinguished in those species with a low ejaculate volume, they are separated as follows (each fraction contain different amounts of sperm and different fluids origins (Sancho and Vilagran, 2013)):

Pre-spermatic fraction

This is the first fraction presenting a watery appearance because it is devoid of spermatozoa (Siqueira *et al.*, 2011; Sancho and Vilagran, 2013). It is formed by secretions from the prostate gland, seminal vesicles and Cowper or bulbourethral glands (Yeste, 2008; Sancho and Vilagran, 2013) and its role is to flush urine and bacteria from the urethra, preparing this conduct for the efflux of the spermatic fraction (Knox, 2010). Represents approximately 5-20% of the total ejaculate volume (CIAP, 2000).

Spermatic (or rich) fraction

Milky-white in appearance, this fraction contains the bulk of ejaculated spermatozoa (80-90% of total sperm) (Yeste, 2008; Knox, 2010; Sancho and Vilagran, 2013). Particularly, a sperm peak-portion with a volume of 10 mL containing high content of epididymal cauda fluid can be differentiated at the beginning (Sancho and Vilagran, 2013). The rest of the spermatic fraction contents a high number of spermatozoa diluted in secretions produced by seminal vesicles and prostate (Siqueira *et al.*, 2011). Corresponds to 30-50% of the total ejaculate volume (CIAP, 2000; Sancho and Vilagran, 2013) and it is the fraction collected when seminal doses are prepared.

Post-spermatic fraction

It is pale-white in appearance and contains decreasing sperm numbers and secretions from vesicular, prostate and, at the end of the ejaculation, from Cowper glands or bulbourethral glands. These glands secrete a gelatinous fraction, characteristic of the boar ejaculate, that avoids sperm to efflux from the sow uterus and cervix after natural matting (Aamdal and Hogset, 1957). When preparing seminal doses, this fraction is discarded not only to prevent over-dilution of the sperm-rich fraction (Knox, 2010;

Sancho and Vilagran, 2013) but also to avoid high content of seminal plasma, since it may be detrimental for sperm preservation in liquid storage (Yeste, 2008; Sancho and Vilagran, 2013).

2.4 The spermatozoon

2.4.1 Boar sperm production

Sperm production takes place in the testis, concretely in the seminiferous tubules, in a cyclical and highly organized process called spermatogenesis (Eddy and O'Brien, 1994; Dyck and Ruvinsky, 2011; Costa *et al.*, 2013). Spermatogenesis comprises the passage from diploid spermatogonia, through a series of biochemical, physiological and morphological changes, to haploid spermatozoa (Eddy and O'Brien, 1994; Luo *et al.*, 2015).

This process is characterized by different cell associations, which are grouped into their nucleus morphologic characteristics. These associations determine the eight different stages of the cycle (França *et al.*, 2005; Bonet *et al.*, 2013; Costa *et al.*, 2013). This cell association is well-arranged in the seminiferous epithelium in segments, so that only one stage or segment is found at each tubular cross-section (França *et al.*, 2005; Hess and França, 2008; Costa *et al.*, 2013). This segmentation is maintained over the process, since cells within each layer of the seminiferous epithelium change in synchrony with the other layers and migrate from the basal region to the apical region of the seminiferous epithelium (Leblond and Clermont, 1952; Hess and França, 2008; Bonet *et al.*, 2013; Costa *et al.*, 2013) **(Fig. 5)**.

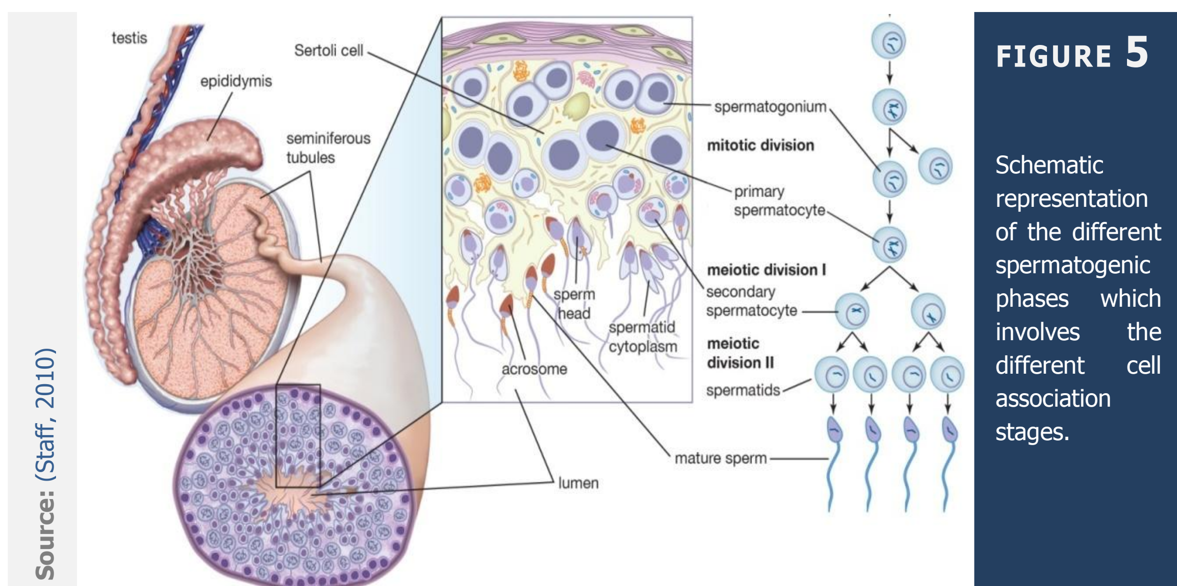


FIGURE 5

Schematic representation of the different spermatogenic phases which involves the different cell association stages.

Source: (Staff, 2010)

Spermatogenesis involves three classes of cells: spermatogonia, spermatocytes and spermatids. First, spermatogonia are divided up through mitosis several times until they become primary spermatocytes in a process called spermatocytogenesis, spermatogonial or proliferative. Subsequently, diploid primary spermatocytes undergo first meiotic division (or reducing division) resulting in secondary spermatocytes which are haploid. These haploid cells go then through a second meiotic division (or equatorial division) and develop into early round spermatids in a process known as meiotic or spermatocytary. In the last, spermiogenic phase, these early spermatids are closely attached to the surface of Sertoli cells and undergo complex morphologic, biochemical and physiological changes resulting in the formation of asymmetric, flagellar spermatozoa. At the end of spermatogenesis, spermatozoa are released from Sertoli cells into the lumen of seminiferous tubules (Eddy and O'Brien, 1994; Garcia-Gil *et al.*, 2002; Bonet *et al.*, 2013; Costa *et al.*, 2013)**(Fig. 5)**.

The cycle of seminiferous epithelium is constituted by the sequence of events that takes place from the disappearance of a given cell association to its reappearance in a given area of the seminiferous epithelium (Leblond and Clermont, 1952; Krestser de and Kerr, 1994). Therefore, the time which comprises the appearance of one complete series of cell associations at a given point within the seminiferous tubule is the cycle of the seminiferous epithelium (Leblond and Clermont, 1952). In mammals, spermatogenesis lasts from 30 to 75 days and takes about 4.5 cycles (França *et al.*, 2005; Costa *et al.*, 2013). In pigs, each spermatogenic cycle lasts 8.6-9.0 days, the duration of the overall spermatogenesis being between 34 and 40 days (França *et al.*, 2005; Zeng, 2006; Bonet *et al.*, 2013).

2.4.2 Maturation of spermatozoa

As aforementioned, testes produce immature spermatozoa that are immotile and infertile. After spermiation, spermatozoa pass through efferent ducts and then the epididymis, where sperm maturation takes place (França *et al.*, 2005; Park *et al.*, 2012; Bonet *et al.*, 2013; Luo *et al.*, 2015).During this process, a series of modifications, including displacement of cytoplasmatic droplet, development and formation of the acrosome, nuclear chromatin condensation, stabilization of dense fibres, and changes in perinuclear theca and plasma membrane components, occur. All these changes are required for spermatozoa to acquire progressive motility and fertilizing ability (i.e. the ability to recognize and bind the oocyte) (Dacheux *et al.*, 2003; França *et al.*, 2005; Bonet *et al.*, 2013; Labas *et al.*, 2015). This extensive cellular differentiation that takes place within the epididymis is not under the genomic control of germ cells (as spermatogenesis in the testis), but rather requires sequential interactions with the medium surrounding sperm during their transit. Such a medium is synthesized and secreted by different cell types of the epididymal epithelium (principal, apical, basal, clear and halo cells) (Cooper *et al.*, 1990; Bonet *et al.*, 2013; Labas *et al.*, 2015).

In mammals, the time required for sperm maturation (i.e. transit through the three epididymal regions, caput, corpus and cauda) ranges from 2 to 5 days. This time ranges from 5 to 5.8 days in mice, 5.5 days are estimated in humans and from 9.0 to 11.8 in pigs (França *et al.*, 2005). Taking into account that the entire longitude of the epididymal duct is 54 m, the distance travelled by a spermatozoon each day is approximately 5 m (Bonet *et al.*, 2013).

Once spermatozoa have become mature, they are stored in epididymal cauda where the duct becomes dilated. Physicochemical conditions of this storage region in the epididymis represent a suitable milieu for maintaining sperm in a quiescent state (Cooper, 1998; França *et al.*, 2005; Jones *et al.*, 2007). When ejaculation takes place, this region has a neuromuscular effector system to recruit sperm, reducing by around 60% the number of sperm stored when is emptied (Strzeżek *et al.*, 1995; França *et al.*, 2005; Bonet *et al.*, 2013).

Ultimate changes for sperm to acquire fertilizing ability are promoted in the female reproductive tract. Sperm reach the oviduct (also known as fallopian or uterine tube), attach to isthmus epithelium, forming the sperm reservoir, and remain in a quiescent state, while waiting for the oocyte to be released (Hunter, 1981; Yeste, 2013). Around ovulation, sperm unattach the isthmus and come into contact with the oviductal fluid (for a review see (Holt and Fazeli, 2015)). The components of this oviductal fluid, including bicarbonate, promote sperm capacitation, a process by which sperm gain the ability to bind the zona pellucida and trigger the acrosome reaction, leading to penetration of zona pellucida and final fusion with the oocyte membrane (Hunter, 1988; Flesch and Gadella, 2000; Gadella, 2008; Yeste, 2013).

2.4.3 The mature spermatozoon

The mature spermatozoon is the specialized male gamete that results from gametogenesis and epididymal maturation (Eddy and O'Brien, 1994; Briz and Fàbrega, 2013). In a boar ejaculation, around 80% to 95% of spermatozoa are mature, 5 to 15% are immature and 1 to 5% are aberrant (Martin, 1982; Bonet *et al.*, 2000; Briz and Fàbrega, 2013).

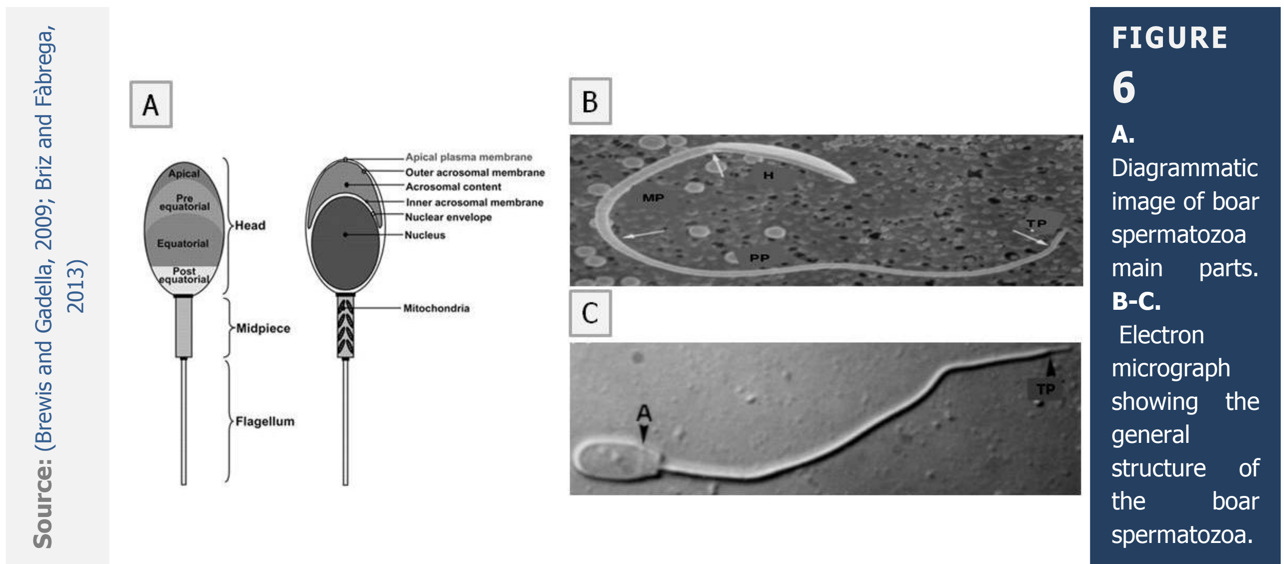
The mature spermatozoon, which measures 43-45 µm in length, has a highly specialized structure to accomplish its basic function. Three regions are distinguished: the head, the connecting piece (or neck) and the tail (Flesch and Gadella, 2000; Gadella, 2008; Briz and Fàbrega, 2013)**(Fig. 6)**.

The sperm head

The head is bilaterally flat and oval (measuring 7µm in length, 3.7µm of maximum wide and 0.4µm of thick). Three major domains of the plasma membrane can be distinguished: apical

segment, pre-equatorial and equatorial regions, composing the acrosomal segment, and the post-acrosomal sheath (Eddy and O'Brien, 1994; Briz and Fàbrega, 2013) **(Fig. 6)**.

The head contains a very low amount of cytosol, the nucleus (with a high compacted chromatin) and the acrosome (Flesch and Gadella, 2000; Briz and Fàbrega, 2013). Amongst the sperm proteins present in the perinuclear theca, one



Source: (Brewis and Gadella, 2009; Briz and Fàbrega, 2013)

FIGURE 6
A. Diagrammatic image of boar spermatozoa main parts. **B-C.** Electron micrograph showing the general structure of the boar spermatozoa.

finds the sperm-borne oocyte activation factor, phospholipase C ζ (PLCζ) (Yeste *et al.*, 2015). The acrosome is a modified Golgi complex that presents two different membrane regions (i.e. internal, in contact with the nucleus and external, in contact with plasma membrane) and contains hydrolytic enzymes that are necessary for penetration of the zona pellucida (Knobil and Neill, 1994; Flesch and Gadella, 2000; Brewis and Gadella, 2010; Briz and Fàbrega, 2013)**(Fig. 6)**.

Therefore, the main roles of the sperm head are bearing the parental DNA, contribute to sperm-oocyte recognition and subsequent fusion, and alleviate the metaphase II-arrest in the oocyte (Flesch and Gadella, 2000; Briz and Fàbrega, 2013; Yeste *et al.*, 2015).

Connecting piece

The neck or connecting piece is a linking segment composed by different structures that firmly attach the end of the head to the apical zone of the tail (i.e. midpiece)(Bonet *et al.*, 2000; Briz and Fàbrega, 2013). Its total length is 0.7 µm and its thickness is 0.5 µm (Bonet *et al.*, 2000) **(Fig. 6)**.

The sperm tail

The tail is filamentous and cylindrical in shape. It measures 38 µm in length, and ranges from 0.7 µm to

0.2 µm in wide (from the apical to the terminal region)(Bonet *et al.*, 2000; Briz and Fàbrega, 2013) **(Fig. 6)**. Three different regions are distinguished:

1. The midpiece, which is a mitochondrial sheath lying beneath the plasmalemma that become activated especially during sperm capacitation (Ramió-Lluch *et al.*, 2011).
2. The main piece, which is the longest region of the tail and is made up of a fibrous sheath and;
3. The terminal piece, constituted by a disorganized axoneme surrounded by the plasma membrane. This piece together with the main one constitutes the propulsive apparatus for initiation and maintenance of sperm motility (Knobil and Neill, 1994; Briz and Fàbrega, 2013).

2.5 The seminal plasma

2.5.1 Generalities about seminal plasma

The boar ejaculation consists of two fractions: the sperm fraction, containing the sperm cells (10-30% of the final volume) and the liquid fraction or seminal plasma in which spermatozoa are suspended (70-90% of the final volume) (Knobil and Neill, 1994; Juyena and Stelletta, 2012; Sancho and Vilagran, 2013).

Seminal plasma is a mixture of secretions from the male accessory sex glands (seminal vesicles, prostate and bulbourethral glands) and epididymis (Davies *et al.*, 1975; Mann and Lutwak-Mann, 1981a; Foxcroft *et al.*, 2008; Song *et al.*, 2010; Sancho and Vilagran, 2013). The exact composition of this fluid is very complex and it varies among species, individuals and even between ejaculations (Mann and Lutwak-Mann, 1981a; Catt *et al.*, 1997; Pérez-Pé *et al.*, 2001; Caballero *et al.*, 2004a; Cardozo *et al.*, 2006; Maxwell *et al.*, 2007; Druart *et al.*, 2013).

2.5.2 Components of seminal plasma

In general terms, seminal plasma is made up of water, inorganic compounds (carbonates and phosphates), energy substrates (glucose, fructose, sorbitol), ions (Na^+, K^+, Zn^{2+}, Ca^{2+}, Mg^{2+}, Cl^-) and organic compounds (amino acids, lipids, peptides and low and high molecular-weight proteins) (Mann and Lutwak-Mann, 1981a; Catt *et al.*, 1997; Juyena and Stelletta, 2012; Sancho and Vilagran, 2013)**(Table 1)**.

Inorganic phosphate (Yi *et al.*, 2012), nickel (Zemanová *et al.*, 2007) or selenium (Lasota *et al.*, 2004) are **inorganic components** that have been found in boar seminal plasma. It is worth noting that inorganic pyrophosphate (PPi), which plays a crucial

role in biosynthetic reactions that utilize ATP, in seminal plasma may serve as an energy source during sperm transport, sperm-oocyte interactions, sperm capacitation and acrosome reaction (Yi *et al.*, 2012).

Seminal plasma compounds	Normal range values (units in mM)
Na^+	125–252
K^+	17–46
Ca^{2+}	1.5–4.6
Mg^+	2.5–2.4
Cl^-	85–105
Phosphate	0.4
Fructose	0.5
Glucose	0.06–0.3
Sorbitol	0.4
Inositol	28
Lactid acid	2.2
Citric acid	2.6–10.4
Glutamic acid	2
Glycerophosphocholine	4
Glycerophosphoinositol	0.26
Arginine	0.01
Creatinine	0.03
Ergothioneine	0.7
Proteins (mg/mL)	30

TABLE 1

Summarized composition of boar seminal plasma and the normal levels of each compound.

Source: (Sancho and Vilagran, 2013)

Apart from this, **energy substrates** in boar seminal plasma, which represent the main sugar apportion for sperm movement and metabolism, are fructose, glucose and sorbitol (López Rodríguez *et al.*, 2013; Sancho and Vilagran, 2013). Glucose is the substrate more rapidly utilized by the boar sperm cell to produce ATP, although other monosaccharides, such as fructose and sorbitol, can be also utilised at a less efficient rate (Medrano *et al.*, 2006). In general, due to its importance for sperm energy production, sugar composition of seminal plasma has been correlated with fertility (Juyena and Stelletta, 2012).

With regard to the **ions** present in boar seminal plasma, zinc (Zn^{2+}) is involved in stabilizing macromolecules and exerts important functions as an antibacterial and antioxidant molecule (Arver and Eliasson, 1980; López Rodríguez *et al.*, 2013). It has to be emphasized the presence calcium (Ca^{2+}) in pig seminal plasma since it is a clue molecule for sperm capacitation and acrosome reaction (Maxwell and Johnson, 1999; Green and Watson, 2001; Bailey *et al.*, 2005; Fernández-Gago *et al.*, 2013).

Finally, boar seminal plasma also contains different **organic compounds**. Concretely, different proteins and amino acids (i.e. gluthamic acid, carnitine, taurine) have been reported to exert important roles as antioxidants (Harrison and White, 1972; Zini *et al.*, 1993) . Recently, Druart and colleagues (2013) have found that fibronectin and spermadhesins are the most predominant proteins in boar seminal plasma, which is in contrast to other domestic mammal species (Druart *et al.*, 2013). Fibronectin-1 present in seminal plasma has been related to sperm midpiece and tail defects (González-Cadavida *et al.*, 2014). Furthermore, spermadhesins represent more than 90% of boar seminal plasma proteins (Song *et al.*, 2010). Among members of this family

of small glycoproteins, the most important are AQN-1, AQN-3, AWN, DQH, PSP-I and PSP-II (Töpfer-Petersen *et al.*, 1998, 2008; Centurion *et al.*, 2003; Calvete and Sanz, 2007; Yeste, 2013), which exert an immunomodulatory effect in the female reproductive tract (for a complete revision (Yeste and Castillo-Martín, 2013)), play a vital role in the formation of oviductal reservoir (See Yeste 2013 for review), become exposed and disattach the sperm plasma membrane during capacitation and acrosome reaction (Calvete *et al.*, 1995; Töpfer-Petersen *et al.*, 1998; Centurion *et al.*, 2003; Caballero *et al.*, 2008), and are involved in sperm-oocyte recognition and interaction (Töpfer-Petersen *et al.*, 1998; Jonáková *et al.*, 2000; Novak *et al.*, 2010; Siqueira *et al.*, 2011).

2.5.3 The role of the seminal plasma

It is important to bear in mind seminal plasma is made up of a wide range of molecules that exert multiple functions during ejaculation, sperm transit throughout the female reproductive tract and fertilization. The roles exerted by seminal plasma could be summarised as follows:

1. Creation of an optimum milieu for sperm, providing suitable osmolarity and pH conditions and complete energy sources for sperm aerobic and anaerobic metabolism (Mann and Lutwak-Mann, 1981b).

2. Seminal plasma exerts an activation and augmentation of the motility of the spermatozoa through a complex and not well determined interaction of seminal plasma coating proteins (Johnson *et al.*, 2000; Caballero *et al.*, 2012; Piehl *et al.*, 2013; Yeste, 2013).

3. Sperm coating with capacitation inhibitors (also known as decapacitation factors (Fraser, 2010)) in order to stabilise the plasma membrane and prevent premature activation during sperm transport (Johnson *et al.*, 2000; Vadnais and Althouse, 2011; Juyena and Stelletta, 2012).

4. In the female reproductive tract, seminal plasma increases the uterine contractions, modulates the immune response of the female against sperm, protecting them from phagocytosis and destruction, induces the ovulation and relaxes the oviduct isthmus (Garner and Johnson, 1995; Waberski *et al.*, 2006; Juyena and Stelletta, 2012; Yeste, 2013; Yeste and Castillo-Martín, 2013).

5. When sperm reaches the oviduct, they bind the oviduct cells forming the sperm reservoir. This allows sperm to be kept in a quiescent state until ovulation occurs. This sperm association with oviductal cells is possible due to seminal plasma sperm-surface attached proteins that recognize and binds specifically to mannosyl-oligosaccharide chains exposed by the oviductal cells (Töpfer-Petersen *et al.*, 2008; Yeste, 2013).

6. Finally, it plays a role for sperm-ooocyte interactions and during oocyte fertilization, since some studies demonstrated that seminal plasma components such as AWN reaches oocyte during insemination developing a function that is not yet well understood (Centurion *et al.*, 2003; Caballero *et al.*, 2008).

2.6 Reproductive technologies in pigs

TABLE 2 Production of meat and sperm doses in each country. It can be observed the AI use and the number of sows and boars which implies in each location.

Nation	Production in million tons pork meat (2008)	Sows millions	AI %	No. of boars	No. of AI centres	Total doses/year millions
China	47.18	44.000	10			24.000
USA	9.52	5.778	90	27.000		34.000
Germany	4.45	2.225		6.611	22	12.470
Spain	3.46	2.200	95	7.500	74	12.500
Brazil	3.02	2.432	66			12.000
Canada	2.84	1.300	80			5.700
Vietnam	2.47	4.159	30.2	4.562	549	4.558
France	2.23	1.160	95	3.709		5.630
Denmark	2.05	1.082	95-98	3.150	13	5.400
Netherlands	2.02	1.001	98			5.006
Russian Federation	2.01					
Poland	1.92	1.320	91.5	1.341	22	7.247
Philippines	1.61	2.100	35			4.200
		1.500	50			
Italy	1.56	0.600	80			3.200
Japan	1.25	0.670	44			1.769
Mexico	1.15	0.950	70			5.500
		0.670	90			
Republic of Korea	1.06					
Belgium	1.01	0.510	87	1989	37	2.319
Thailand	0.82	0.900	70			4.000
United Kingdom	0.69	0.450	60			2.200
Taiwan		0.600	90			3.000
Chile	0.52	0.300	99			
Australia	0.38	0.200	85			0.850
South Africa	0.30	0.200	40			0.400
Czech Republic	0.28	0.105	95			0.147
Sweden	0.27	0.140	95	500	3	0.800
Argentina	0.23	0.180	85			1.332
Finland	0.22	0.180	80			0.440
Norway	0.12	0.075	98	280	1	0.428
Croatia	0.08	0.132				0.170
Bulgaria	0.07	0.06	80			0.162

Source: (Riesenbeck, 2011)

Pork is the most consumed meat worldwide and many developed countries are global leaders in its production and exportation (See. 2.1.2 Livestock and porcine markets). For this reason, there is mounting interest for development and implementation of several reproductive technologies since their use may make swine production more efficient (Day, 2000; Gerrits *et al.*, 2005). These reproductive technologies have dramatically changed the way that pigs are bred for pork production, and artificial insemination has represented a huge success for this market when its use started to be widespread during the XX century. Indeed, AI in swine has developed exponentially since 1980s, when the commercial application of this technique was increased in production centres (Gerrits *et al.*, 2005; Rodríguez-Gil and Estrada, 2013). Nowadays, in European countries, USA and Canada, AI is used in around 90% of pig farms. In Spain, this figure reaches up to 95% (Riesenbeck, 2011; Rodríguez-Gil and Estrada, 2013). **Table 2** shows the percentage of piglets conceived through AI and intended for meat

production and the number of sperm doses produced in the biggest pork producers worldwide.

Such wide application of AI is due to its developments and improvements that have allowed to obtain similar or better results than those obtained with natural mating (Gadea, 2004). In addition, AI also offers other advantages, such as: high spreading of selected genetic material from a given boar, decreasing the boar-to-sow ratio (Gadea, 2004; Rodríguez-Gil and Estrada, 2013); better sanitary and hygienic measures, avoiding the direct contact between the boar and the sow (Gerrits *et al.*, 2005; Rodríguez-Gil and Estrada, 2013); use of quality genetics material since semen can be extended in long-term- storage media (Levis, 2000; Gerrits *et al.*, 2005); decrease in the time and costs required for natural mating; and high availability of selected genetic lines, which facilitates its use and increases cross-breeding possibilities (Rodríguez-Gil and Estrada, 2013).

In the last years, substantial advances have been made regarding: a) the better place for deposition of insemination doses (Wongtawan *et al.*, 2006; Rodríguez-Gil and Estrada, 2013; Gonzalez-Peña *et al.*, 2014), b) the optimal number of spermatozoa per dose (Martinez *et al.*, 2005; Spencer *et al.*, 2010), and c) the time of insemination relative to ovulation (Soede *et al.*, 1995; Abad *et al.*, 2007). Furthermore, other investigations have focused upon evaluating sperm function and quality in order to better predict reproductive performances (Tardif *et al.*, 1999; Ruiz-Sánchez *et al.*, 2006; Amann and Waberski, 2014). Finally, the relevance of pre-selection of X and Y bearing spermatozoa and sperm cryopreservation should also be highlighted. Sperm sex-sorting has been studied all over the world since represents an important tool for maintaining a high sow-to-boar ratio as requested by pig industry and pork meat market (Rodríguez-Gil and Estrada, 2013). This technology is still in its infancy and is far from solving its handicaps in swine, i.e. time-consuming, expensive, low yield, and low sperm count of sexed doses (Rath *et al.*, 2009; Rodríguez-Gil and Estrada, 2013). With regard to sperm cryopreservation, this reproductive biotechnology is fully discussed in the next section (See 2.7 Boar sperm cryopreservation).

2.7 Boar sperm cryopreservation

2.7.1 The origins, developments and uses of sperm cryopreservation until today

As early as 1776, Spallanzani made the first attempts in sperm cryopreservation when exposed human and stallion sperm to snow and found they were motile at post-thawing (Spallanzani, 1979). Cryopreservation was devoid of a interest for over a

century, but in 1866, a second study reported that human spermatozoa survived after freezing at -17ºC (Mantegazza, 1866).

In 1949, semen cryopreservation made a clue advance since Polge and colleagues in Great Britain reported the successful use of glycerol as a cryoprotective agent (Polge *et al.*, 1949). Such important discovery not only represented a forward step for cryopreservation of mammalian sperm but also for preservation of other somatic cells and tissues (Gobfeld *et al.*, 2008; Casas and Flores, 2013). During the next decade, experiences involving cryopreservation of horse (Watson, 1990a) and bull sperm (Curry, 2000) took place, but was not until 1957 when Polge's laboratory successfully cryopreserved pig sperm (Polge, 1957). At the same date, first piglets were born from the use of frozen-thawed boar sperm (Hess *et al.*, 1957). This experience was subsequently repeated by independent laboratories (Polge *et al.*, 1970; Crabo and Einarsson, 1971; Graham *et al.*, 1971).

Apart from these studies, 1970s were especially relevant for the development of boar sperm cryopreservation technology, since the intensive efforts conducted by different laboratories resulted in the publication of the two land-marking procedures. On the one hand, Pursel and Johnson in EEUU set the American or Beltsville method which is characterized by adding glucose to the freezing extender. Sperm are then frozen in the shape of round pellets on carbonic ice (Pursel and Johnson, 1975). On the other hand, Westendorf and colleagues authored the German or Hülsenberger method which uses lactose as the main component in freezing extenders and packs the sperm in straws before their exposure to vapours of liquid nitrogen (Westendorf *et al.*, 1975). Today, rather than submitting the samples to carbonic ice or liquid nitrogen vapours, controlled-rate freezers are used.

Following the establishment of these two freezing methods, research has been focused upon optimization of freezing and thawing protocols (Hammitt and Martin, 1989; Berger and Fischerleitner, 1992; Holt *et al.*, 2005; Yeste *et al.*, 2014) , sperm preparation and selection (Peña *et al.*, 2006; Matás *et al.*, 2007; Siqueira *et al.*, 2011), formulation and supplementation of freezing and thawing extenders (Gutiérrez-Pérez *et al.*, 2009; Hu *et al.*, 2009; Yeste *et al.*, 2013b), searching of freezability markers (Thurston *et al.*, 2002; Casas *et al.*, 2010a; Vilagran *et al.*, 2013) and optimization of AI techniques (Roca *et al.*, 2003; Casas *et al.*, 2010b; Spencer *et al.*, 2010).

In spite of this, boar sperm cryopreservation has not been integrated in the current porcine AI techniques at a rate similar to that observed in other species, such as bovine and equine (Curry, 2000). In fact, although reproductive performance of frozen-thawed boar sperm is higher than that observed in dairy bovines and horses, its fertility rates are significantly lower than those obtained with refrigerated boar sperm (Johnson, 2011). Thus, as Curry and colleagues stated, a new technique is only successful when, at least, gives results comparable to those of the existing current method, in this case the use of refrigerated semen for pig AI at a similar economic cost (Curry, 2000). Figures reveal that while average farrowing rates for AI using refrigerated boar semen are equal or above of 90%, at an approximate mean cost per

sow pregnancy of less than €20, those of frozen-thawed semen are significantly lower, in the best case being of about 80%, with an approximate mean cost per sow pregnancy of more than €35 (Rodríguez-Gil and Estrada, 2013). The same occurs with regard to litter sizes. In 2000, Johnson *et al.* reported seven alive piglets born per litter following the use of frozen-thawed boar sperm (Johnson *et al.*, 2000). Nowadays, figures are far more optimistic since ten piglets per litter, or even more, may be achieved (Estrada *et al.*, 2014).

However, commercial farms and the industry are used to work with conception rates higher than 85% and litter sizes bigger than 11 piglets with the use of refrigerated boar sperm (Knox, 2011; Men *et al.*, 2012). Nevertheless, scientific community reaches every year new advances in the use of cryopreserved boar sperm for AI. Studies such as that authored by Didion and colleagues (2013) are more than encouraging. These investigators used frozen-thawed sperm for more than 2,600 AI services during a 4-year period in a single breeding farm. Pregnant females were 78.7% and the average of total piglets born per litter was 12.5 (Didion *et al.*, 2013).

Apart from this, more studies and developments have to be made in order for cryopreserved boar sperm to be more utilised in AI breeding programs. However, frozen-thawed boar sperm has an advantage that has already been exploited from a specific niche in swine sector: unlimited durability than fresh semen, which allows both its use for genetic revitalisation/improvement in remote regions and gene banking of the most valuable studs (Riesenbeck, 2011; Benson *et al.*, 2012; Rodríguez-Gil and Estrada, 2013). Frozen sperm can also be used for maintaining swine models for human diseases and health, especially those involving a single locus on a chromosome (Men *et al.*, 2012). In this context, rather than speaking about unlimited durability, we should say extensive durability since Fraser and colleagues (2014) have recently demonstrated that long-term storage in liquid nitrogen affects the quality of boar sperm (Fraser *et al.*, 2014).

Finally, it is worth mentioning that reproductive performance of frozen-thawed boar sperm may have reached a plateau. However, there is an increasing demand for improvement that deserves our attention (Johnson, 2011). Moreover, other new technologies, such as sex-sorted sperm, arise in swine reproduction. Those technologies may require or benefit from the use of frozen-thawed sperm.

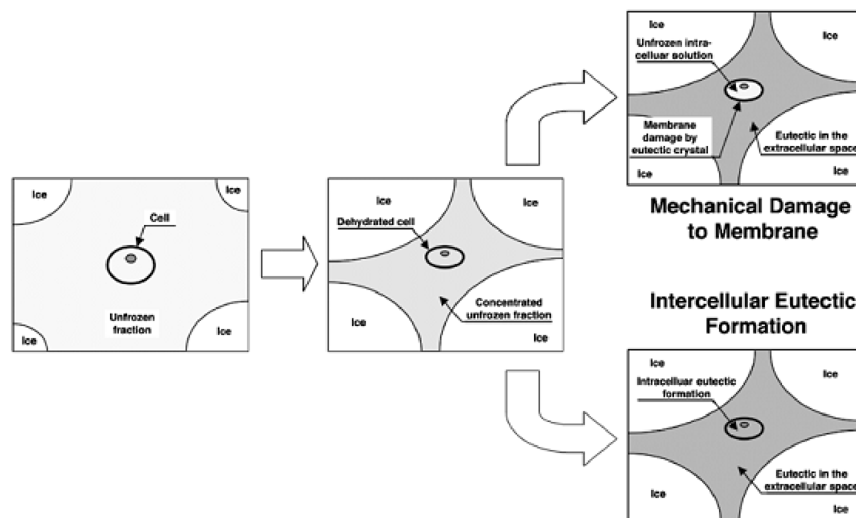
2.7.2 Principles of cryopreservation and sperm cryoinjury

When cells are frozen, they are subjected to stress resulting from water-solute interactions that may lead to ice crystallization (Holt, 2000b; Watson, 2000). Cooling a given solution below its freezing point provokes ice crystal formation in a focus (process called nucleation) from which ice expands like a wave. Under such physical phenomenon, the cell must deal with water efflux from the cytosol to the increasing hyperosmotic medium which has not yet been crystallized, and ice formation inside

the cell which causes organelles and membranes shrinkage (Mazur, 1984; Watson, 2000). Taking all the aforementioned into account and as stated by Mazur *et al.* in *The Two-Factor Hypothesis*, equilibrium should be reached between slow and fast freezing. Indeed, while slow freezing can damage the cell because prolonged exposure to hyperosmotic medium cause dehydration and pH changes and cryopotectants are cytotoxic, fast freezing involves intracellular ice formation and, thus, deleterious effects since water has not enough time to flow out (Mazur *et al.*, 1972; Han and Bischof, 2004)**(Fig. 7)**. It is important to mention that thawing represents the reversal of these effects with the consequent inward of water flux that may cause cell membrane disruption and osmotic stress (Holt, 2000b; Casas and Flores, 2013). For these reasons, many efforts have been directed to the optimization of cooling rates for boar sperm (Bwanga *et al.*, 1991; Woelders, 1997; Kumar *et al.*, 2003; Holt *et al.*, 2005; Juarez *et al.*, 2011). However, in this context, there is another variable that has to be taken into consideration: cryoprotectant concentration. This will be tackled in the following lines.

FIGURE 7

Schematic freezing injury mechanisms associated to osmotic effects and eutectic crystallization.



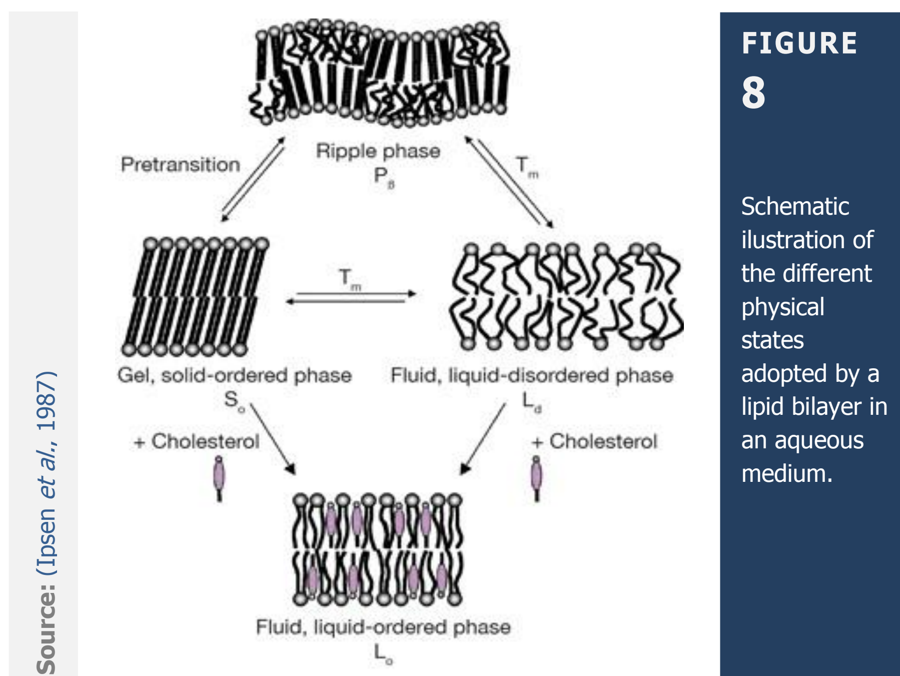
Source: (Han and Bischof, 2004)

Cryoprotectants or cryoprotective agents (CPAs) are included in cryopreservation media to reduce stress derived from freezing and thawing protocols. In the case of boar sperm, egg yolk, a non-penetrating cryoprotectant that does not cross plasma membrane and develop its role extracellularly (Barbas and Mascarenhas, 2009; Benson *et al.*, 2012), is routinely included in cryopreservation extenders. This CPA is able to lower freezing temperature of medium and decrease the extracellular ice formation. Thus, the unfrozen fraction is high and salt concentration more diluted resulting in less osmotic stress for sperm (Woelders, 1997; Barbas and Mascarenhas, 2009; Benson *et al.*, 2012). When combined with surfactant Orvus ES Paste®, egg yolk provides a better protection effect, since this detergent modifies egg yolk particles, thereby facilitating their interaction with sperm plasma membrane (Holt, 2000b; Rodríguez-Martinez and Wallgreen, 2011).

In addition to this, and as aforementioned, glycerol is the main CPA used for boar sperm and no CPA has demonstrated to yield better results thus far (Holt, 2000b; Johnson *et al.*, 2000; Yeste, 2015). Hence, glycerol, as penetrating cryoprotectant (i.e. permeate into the cellular cytoplasm), has been extensively used because of its protective effects. These effects are mainly reliant upon its ability to depress the freezing point of the unfrozen fraction, to affect cytoplasmic viscosity and change diffusion rates, to alter cell membrane properties via its insertion across the lipid bilayer and to become a sperm energy substrate which can interfere in the ATP balance (Holt, 2000a, 2000b; Barbas and Mascarenhas, 2009). However, in pigs high glycerol concentrations have been largely observed to damage the sperm cell causing a loss of fertility (Holt, 2000a; Johnson *et al.*, 2000; Watson, 2000; Barbas and Mascarenhas, 2009; Benson *et al.*, 2012; Casas and Flores, 2013). For this reason, many efforts have been focused on setting the optimal level of glycerol in semen extenders, combining different cooling and freezing rates, extender composition and the type of glycerol added (Holt, 2000a; Barbas and Mascarenhas, 2009). As the bulk of research conducted shows, this realm is yet to be closed (Corcuera *et al.*, 2007; Hernández *et al.*, 2007b; Okazaki *et al.*, 2009a; Arena Nuñez *et al.*, 2013; Yeste, 2015).

Apart from the harmful effects related to the cryopreservation process and extender composition, boar sperm must deal with their high sensibility to cold shock. The name of "cold-shock" includes all the dramatic effects resulting from plasma membrane destabilization and loss of selective permeability at temperatures equal or lower than 5ºC (Watson, 2000; Bailey *et al.*, 2008; Casas and Flores, 2013).

For better understanding this phenomenon, it is worth noting that due to its composition, phase events in lipid bilayers of boar sperm largely explain membrane injuries (Johnson *et al.*, 2000; Watson, 2000). In pigs, phospholipids represent 65-70% of



Source: (Ipsen *et al.*, 1987)

FIGURE 8

Schematic ilustration of the different physical states adopted by a lipid bilayer in an aqueous medium.

plasmalemma and confer fluidity. In addition a variable amount of sterols, such as cholesterol, give rigidity and stability to the membrane, thus complementing the phospholipids role. In this system, lipids may suffer alterations in physical phases (i.e. fluid- and gel-phase lipids) at specific temperatures. Indeed, when temperature is reduced, regions of gel phases are increased compared to those liquid-crystalline. However, the presence of sterols is able to inhibit these phase changes (Holt, 2000a; Alberts *et al.*, 2008), so that when sperm membrane presents low sterol concentrations and is rich in polyunsaturated fatty acids, cryoinjuries induced by cooling are more severe (Holt, 2000b; Leahy and Gadella, 2011; Yeste, 2015) **(Fig. 8)**.

Boar sperm membrane presents a low cholesterol:phospholipid ratio and cholesterol molecules are distributed asymmetrically, so that the outer monolayer contains more cholesterol than the inner. This specific composition of plasma membrane explains the high sensitivity to cold shock of boar sperm (Johnson *et al.*, 2000; Cerolini *et al.*, 2001; Chen and Liu, 2007). Therefore, restriction of lateral movements of membrane phospholipids occur when temperatures are lower than 5ºC. This phenomenon results in a transition from fluid to gel phase. Since different membrane lipids present different transition temperatures, some unsaturated phospholipids being jellified earlier than others, phase separations may occur. Following this phenomenon, integral membrane proteins become irreversibly clustered by lipid phase separations. The result of all these structural alterations is membrane leakiness due to ruptures among lipids bonds and loss of protein function, such as ion channel proteins (Drobnis *et al.*, 1993; Johnson *et al.*, 2000; Watson, 2000; Bailey *et al.*, 2008; Casas and Flores, 2013). At this point, membrane loses its selective permeability, enzyme activity is reduced and there are changes in lateral motion of ion channels (De Leeuw *et al.*, 1990). Together, this triggers an influx of ions from the extracellular space into the cell and this ultimately results in the activation of a metabolic cascade that resembles, but is not identical, to true sperm capacitation (Green and Watson, 2001; Petrunkina *et al.*, 2005; Casas and Flores, 2013; Yeste, 2015). Capacitation-like changes that may occur during cryopreservation include: increases of plasma membrane permeability to ions such as calcium and bicarbonate (White, 1993; Kumaresan *et al.*, 2011), restructuration of membrane lipids and cholesterol release (Harrison and Miller, 2000; Vadnais and Althouse, 2011) and alterations in phosphorylation patterns of sperm proteins (Tardif *et al.*, 2001; Kumaresan *et al.*, 2012). Other cryoinjuries are destabilisation of nucleoprotein structure and DNA damage (Flores *et al.*, 2008; Yeste *et al.*, 2013a), decrease of mitochondria membrane potential (Flores *et al.*, 2009; Casas and Flores, 2013), and increase in intracellular levels and release of reactive oxygen species (ROS) (Peña *et al.*, 2009), although in the case of boar sperm this is yet to be fully addressed (Guthrie and Welch, 2006; Awda *et al.*, 2009).

Because of all aforementioned, numerous advances have been made to counteract the damage that sperm may suffer due to cold-shock during cryopreservation protocol. There are technical procedures such as slow cooling, and use of cryoprotectants, such as egg yolk, that help to maintain membrane integrity (Holt, 2000b; Casas and Flores, 2013). Another component added to the media are

antioxidants, as they prevent the damage of the cellular structure (Kim *et al.*, 2011; Zhang *et al.*, 2012; Yeste *et al.*, 2013b) for an updated revision see (Yeste, 2015).

Finally, sperm survival following cryopreservation protocols not only implies cryoinjuries during freezing, but also along thawing. Indeed, sperm may be subjected to "recristalization" during thawing as a result of low heating rates (Mazur and Cole, 1985). Furthermore, osmotic stresses may appear when thawing rates are too fast since CPAs are unable to leave the cell. This provokes an increase of its osmolarity resulting in high water entrance to the cell (Watson, 1990b). For this reason, optimal thawing rates must be a balance between the two scenarios described above, since this diminishes cell injuries. While a fast warming/thawing protocol is usually followed for boar sperm, thawing rates and composition of thawing medium still remains an open question (Casas *et al.*, 2012; Okazaki and Shimada, 2012; Tomás *et al.*, 2014; Knox *et al.*, 2015).

In conclusion, cryoinjuries occurring during freeze—thawing protocols shorten sperm lifespan and interfere with their response to the signalling events required for fertilization (Watson, 2000; Bailey *et al.*, 2008; Vadnais and Althouse, 2011; Casas and Flores, 2013). For this reason, and even under the most optimized protocols, only half of boar sperm population survive after freeze-thawing (Holt, 2000b; Watson, 2000; Casas and Flores, 2013; Yeste, 2015).

2.8 Variability on sustaining cryopreservation

In addition to all the aforementioned, there is another factor that also explains why frozen-thawed sperm is not often used for AI: the great variability that exists in boar sperm survival following freeze-thawing (i.e. freezability) (Larsson and Einarsson, 1976; Holt *et al.*, 2005; Hernández *et al.*, 2007b; Benson *et al.*, 2012; Casas and Flores, 2013; Yeste, 2015). This variability, that also exists in other mammalian species, has been described between pig breeds (Thurston *et al.*, 2002; Waterhouse *et al.*, 2006), among individuals (Watson, 1995; Thurston *et al.*, 1999; Hernández *et al.*, 2007b) and even among ejaculations from the same boar, and has led to classify sperm samples into good freezability ejaculates (GFE) and poor freezability ejaculates (PFE) (Watson, 1995; Casas *et al.*, 2009; Casas and Flores, 2013; Yeste *et al.*, 2013a).

There are few studies that have tried to elucidate which factors may influence the sperm variability to withstand freeze-thawing (Larsson and Einarsson, 1976; Roca *et al.*, 2006). According to Roca and colleagues, more than 70% of variation in sperm motility and viability among ejaculates at post-thawing is due to the boar effect (Roca *et al.*, 2006). Differences in freezability between individuals seem to have a genetic

origin. This was first demonstrated by Thurston and colleagues who compared bad and good freezer Landrace boars through Amplified Fragment Length Polymorphisms (AFLPs). Their work found a relationship between AFLPs profiles (genetic variation) and ejaculate freezability (Thurston *et al.*, 2002). However, it is not known by which mechanism genetic variation underlies differences in ejaculate freezability. In this context, it is important to notice that previous studies have compared the relative amount of some sperm proteins between GFE and PFE (Casas *et al.*, 2010a) and it is the focus of the present dissertation. Such studies have found that conventional sperm quality parameters evaluated in fresh/extended ejaculations are not able to predict boar sperm cryopreservation capacity. For this reason, markers of boar sperm freezability that distinguish between GFE and PFE may predict ejaculate freezability prior to starting cryopreservation procedures (Casas *et al.*, 2010a). Apart from protein composition, it has been reported that differences in subpopulation patterns of motile spermatozoa, sperm kinetic parameters in the cooling step and acrosin activity may also be used as freezability markers (Yeste, 2015).

The widespread use of frozen-thawed boar sperm would benefit from developing new tests that would allow the detection of variations at a molecular level between different freezability ejaculations. This requires urgent attention, since unnecessary costs of money and time could be avoided and bad freezability ejaculates could be discarded prior to starting cryopreservation practices. The present dissertation seeks to tackle these issues.

2.9 The role of seminal plasma during cryopreservation: an open debate

Ejaculate variability in sperm quality following freeze-thawing has been observed in ejaculated sperm, but is yet to be reported in epididymal spermatozoa (Rath and Niemann, 1997). Thus, one could suggest that seminal plasma could make the difference between GFE and PFE. Supporting this hypothesis, supplementing PFE with seminal plasma from good freezer boars has been reported to improve sperm quality at post-thawing (Hernández *et al.*, 2007a). Composition of seminal plasma differs between individuals from the same species (Strzeżek *et al.*, 2005) and previous reports have shown that proteome differences between GFE and PFE exist (Jobim *et al.*, 2004, 2011; Zahn *et al.*, 2005; Novak *et al.*, 2010). For this reason, establishing which seminal plasma factors correlate with sperm freezability may contribute to counteract the significant drop of sperm quality of PFE at post-thawing (Strzeżek *et al.*, 2005; Caballero *et al.*, 2012).

The effect of seminal plasma on cryopreserved boar sperm has reported conflicting results (Caballero *et al.*, 2012). While some authors have reported beneficial effects on sperm function after adding seminal plasma to cryopreservation/thawing media (Pursel *et al.*, 1973; Rath and Niemann, 1997; Okazaki *et al.*, 2009b; Mogielnicka-Brzozowska *et al.*, 2011; Vadnais and Althouse, 2011; Fernández-Gago *et al.*, 2013), others have rather observed a detrimental effect (Maxwell and Johnson, 1999; Eriksson *et al.*, 2001; Kawano *et al.*, 2004). This divergence could be attributed to differences in the composition of seminal plasma as mentioned before, since these differences have been found not only amongst males and ejaculates from the same males but even between fractions of the same ejaculation (Zhu *et al.*, 2000; Rodríguez-Martinez and Wallgreen, 2011; Siqueira *et al.*, 2011). In this regard, when studying this divergence in the results obtained following the use of seminal plasma during cryopreservation, the focus has been usually been on proteins (Caballero *et al.*, 2012). Among the different proteins studied, spermadhesins have attracted most of the attention. For example, heparin-binding spermadhesins (AQN-1, AQN-3 and AWN) are able to prevent in vitro capacitation as well as cooling-induced capacitation-like changes (Vadnais and Roberts, 2010). According to these results, it has been shown that damaging effects of cold shock could be avoided by low-weight zinc-binding seminal plasma proteins (Mogielnicka-Brzozowska *et al.*, 2011). Moreover, positive results on sperm survival to cryopreservation are reported when PSP-I and PSP-II heterodimer are added to the media, since these aggregated proteins are able to increase sperm viability and motility (Caballero *et al.*, 2004b). Thus, more research about which seminal plasma proteins could be valuable additives for cryopreservation/thawing media is warranted.

3

objectives

objectives
objectives

Based on all exposed in the previous sections, the three aims of the present Thesis matched with one of the current challenges in boar sperm cryopreservation: evaluation of sperm freezability. Addressing these three aims led to the publication of three papers, as referred at the end of each aim:

1. To test the reliability of two proteins involved in cryopreservation injuries to the cell: ODF1/HSPB10 and VDAC2 as freezability markers in extended semen using Western Blot techniques **(PAPER I)**.

2. To identify potential freezability markers in boar spermatozoa by 2D-DIGE through comparing the proteome of refrigerated (17ºC) GFE and PFE, and to validate these results using Western Blotting **(PAPER II)**.

3. To compare the proteome of fresh seminal plasma between GFE and PFE by 2D-DIGE, seeking potential markers for boar sperm freezability, and to conduct a further validation step through Western Blotting **(PAPER III)**.

4

Paper Compendium

Paper Compendium
Paper Compendium

Paper I

Relationship of sperm small heat-shock protein 10 and voltage-dependent anion channel 2 with semen freezability in boars

Ingrid Vilagran, Marc Yeste, Sílvia Sancho, Isabel Casas, Maria M. Rivera del Álamo, Sergi Bonet

Theriogenology **2014;** 82: 418-426

DOI: 10.1016/j.theriogenology.2014.04.023

Theriogenology 82 (2014) 418–426

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Relationship of sperm small heat-shock protein 10 and voltage-dependent anion channel 2 with semen freezability in boars

Ingrid Vilagran [a,*], Marc Yeste [b], Sílvia Sancho [a], Isabel Casas [c], Maria M. Rivera del Álamo [b], Sergi Bonet [a]

[a] Biotechnology of Animal and Human Reproduction (TechnoSperm), Department of Biology, Institute of Food and Agricultural Technology, University of Girona, Girona, Spain
[b] Unit of Animal Reproduction, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Bellaterra (Barcelona), Spain
[c] Andrology Laboratory, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, Pennsylvania, USA

A R T I C L E I N F O

Article history:
Received 4 December 2013
Received in revised form 28 April 2014
Accepted 28 April 2014

Keywords:
Boar sperm
Cryopreservation
Freezability marker
ODF1/HSPB10
VDAC2

A B S T R A C T

Freezability differences between boar ejaculates exist, but there is no useful method to predict the ejaculate freezability before sperm cryopreservation takes place. In this context, the present study sought to determine whether the amounts of small heat-shock protein 10 (also known as outer dense fiber protein 1) (ODF1/HSPB10) and voltage-dependent anion channel 2 (VDAC2) may be used as boar sperm freezability markers. With this aim, 26 boar ejaculates were split into two fractions: one for protein extraction and the other for cryopreservation purposes. Ejaculates were subsequently classified into two groups (good freezability ejaculates [GFE] and poor freezability ejaculates [PFE]) based on viability and sperm motility assessments after 30 and 240 minutes of after thawing. Although the VDAC2 amounts, analyzed through Western blot, were significantly higher ($P < 0.01$) in GFE (1.15 ± 0.18 density mm^2) than in PFE (0.16 ± 0.03 density mm^2), no significant differences were observed in ODF1/HSPB10 between both groups (i.e., 1.97 ± 0.38 density mm^2 in GFE vs. 1.87 ± 1.54 density mm^2 in PFE). In addition, principal component and multiple regression analyses indicated that the component explaining most of the variance (78.41%) in ejaculate freezability at 240 minutes after thawing resulted to be significantly ($P < 0.05$) correlated with VDAC2 content. This result revealed that the amounts of VDAC2 but not those of ODF1/HSPB10 may be used to predict the freezability of a given boar ejaculate before starting cryopreservation procedures.

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1. Introduction

Cryopreservation of boar spermatozoa is not yet integrated in swine artificial insemination programs, and the use of frozen-thawed boar sperm currently represents less than 1% of all inseminations conducted worldwide [1]. Compared with other mammalian species, boar spermatozoa present low freezability because of their high cold shock sensitivity [2,3] that results in low fertility output compared with liquid-stored semen [1,4,5]. In addition, not all boars present the same capacity to withstand the freeze-thawing process but rather a great variability between ejaculates has largely been reported in the literature [6–8]. This consistent variation has been described between different pig breeds [9–11], between ejaculates [6,12], and even between fractions from the

* Corresponding author. Tel.: +34 972 41 95 14; fax: +34 972 41 81 50.
E-mail addresses: ingrid.vilagran@udg.edu, ingrid.vilagran@gmail.com (I. Vilagran).

0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.04.023

same ejaculate [13] and leads to classify boar ejaculates between good freezability ejaculates (GFE) and poor freezability ejaculates (PFE).

In the freezability prediction scenario, the low correlation between the values of conventional sperm quality parameters before cryopreservation and the ejaculate freezability is well documented [7,12]. However, Thurston et al. [14] found genomic markers of ejaculate freezability by evaluating amplified fragment length polymorphisms. Another approach to find boar freezability markers consists of identifying relevant proteins that may predict the cryotolerance of boar ejaculates before starting cryopreservation procedures. In this regard, our group has found that the amounts of heat-shock protein 90 (HSP900AA1), acrosin-binding protein, and triose-phosphate isomerase in extended semen can predict the freezability of boar ejaculates [15,16]. With this approach in mind, the present study aimed to evaluate two new proteins as cryopreservation markers of porcine semen: small heat-shock protein 10 (HSPB10), also known as outer dense fiber protein 1 (ODF1), and voltage-dependent anion channel 2 (VDAC2).

Small heat-shock protein 10 (ODF1/HSPB10) is a member of the small heat-shock proteins (sHSPs) family [17] that could exert strong cryoprotective effects through its role as chaperone. In addition, the ODF1/HSPB10 being the major protein of mammalian sperm tail outer dense fibers, it plays a key role in the maintenance of elastic structures and elastic recoil of the sperm tail [18], thereby protecting sperm against shear forces [19]. This protein has also been related to low human sperm motility [20] and to infertility in humans and mice [21,22].

Voltage-dependent anion channel 2 is a pore-forming protein described in bull [23], buffalo [24], mouse [25], and human [26] spermatozoa. Despite its presence and role in boar spermatozoa still remaining unknown, this protein has been reported to be involved in the regulation of sperm motility and membrane permeability to small ions and molecules in human, mouse, and bovine spermatozoa [23,27–29]. For this reason, we suggest that it could also be related to permeability changes of a boar sperm membrane during cold shock and freeze-thawing procedures.

Therefore, the aim of the present study was to test whether, before starting boar sperm cryopreservation, differences in the amounts of ODF1/HSPB10 or VDAC2 existed between GFE and PFE and whether the levels of these two proteins in extended semen could be used for predicting boar ejaculate freezability through linear regression models.

2. Materials and methods

2.1. Animals and semen collection

Thirty-four adult healthy boars from Piétrain breed were stalled in commercial herds at a local farm (Selecció Batallé S.A., Riudarenes, Girona, Spain), fed according to standard protocols and provided with water *ad libitum*. Ejaculates were obtained following the EU Directive 2010/63/EU for care and use of animal for scientific purposes. One ejaculate per boar was obtained for this study, whereas the animals were submitted to a regular rhythm of semen collection of twice a week. Ejaculate collection was done with the gloved-hand method, filtering the ejaculation through a gauze to remove the gel, and discarding the first part to collect the rich fraction (100 mL approximately). This rich fraction was diluted 1:2 (v:v) using a long-term commercial extender free from bovine serum albumin (Vitasem LD; Magapor S.L., Zaragoza, Spain). Finally, semen samples were packed in bags and transported at 17 °C to our laboratories within 2 hours after collection.

2.2. Assessment of sperm quality

The assessment of sperm quality parameters was carried out three times at different steps of this study: point I, before cryopreservation and after extended ejaculates being held for a holding time of 24 hours at 17 °C; point II, after 30 minutes of after thawing at 37 °C; and point III, after 240 minutes after thawing at 37 °C. The evaluation of sperm quality performed at point I also served to check that all ejaculates fulfilled the minimum requirements to be included in this study. These minimal quality standards were 80% of morphologically normal spermatozoa, 80% of total motile spermatozoa, 80% of viable spermatozoa, and 80% of spermatozoa with non-altered acrosomes in the osmotic resistance test (ORT) [7]. Following this assessment, and according to these standards, 26 ejaculates from the 34 evaluated were included in the study, whereas the other eight were discarded.

On the other hand, according to Casas et al. [7], data from sperm motility, viability, and ORT assessment at points II and III were used to classify the ejaculates into GFE or PFE. These two time points were set because they correspond to the insemination-to-ovulation interval recommended for cryopreserved doses [1,15].

2.2.1. Sperm morphology

Sperm morphology was evaluated after fixation of 0.5 mL from each sperm sample with 0.5 mL of prewarmed PBS solution containing 2% formaldehyde (1:1, v:v) at room temperature and for 5 minutes. Then, 5 μL of each sample was placed on a slide mounted with a cover slip and observed under a phase contrast microscope (Olympus BX41; Olympus, Hamburg, Germany) at a magnification of ×200 (Olympus 20X 0.40 PLAN objective; positive phase-contrast field). Three replicates of 100 spermatozoa each were made per sample, and sperm cells were subjectively classified as mature, immature with proximal or distal cytoplasmic droplets, or aberrant (coiled tails, tails folded at the connecting piece, at the intermediate piece, or at the Jensen ring) [30]. Sperm morphology was analyzed at point I, but not at points II and III, because osmotic alterations that sperm suffer during freeze-thawing account for main morphologic changes and are thus not a proper marker of boar sperm cryotolerance [31,32].

2.2.2. Sperm motility

Sperm motility was determined with a computer-assisted sperm analysis system after incubating samples

at 37 °C for 20 minutes (point I), after 30 minutes (point II), or 240 minutes (point III) after thawing. With this purpose, 20 μL of each sperm sample was placed on a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and observed under a phase contrast microscope (Olympus BX41) at a magnification of ×100 (Olympus 10X 0.30 PLAN objective lens; a negative phase-contrast field). This microscope was connected to a computer equipped with the Sperm Class Analyzer software (SCA5; Microptic S.L., Barcelona, Spain). Three replicates per sample were evaluated before calculating the corresponding mean ± standard error of the mean (SEM). In each analysis, a minimum of 1000 spermatozoa were counted per replicate and the following sperm kinematic parameters were recorded: total sperm motility (TMOT, %), progressive sperm motility (PMOT, %, with straightness [STR] > 45%), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), percentage of straightness (%, STR = VSL/VAP × 100), motility parameter wobble (%, WOB = VAP/VCL × 100), amplitude of lateral head displacement (μm), beat cross frequency (Hz), and percentage of linearity (%, LIN = VSL/VCL × 100) [33].

2.2.3. Sperm viability

Sperm viability was evaluated following the Garner and Johnson [34] protocol. Briefly, sperm samples were stained with SYBR14 (LIVE/DEAD Sperm Viability Kit, SYBR14/PI; Molecular Probes, Eugene, OR, USA) at a final concentration of 100 nM and incubated at 38 °C for 10 minutes. Next, they were incubated at 38 °C for 5 minutes with propidium iodide (PI) at a final concentration of 10 μM. Three replicates of 100 spermatozoa each were assessed per sample under an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss AG, Göttingen, Germany) at a magnification of ×400, before calculating the corresponding mean ± SEM.

2.2.4. Osmotic resistance of spermatozoa (ORT)

Osmotic resistance of spermatozoa (osmotic resistance test [ORT]) was evaluated following Pérez-Llano et al. [35] after staining sperm samples with lectin from *Arachis hypogaea* (peanut agglutinin [PNA]) conjugated with fluorescein isothiocyanate (FITC) and PI, according to the procedure described by Nagy et al. [36]. Briefly, two aliquots of 0.5 mL from each sperm sample were incubated at 37 °C for 15 minutes either with 1.5 mL of isotonic solution containing 3.2% (wt/vol) sodium citrate in distilled water (pH = 7.4; osmotic pressure of 304 ± 8 mOsm/kg) or with 1.5 mL of hypotonic solution containing 1% (wt/vol) sodium citrate in distilled water (pH = 7.4; osmotic pressure of 100 ± 3 mOsm/kg). In both cases, samples were subsequently stained with PNA-FITC (final concentration of 2.5 μg/mL) and PI (final concentration of 10 μM), incubated at 37 °C for 10 minutes, and finally observed under an epifluorescence microscope (Zeiss Axiomager Z1; Zeiss) at a magnification of ×400. Three counts of 100 spermatozoa each were performed per aliquot and sample, and spermatozoa were classified as with an intact ($PNA\text{-}FITC^{-}$) or damaged ($PNA\text{-}FITC^{+}$) outer acrosome membrane. The percentage of spermatozoa with an intact outer acrosome membrane was calculated per sample as the mean between values in isotonic and hyptonic solutions [7].

2.3. Sperm cryopreservation and thawing

Twenty-six ejaculates, which fulfilled the minimal requirements of sperm quality, were held at 17 °C for 24 hours after collection because spermatozoa are known to acquire a better protection against cold shock injuries during this holding time [37,38]. After one aliquot from all ejaculates was taken and processed (see Section 2.4), the remaining ejaculate volume was cryopreserved according to the Westendorf method for porcine semen [39]. Briefly, ejaculates were first centrifuged at 640× *g* for 5 minutes at 17 °C. After discarding the supernatants, soft pellets were diluted at 1.5×10^9 spermatozoa/mL in a freezing medium (LEY) containing lactose (80%, v:v; 310 mM) and egg yolk (20%, v:v). A Makler counting chamber (Sefi Medical Instruments) was used to adjust sperm concentration. Samples were then cooled down for 120 minutes up to 5 °C in a programmable liquid nitrogen freezer (IceCube 14S-B; Minitüb, Tiefenbach, Germany and SY-LAB software, version 1.0; Minitüb; cooling ramp of 0.1 °C/min), and subsequently rediluted to a final concentration of 1×10^9 spermatozoa/mL in a second freezing medium (LEYGO) containing LEY supplemented with 6% glycerol and 1.5% Orvus ES Paste (Equex STM; Nova Chemical Sales Inc., Scituate, MA, USA). Sperm samples were finally packed in 0.5-mL plastic straws (Minitüb) using a semi-automatic filling engine (SFS 133; Minitüb), and a freezing ramp as described by Casas et al. [7] was subsequently applied using the aforementioned programmable freezer (IceCube 14S-B). The cooling rates consisted of −6 °C/min from 5 °C to −5 °C (100 s), −39.82 °C/min from −5 °C to −80 °C (113 s), 0 °C/min at −80 °C (30 s), and finally −60 °C/min from −80 °C to −150 °C (70 s). Straws were finally immersed and stored in liquid nitrogen (−196 °C) for a minimum of 5 days.

Following Casas et al. [40], four straws per ejaculate were thawed. Straws were plunged individually in a water bath at 37 °C for 20 seconds and immediately diluted with three volumes of prewarmed Beltsville thawing solution [41] at 37 °C (final dilution rate of 1:4, v:v). Quality parameters of frozen-thawed sperm were determined after incubating samples at 37 °C for 30 minutes (point II) or 240 minutes (point III).

2.4. Protein extraction, quantification, and processing

Before starting the cryopreservation protocol and after samples being held at 17 °C for 24 hours after collection (see Section 2.3), an aliquot of 500×10^6 spermatozoa (adjusted with a Makler counting chamber) was taken per ejaculate and centrifuged at 640× *g* for 3 minutes at 17 °C. After supernatants were discarded, sperm pellets were washed with 10 mL of PBS and centrifuged again at 640× *g* for 3 minutes at 17 °C to ensure that sperm samples were cleaned from extender medium. Cleaned pellets were then diluted with 8 mL of HAM F-10 1X (Gibco BRL, Life Technologies Ltd., Paisley, UK) and washed through 50% Percoll solution (GE Healthcare, Uppsala, Sweden).

Protein extraction was carried out following Vilagran et al. [16]. Briefly, 100×10^7 sperm cells per sample

(counted using a Mackler chamber) were solubilized in 1 mL of lysis buffer (7 M urea [BioRad, Richmond, CA, USA], 2 M thiourea [Sigma Aldrich, Saint Louis, MO, USA], Tris-HCl 30 mM [BioRad], 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate [BioRad], 2.4 μM phenylmethanesulfonylfluoride [Sigma], and 18 mM dithiothreitol [Sigma]; at pH = 8) for 1 hour at room temperature. Solubilized samples were then centrifuged at 3000× *g* for 5 minutes at 4 °C to precipitate insoluble debris, and supernatants were divided into aliquots which were stored at −20 °C for subsequent analysis.

To precipitate the protein fractions, samples were thawed and incubated in 80% cold acetone at −20 °C for 15 minutes. Protein pellets were obtained through centrifugation at 17,530× *g* for 10 minutes at 4 °C and solubilized in lysis buffer without phenylmethanesulfonylfluoride and dithiothreitol. Quantification of protein samples was carried out using a commercial kit based on the Bradford method (Quick Start Bradford Protein Assay; BioRad) [42]. Three replicates per ejaculate were evaluated, before calculating the corresponding mean ± SEM. These results were confirmed using SDS-PAGE gel analysis (data not shown).

2.5. SDS-PAGE and Western blotting

Fifteen micrograms of protein in the case of Western analysis of ODF1/HSPB10 and 10 μg in the case of VDAC2 were resuspended in 10 μL of Laemmli reducer buffer 1× (65 mM Tris [Serva, Heidelberg, Germany], 10.5% glycerol [Panreac, Castellar del Vallès, Spain], 2.15% SDS [Serva], 2.5% beta-mercaptoethanol [BioRad], and traces of bromophenol blue [Panreac]) and stored at −20 °C until the beginning of the assay. Thawed samples were boiled at 90 °C for 5 minutes and cooled to 4 °C, and proteins were separated into 1-mm SDS electrophoresis gels containing 15% acrylamide (BioRad) in those for separating ODF1/HSPB10 and 12% acrylamide in gels for VDAC2 evaluation. Stacking gels contained 4% acrylamide. Electrophoretic protein separation was performed according to standard procedures [43], and proteins from the gel were transferred to polyvinyl fluoride membranes (Immobilion-P; Millipore, Darmstadt, Germany) at 120 mA for 120 minutes. Membranes were then washed with agitation at room temperature for 5 minutes with TBS 1X-Tween-20 (10 mM Tris [Panreac], 150 mM NaCl [labKem, Mataró, Spa], and 0.05% Tween-20 [(Panreac]; pH = 7.3) and incubated overnight with agitation at 4 °C with a blocking solution of TBS 1X-Tween20 and 5% bovine serum albumin (Roche Diagnostics, S.L., Basel, Switzerland). Then, membranes with primary antibodies were incubated for 1 hour with agitation at room temperature. These primary antibodies were anti-ODF1 polyclonal rabbit antibody (ref. ARP53765-P050; Aviva Systems Biology, San Diego, CA, USA) and anti-VDAC2 goat polyclonal antibody (ref. AP16225PU-N; Acris Antibodies, San Diego, CA, USA), diluted 1:5000 (v:v) and 1:1000 (v:v), respectively, in blocking solution. Next, membranes were washed with agitation thrice with TBS 1X-Tween20 at room temperature for 5 minutes before incubating for 1 hour with secondary antibodies, goat anti-rabbit polyclonal horseradish peroxidise (HRP)-conjugated antibody (for ODF1/HSPB10, ref. P0161; Dako, Glostrup, Denmark) and rabbit anti-goat polyclonal HRP–conjugated antibody (for VDAC2, ref. AP106P; Millipore), diluted 1:20000 (v:v) and 1:5000 (v:v), respectively. Chemiluminescent reaction was developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and membranes were scanned with G:BOX Chemi XL 1.4 (SynGene, Frederick, MT, USA). The protein patterns were quantified using Quantity One Version 4.6.2 software package (BioRad). Protein quantification was expressed as adjusted volume, which was defined as the sum of pixel intensities inside the volume boundary × area of a single pixel minus the background volume (in density per square millimeter). These adjusted protein band volumes were normalized using alpha-tubulin as an internal standard. For this reason, membranes were subsequently stripped and each membrane was incubated at 37 °C with glycine buffer (0.2 M glycine; Serva and 0.05 mM Tween20; Panreac; pH adjusted at 2.2). Stripped membranes were subsequently incubated with anti-alpha-tubulin mouse monoclonal antibody (ref. MABT205; Millipore), diluted 1:1500 (v:v) at room temperature with agitation for 1 hour, and then with a secondary antibody (rabbit anti-mouse HRP–conjugated polyclonal antibody, ref. P0260; Dako) diluted 1:2000 (v:v) again for 1 hour at room temperature with agitation.

Relative amounts of ODF1/HSPB10 and VDAC2 in each ejaculate before starting cryopreservation protocols were determined per triplicate in all those ejaculates that fulfilled the minimum sperm quality standards (N = 26; see Section 2.2).

2.6. Statistical analyses

Data were managed using Microsoft Excel (Microsoft Office 2003 Professional Edition; Microsoft Corp., Redmond, WA, USA) and statistical package SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA). In all the statistical analyses, the significance level was set as $P < 0.05$.

2.6.1. First approach: general linear models for evaluating differences of ODF1/HSPB10 and VDAC2 between GFE and PFE

Results obtained for sperm quality parameters and protein band quantification are expressed as the mean ± SEM. Data in percentages (x) were previously transformed to arcsine square root ($\arcsin\sqrt{x}$) to accomplish parametric assumptions checked using Kolmogorov-Smirnov (normality) and Levene (homogeneity of variances) tests.

Twenty-six ejaculates included in this study were classified into GFE or PFE groups after a hierarchical cluster analysis for dissimilarities including a complete linkage (furthest neighbor) clustering algorithm application. Data used came from PMOT and sperm viability assessed at points II and III, following the procedure described by Casas et al. [7]. The dissimilarity dendrogram obtained was compared with one constructed using 12 parameters obtained in the assessment of sperm quality at points II and III, so as to test the reliability of the classification on the basis of PMOT and viability.

Table 1
Percentages of TMOT, PMOT, viable spermatozoa (SYBR-14$^+$/PI$^-$), and acrosome-intact spermatozoa in ORT (PNA-FITC$^-$) during three different points (I–III) of the freeze-thawing protocol.

Step	TMOT (%)		PMOT (%)		Viability, SYBR-14$^+$/PI$^-$ (%)		ORT, PNA-FITC$^-$ (%)	
	GFE	PFE	GFE	PFE	GFE	PFE	GFE	PFE
Fresh extended semen (point I)	96.6 ± 1.1^{a,1}	92.1 ± 2.0^{a,1}	61.8 ± 3.0^{a,1}	57.1 ± 2.9^{a,1}	93.8 ± 1.0^{a,1}	91.8 ± 0.9^{a,1}	89.5 ± 3.3^{a,1}	88.1 ± 3.5^{a,1}
30 min after thawed semen (point II)	82.2 ± 2.9^{b,2}	24.8 ± 5.5^{b,2}	42.9 ± 2.8^{a,2}	6.3 ± 1.9^{b,2}	61.5 ± 3.2^{a,2}	30.4 ± 3.1^{b,2}	75.8 ± 3.1^{a,2}	68.5 ± 2.7^{b,2}
240 min after thawed semen (point III)	65.0 ± 4.5^{a,3}	13.1 ± 1.7^{b,3}	27.7 ± 3.4^{a,3}	2.4 ± 0.6^{b,3}	45.0 ± 2.3^{a,3}	19.4 ± 3.2^{b,3}	62.0 ± 2.4^{a,3}	54.6 ± 2.0^{b,3}

Different letters (a and b) indicate significant differences ($P < 0.05$) between ejaculate groups (columns; GFE vs. PFE) within a given cryopreservation step (I, II, and III) and sperm parameter, whereas different numbers (1–3) mean significant differences ($P < 0.05$) between cryopreservation steps (rows; points I, II, and III) within a given ejaculate group and sperm parameter. All data are the mean ± SEM from 26 replicates.
Abbreviations: FITC, fluorescein isothiocyanate; GFE, good freezability ejaculates; ORT, osmotic resistance test; PFE, poor freezability ejaculates; PI, propidium iodide; PMOT, progressive sperm motility; PNA, peanut agglutinin; SEM, standard error of the mean; TMOT, total sperm motility.

After classifying ejaculates into GFE and PFE groups, the normalized band volumes of ODF1/HSPB10 and VDAC2, previously tested for normality and homogeneity of variances, were compared using a *t* test for independent samples. In addition, all sperm parameters were compared with a repeated measures analysis of variance, in which the dependent variable was the sperm parameter, the intersubject factor was the freezability ejaculate group (i.e., GFE vs. PFE), and the intrasubject factor was the cryopreservation step (i.e., points I, II, and III).

2.6.2. Second approach: principal component analyses and linear regression models for prediction of the ejaculate freezability

In the second approach for evaluating the reliability of ODF1/HSPB10 and VDAC2 as freezability markers, two factorial analyses were run using the values obtained for all sperm quality parameters evaluated at points II and III. In each factorial analysis, sperm quality parameters were sorted into some components extracted by principal component analysis (PCA), and the obtained data matrix was rotated using the Varimax procedure with Kaiser normalization. Only those variables with a square factor loading (a_{ij}^2) higher than 0.3 with its respective component and lower than 0.1 with respect to the other components in the rotated matrix were selected from the linear combination of j variables (z) in each component y_i. Regression factors for each component after PCA were used for multiple regression analyses.

Correlations of ODF1/HSPB10 or VDAC2 normalized band volumes with all sperm parameters and regression factors after 30 and 240 minutes after thawing were calculated using the Pearson correlation. In addition, multiple regression analyses were carried out to determine the ability of ODF1/HSPB10 or VDAC2 normalized band volumes to predict the sperm quality after 30 and 240 minutes after thawing. The procedure used (the forward stepwise model) was the same described by Yeste et al. [44] and consisted of optimizing the regression equation to increase the determination coefficient (r^2). The dependent variables (y's) in all cases were the regression factors PCA at points II and III, and the independent variables were the normalized band volumes of ODF1/HSPB10 or VDAC2. The significance level for introducing each parameter in the multiple regression model was 10% and the significance level (α) for the model was 5%.

3. Results

3.1. Classification of boar ejaculates between GFE and PFE groups and their differences before and after freeze-thawing

Twenty-six ejaculates, each coming from a different boar, were classified into two clusters with maximal dissimilarities for PMOT (%) and sperm viability (SYBR14$^+$/PI$^-$, %) at points II and II. This classification resulted in 10 GFE and 16 PFE (Supplementary data, Fig. 1), and agreed with that obtained using the 12 sperm quality parameters as described in Section 2 (data not shown).

Referring to differences between GFE and PFE, Table 1 summarizes main sperm quality parameters assessed at three different points before and after freeze-thawing (i.e., points I, II, and III). The TMOT (%), PMOT (%), viability (SYBR-14$^+$/PI$^-$, %), and spermatozoa with an intact outer acrosome membrane in ORT (PNA-FITC$^-$, %) were similar in GFE and PFE at point I. However, values from these parameters differed between these two groups (GFE vs. PFE) at points II and III, the sperm viability, motility, and osmotic resistance being significantly higher ($P < 0.05$) in GFE than in PFE. In addition, TMOT, PMOT, viability, and osmotic resistance decreased significantly ($P < 0.05$) at points II and III when compared with point I. Other motility parameters analyzed by computer-assisted sperm analysis (see Section 2.2.2) do not present significant differences between GFE and PFE at the three analyzed points.

3.2. Differences between GFE and PFE in ODF1/HSPB10 and VDAC2 amounts before freeze-thawing

The most representative protein band patterns of ODF1/HSPB10 and VDAC2 obtained before freeze-thawing, as well as alpha-tubulin are shown in Figure 2 of Supplementary data. Normalized protein amounts, represented in Figure 1, were similar in both GFE and PFE groups in the case of ODF1/HSPB10. On the contrary, VDAC2 amounts were significantly higher ($P < 0.01$) in GFE than in PFE.

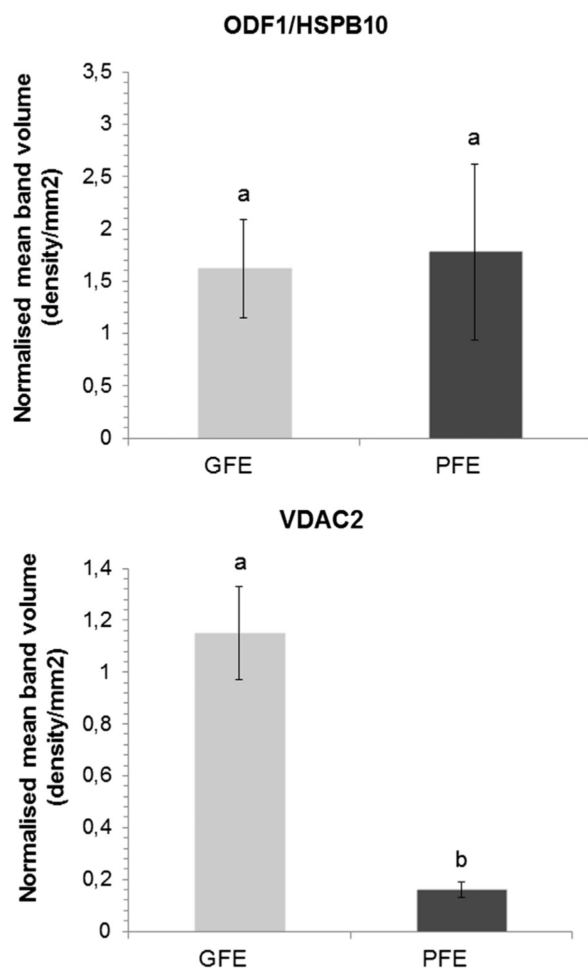

Fig. 1. Normalized band volume (expressed in density/mm^2) of ODF1/HSPB10 and VDAC2 in GFE and PFE at point I using 10 replicates from GFE group and 16 from PFE group. Bars represent the mean ± SEM of the band volume ratio of ODF1/HSPB10 or VDAC2 to alpha-tubulin. Different letters (a and b) indicate significant differences ($P < 0.05$) between ejaculate groups (GFE vs. PFE). Abbreviations: GFE, good freezability ejaculates; PFE, poor freezability ejaculates; SEM, standard error of the mean; VDAC2, voltage-dependent anion channel 2.

3.3. Principal component analyses of sperm quality parameters and multiple regression analyses between VDAC2 volume and quality parameters

Tables 2 and 3 present the results of PCAs from all sperm quality parameters obtained at points II and III, respectively. Regarding sperm quality parameters assessed at point II, a total of two components, explaining 91.72% of variance, were extracted. The first component included most of the parameters describing sperm function and integrity, and explained 74.23% of variance (Table 2).

In PCA performed with sperm quality parameters evaluated at point III (Table 3), there were also two extracted components and the total explained variance was 89.97%. Again, the first component, which explained 78.41% of variance, included most of the parameters describing sperm function and integrity after 240 minutes of thawing, similarly to that observed for PCA after 30 minutes after thawing.

Table 2
Principal component analysis of sperm freezability with all quality parameters assessed after 30 min after thawing (point II).

Component	Variance	Combinations of variables	a_{ij}	a_{ij}^2
1	74.23%	Viability (SYBR-14$^+$/PI$^-$, %)	0.94	0.89
		VSL (μm/s)	0.89	0.78
		VAP (μm/s)	0.87	0.76
		VCL (μm/s)	0.82	0.67
		WOB (%)	0.82	0.67
		PMOT (%)	0.81	0.66
		BCF (Hz)	0.81	0.66
		TMOT (%)	0.81	0.65
		LIN (%)	0.78	0.61
		ORT (PNA-FITC$^-$, %)	0.77	0.60
		STR (%)	0.72	0.52
2	17.49%	ALH (μm)	0.91	0.83
Total	91.72%			

[a]Variance explained by each component and total variance represented as percentage; a_{ij} values correspond to factorial coefficients from each variable and its two square values (a_{ij}^2).
Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; FITC, fluorescein isothiocyanate; LIN, linearity; ORT, osmotic resistance test; PI, propidium iodide; PMOT, progressive sperm motility; PNA, peanut agglutinin; STR, straightness; TMOT, total sperm motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, motility parameter wobble.

Pearson correlation coefficients between extracted components from PCA described previously and normalized band volumes of VDAC2 and ODF1/HSPB10 assessed at point I revealed that only VDAC2 amount was significantly ($P < 0.05$) and positively correlated with the first component of PCA at 240 minutes after thawing. In this case, a regression equation using VDAC2 amount as an independent variable (x) and the first component of PCA at 240 minutes after thawing as a dependent variable (y) was worked out, with P values for regression coefficient and the model less than 0.05 (Table 4). In contrast, no significant correlation between any PCA component at 30 minutes after thawing and VDAC2 amounts was found (Table 4). Finally, there was no correlation between ODF1/HSPB10 amounts and PCA components at 30 or 240 minutes after thawing (data not shown).

4. Discussion

In this study, we have found for the first time that VDAC2 is present in boar sperm and it may be used as a freezability marker in boar ejaculates because their amounts are significantly ($P < 0.05$) and positively correlated with sperm quality after 240 minutes after thawing. In contrast, and from the data obtained in the present study, ODF1/HSPB10 cannot be used as a freezability marker for boar ejaculates. These results are of paramount importance, especially if we take into account that, so far, sperm conventional parameters evaluated before freeze-thawing have been proven to be nonuseful for predicting boar sperm freezability [7,12,15]. This has been confirmed in our study as GFE and PFE did not differ in terms of sperm quality at point I.

The sHSP ODF1/HSPB10 is a member of the sHSP family[17]. Because other HSPs have protective functions and one member of this family, HSP90AA1, has been revealed as a molecular marker of boar sperm freezability

Table 3
Principal component analysis of sperm freezability with all quality parameters assessed after 240 minutes of after thawing (point III).

Component	Variance	Combinations of variables	a_{ij}	a_{ij}^2
1	78.41%	Viability (SYBR-14$^+$/PI$^-$, %)	0.92	0.85
		VAP (μm/s)	0.89	0.79
		LIN (%)	0.85	0.72
		VSL (μm/s)	0.84	0.71
		STR (%)	0.82	0.67
		TMOT (%)	0.82	0.66
		PMOT (%)	0.80	0.65
		ORT (PNA-FITC$^-$, %)	0.79	0.62
		VCL (μm/s)	0.78	0.61
		WOB (%)	0.77	0.59
2	14.57%	ALH (μm)	−0.85	0.72
		BCF (Hz)	0.80	0.64
Total	89.97%			

[a]Variance explained by each component and total variance are represented as percentage. a_{ij} values correspond to factorial coefficients from each variable and its two square values (a_{ij}^2).
Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; FITC, fluorescein isothiocyanate; LIN, linearity; ORT, osmotic resistance test; PI, propidium iodide; PMOT, progressive sperm motility; PNA, peanut agglutinin; STR, straightness; TMOT, total sperm motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, motility parameter wobble.

[15], we hypothesized that ODF1/HSPB10 could be involved in some way in the preservation of sperm function and survival during freeze-thawing. However, this presumed role has not been reported in the present study, because no differences in the amounts of this protein were seen between GFE and PFE, and no regression equation was worked out when trying to predict the sperm quality at post-thawing from ODF1/HSPB10 amounts in extended semen. Apart from structural and mechanical roles of this protein in sperm tail [19,45], ODF1/HSPB10 has been described as a sperm motility marker in human spermatozoa [20] and defects in its expression have been related to an impaired sperm development [22] and to male infertility [21,46]. However, in our study, GFE and PFE did not differ in the amounts of this protein either before or after freeze-thawing, so that ODF1/HSPB10 cannot be considered as a marker of sperm motility, at least in porcine species. Unfortunately, and as far as the use of this protein as a freezability marker is concerned, there are no similar studies in other species and we cannot thus make other comparisons. In short, and to the best of our knowledge, no previous study has evaluated the reliability of ODF1/HSPB10 as a freezability marker in mammalian spermatozoa. From our results using the porcine as a model, we can conclude that this protein is not involved in the sperm resistance of boar to freeze-thawing.

Regarding VDAC2, it was first identified in an outer mitochondrial membrane of *Paramecium aurelia* [47]. This pore-forming porine has also been found in other cell compartments, such as sarcoplasmic reticulum or plasma membrane. VDAC2 is present in mature spermatozoa from bulls [23], buffalos [24], humans [26], and mice [25]. In the present work, we have reported for the first time that VDAC2 is also present in mature spermatozoa of pigs. However, further studies shall address the specific location of VDAC2 in acrosome, midpiece, or tail of boar spermatozoa.

In mammalian spermatozoa, VDAC2 has been suggested to play relevant roles during spermatogenesis, sperm maturation, motility, and fertilization [48]. From our results, we can suggest that this protein is also involved in the cryotolerance of boar semen because (1) VDCA2 amounts in extended semen differ between GFE and PFE and (2) a regression equation using VDCA2 amounts as an independent variable and a PCA component as a dependent variable can predict boar sperm quality after 240 minutes after thawing (Table 4). It is worth noting that there is no correlation between VDAC2 amounts in extended semen and sperm quality at point II, and this may be explained because, according to other studies [7,16], differences between GFE and PFE quality parameters are higher at 240 minutes than at 30 minutes after thawing.

To explain the involvement of VDAC2 in boar sperm cryopreservation, we have to keep in mind that this protein has been reported to form a channel structure in the lipid bilayer that mediates transport of ions and small molecules (Na^+, Ca^{2+}, Cl^-, HCO^{3-}, ATP, glutamate, etc.) through the plasma membrane in mammalian spermatozoa [28]. After this, coincubating bull spermatozoa with anti-VDAC2 antibody results in a time- and dose-dependent reduction of ion transport [49]. These evidences may allow us to explain why VDAC2 is a marker of boar sperm freezability, because there is an important ion flux in the great osmotic-changes that

Table 4
Regression equations between principal component 1 from PCA resulting from all sperm quality parameters assessed at points II and III after thawing (y) and VDAC2 amounts determined before cryopreservation (i.e., at point I).

Step	Regression equation	R^2	R	P value model
30 min after thawing (point II)	$y = 1.36$ (VDAC2) $- 0.64$	0.34	0.58	>0.05
240 min after thawing (point III)	$y = 1.57$ (VDAC2) $- 0.85$	0.59	0.77	<0.05

Abbreviations: PCA, principal component analysis; VDAC2, voltage-dependent anion channel 2.

take place during the freeze-thawing process [50]. Related to this, VDAC2 might play an important role during cold shock events that take place in cryopreservation protocols. As such, cold shock occurs when ejaculated boar spermatozoa are cooled quickly to temperatures less than 15 °C [1], as this induces lipid and protein membrane rearrangements that compromise the selective permeability of a membrane lipid bilayer [51]. These changes in membrane architecture provoke a fast entrance of ions and water resulting in capacitation-like changes that may finally lead to cell death. Such Ca^{2+}-dependent events have been suggested as one of the reasons of the low fertility output of boar sperm cryopreserved doses [52]. Bearing these evidences in mind, in our study, VDAC2 has been significantly ($P < 0.05$) and positively correlated with boar sperm cryotolerance, as GFE presented significant higher levels of VDAC2 than PFE. Taking together, these data prompt us to hypothesize that higher amounts of VDAC2 in GFE protect sperm from changes in membrane fluidity through a better regulation of ion transmembrane flux [28] during freeze-thawing, thereby partially avoiding the negative effects that cold shock inflicts on PFE samples.

Apart from this, VDAC2 has been related with capacitation of mouse [25,53], human [26], and buffalo spermatozoa [24]. In addition, studies conducted with human spermatozoa have found that VDAC2-mRNA levels are a marker of idiopathic asthenozoospermia, because infertile patients have significantly lower ($P < 0.05$) content of VDCA2-mRNA than normozoospermics [48,54]. In bull spermatozoa, there are also evidences that this protein plays an important role in regulating sperm motility [23]. In this context, it is important to note that, in the present study, boar PFE presented lower percentages of progressive motile spermatozoa than GFE after freeze-thawing but not before. Taking into account the aforementioned studies in other species, thus one could also suggest that higher VDAC2 protein amounts in GFE could contribute to a better regulation of sperm motility, which could in turn maintain this sperm parameter better after freeze-thawing. Previous studies [23,28] have suggested the mechanisms underlying this VDAC2-mediated effect on sperm motility could be related to the ATP transport that should take place through VDAC2 pore. Finally, VDAC2 has also been related to the sperm ability to bind the zona pellucida in humans [29], thereby correlating this protein with fertilization. In agreement to this, a lower content of VDAC2 in boar PFE could also be related to the lower fertilizing ability of these ejaculates when compared with GFE [15,55,56], as an insufficient VDAC2 content could hamper sperm to bind the zona pellucida properly.

In conclusion, in the present study, we have found the ability of boar spermatozoa to withstand cryopreservation protocols can be predicted by analyzing the VDAC2 content in extended ejaculates, whereas ODF1/HSPB10 amounts cannot be used with this purpose. Because VDAC2 is involved in sperm function and survival and has been described as a marker of boar sperm freezability, further research is warranted to shed light on the molecular mechanisms by which this protein is related to boar sperm cryotolerance.

Acknowledgments

This research was supported by project RZ 2011-00001-00-00 of the Spanish Ministry of Science and Innovation to Sergi Bonet and by a Predoctoral fellowship granted to Ingrid Vilagran from University of Girona (BR-2012).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2014.04.023.

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Paper II

Acrosin-binding protein (ACRBP) and triosephosphate isomerase (TPI) are good markers to predict boar sperm freezing capacity

Ingrid Vilagran, Judit Castillo, Sergi Bonet, Sílvia Sancho, Marc Yeste, Josep M. Estanyol, Rafael Oliva

Theriogenology **2013;** 80: 443-450

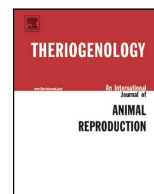
DOI: 10.1016/j.theriogenology.2013.05.006

Theriogenology 80 (2013) 443–450

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com



Acrosin-binding protein (ACRBP) and triosephosphate isomerase (TPI) are good markers to predict boar sperm freezing capacity

Ingrid Vilagran [a], Judit Castillo [b,c], Sergi Bonet [a], Sílvia Sancho [a], Marc Yeste [d], Josep M. Estanyol [e], Rafael Oliva [b,c,*]

[a] Biotechnology of Animal and Human Reproduction (TechnoSperm), Department of Biology, Institute of Food and Agricultural Technology, University of Girona, Girona, Spain
[b] Human Genetics Research Group, IDIBAPS, Faculty of Medicine, University of Barcelona, Barcelona, Spain
[c] Biochemistry and Molecular Genetics Service, Clinic Hospital, Barcelona, Spain
[d] Unit of Animal Reproduction, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Barcelona, Spain
[e] Scientific and Technological Centers (CCiTUB), University of Barcelona, Barcelona, Spain

ARTICLE INFO

Article history:
Received 14 February 2013
Received in revised form 26 April 2013
Accepted 4 May 2013

Keywords:
Cryopreservation
Boar sperm
GFE
PFE
ACRBP
TPI

ABSTRACT

Sperm cryopreservation is the most efficient method for storing boar sperm samples for a long time. However, one of the inconveniences of this method is the large variation between and within boars in the cryopreservation success of their sperm. The aim of the present work was thus to find reliable and useful predictive biomarkers of the good and poor capacity to withstand the freeze-thawing process in boar ejaculates. To find these biomarkers, the amount of proteins present in the total proteome in sperm cells were compared between good freezability ejaculates (GFE) and poor freezability ejaculates (PFE) using the two-dimensional difference gel electrophoresis technique. Samples were classified as GFE and PFE using progressive motility and viability of the sperm at 30 and 240 minutes after thawing, and the proteomes from each group, before starting cryopreservation protocols, were compared. Because two proteins, acrosin binding protein (ACRBP) and triosephosphate isomerase (TPI), presented the highest significant differences between GFE and PFE groups in two-dimensional difference gel electrophoresis assessment, Western blot analyses for ACRBP and TPI were also performed for validation. ACRBP normalized content was significantly lower in PFE than in GFE ($P < 0.05$), whereas the TPI amounts were significantly lower in GFE ($P < 0.05$) than in PFE. The association of ACRBP and TPI with postthaw sperm viability and motility was confirmed using Pearson's linear correlation. In conclusion, ACRBP and TPI can be used as markers of boar sperm freezability before starting the cryopreservation procedure, thereby avoiding unnecessary costs involved in this practice.

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1. Introduction

Sperm cryopreservation is currently the most efficient method for storing boar sperm samples for a long period of time. Despite the improvements in freeze-thawing technology so far, one of the critical limitations of this technique concerns the sensitivity of boar spermatozoa to cold shock because of the composition of their plasma membrane [1,2].

However, not all the ejaculates present the same sensitivity to cold shock, but a great variation between and within boars exists in the cryopreservation process [3–6]. This allows the classification of boars and their ejaculates as good (GFE) or poor freezability (PFE) boars and/or ejaculates [6,7]. Related to this, it is of great interest to predict the freezability (i.e., the ability to withstand freeze-thawing) of a given ejaculate before carrying out its

* Corresponding author. Tel.: +34 93 4021877; fax: +34 93 4035278.
E-mail address: roliva@ub.edu.edu (R. Oliva).

0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2013.05.006

cryopreservation. For this reason, previous reports sought to find freezability markers that could predict the ejaculate freezability before the cryopreservation procedures. It has been found that routine analysis of semen quality (conventional spermiogram) performed before freeze-thawing is not a valid method to predict sperm survival variability during the cooling and freeze-thawing processes, and cannot thus be used as a freezability marker [5,8,9]. Conversely, other studies have found that the structure of sperm motility subpopulations in boar semen samples is related to ejaculate freezability [10]. In addition, the sperm motility indexes of linearity and straightness (STR) at the cooling step (5 °C) have also been suggested as freezability predictors in boar sperm [6]. Other freezability markers have been related to genetic differences between boars [11], and amplified fragment length polymorphisms have been associated with variation of post-thaw sperm quality [12].

Another approach has been the assessment of relative amounts of some heat shock proteins, such as HSP90AA1 [6,13], before starting the cryopreservation protocol (i.e., in refrigerated semen at 17 °C). These findings thus suggest that good predictive markers of boar sperm freezability can be found using proteomic assessment of refrigerated semen, and additional research on this topic is warranted.

In the andrology scenario, proteomic techniques have been extensively applied and they have made a relevant forward step in identifying male infertility markers in human [14,15]. In such experiments, two-dimensional (2-D) separation of proteins has been applied followed by protein identification using mass spectrometry. Two-dimensional approaches, however, have some limitations, such as low sensitivity that can be related to a low throughput power, and the complexity of matching the same spots in all of the gel replicates [16]. The 2-D difference gel electrophoresis (2D-DIGE) is an alternative method that can overcome gel to gel variation and improve the sensitivity of protein spot detection, making it a good choice to surmount these limitations [17].

Against this background, the present study had two aims. First, to identify potential markers of boar sperm freezability by applying the 2D-DIGE technique, thereby comparing the protein composition between GFE and PFE (after a proper identification of both groups using postthaw quality) in refrigerated semen (17 °C). Second, to validate the reliability of those freezability markers with the highest difference between GFE and PFE, using Western blot assessments.

2. Materials and methods

2.1. Animals and samples

This study was carried out from May 2011 to January 2012 (From May 2011 to July 2011 the cryopreservation and thawing experiments were carried out, and from September 2011 to January 2012, the 2D-DIGE and Western blot analyses were performed). Ejaculates were obtained following the EU Directive 2010/63/EU for animal experiments. Thirty-four mature healthy Piétrain boars, which were stalled in commercial herds (Selecció Batallé S.A., Girona, Spain), were submitted to regular rhythm of semen collection (twice a week) while they were fed according to standard protocols and provided with water *ad libitum*. One single ejaculate per male was used in this study. The sperm-rich fractions, obtained by discarding the first part of the ejaculate and re-collecting approximately 100 mL from each ejaculate, were diluted at the rate 1:2 (v:v) using a long-term commercial extender free from bovine serum albumin (Vitasem LD; Magapor SL, Zaragoza, Spain) with the gloved-hand method and filtered through gauze to remove the gel. Semen samples were finally packed in bags and transported at 17 °C to our laboratories.

2.2. Sperm quality assessment

The assessment of sperm quality was carried out during the study in three different stages: in refrigerated semen before cryopreservation at 17 °C (Stage 1), in frozen-thawed spermatozoa at 30 minutes after thawing (Stage 2), and in frozen-thawed spermatozoa at 240 minutes after thawing (Stage 3). In Stage 1, the ejaculates were evaluated and only those that satisfied the quality standard (80% of sperm without morphoanomalies, 80% of total motile sperm, 80% of viable spermatozoa, and 80% of nondamaged acrosome spermatozoa in the Osmotic Resistance Test [ORT] [6,18]) which corresponded to 26 of the 34 included in the study, were cryopreserved according to the protocol described later in text. In Stage 2 and 3, sperm motility and viability, and the ORT of the spermatozoa were evaluated to classify the ejaculates as GFE or PFE.

To assess sperm morphology in Stage 1, spermatozoa were fixed with prewarmed 2% formaldehyde in PBS for 5 minutes at room temperature (RT), and 5 μL of each semen sample was then placed on a slide and mounted with a coverslip. Sperm morphology was evaluated subjectively by making three counts of 100 spermatozoa each, differentiating between mature, immature with cytoplasmic droplets, and aberrant spermatozoa (coiled tails, tails folded at the connecting piece, at the intermediate piece, or at the Jensen's ring) [19]. A phase contrast microscope (Olympus BX41, Hamburg, Germany) was used, and the samples were observed at magnification ×200 (Olympus X 20 0.40 PLAN objective, positive phase-contrast field).

To evaluate sperm motility, the samples were first incubated at 37 °C for 20 minutes and then assessed in triplicate using a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) at magnification ×100 in a phase contrast microscope (Olympus BX41) connected to a computer equipped with the Sperm Class Analyzer software (SCA 2002, motility module, Microptic S.L., Barcelona, Spain). A minimum of 1000 spermatozoa were counted for each replicate. In each assessment, the following motility parameters were obtained: overall motility (%), progressive motility (% with STR > 45%, classified as fast and low), circular trips, curvilinear velocity (μm/s), straight line velocity (μm/s), average path velocity (μm/s), percentages of linearity (%), STR (%), and oscillation (%), lateral head displacement (μm), and beat cross frequency (Hz).

The viability assessment was performed using a double DNA-specific fluorochrome probes kit (LIVE/DEAD Sperm viability kit, Molecular Probes, Eugene, OR, USA), applying

the protocol described by Garner and Johnson [20] and adapted by Casas et al. [6]. Samples were observed using an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss) at magnification ×400, and three counts of 100 each were made and the corresponding mean ± standard error of the mean (SEM) was calculated.

Finally, the osmotic resistance of sperm was assessed as described by Casas and colleagues [6], using a modified ORT [18,21]. Samples were observed using a phase contrast microscope (Olympus BX41) at magnification ×200. In all of the samples, three counts of 100 each were performed and the corresponding mean ± SEM was subsequently calculated.

2.3. Sperm cryopreservation and thawing

Ejaculates were stored for 24 hours at 17 °C, so that sperm could acquire a better resistance to cold shock [22,23], and a cryopreservation protocol was applied in each ejaculate using the Westendorf method for porcine [24], modified by Carvajal et al. [25], and adapted in our laboratory [6]. Briefly, all of the ejaculates were centrifuged at 17 °C and 600 × g for 5 minutes. Pellets were recovered with 3 to 4 mL of supernatant and diluted at 1.5×10^9 spermatozoa per mL (using a Makler counting chamber) in a freezing medium containing lactose and egg yolk. Spermatozoa were then cooled down to 5 °C for 120 minutes in a programmable freezer (Icecube14S-B; Minitüb Ibérica SL, Tarragona, Spain) with a cooling ramp of 0.1 °C/min. Afterward, sperm was diluted at 1×10^9 spermatozoa/mL in a second medium (LEYGO) containing lactose and egg yolk with 6% glycerol and 1.5% Orvus ES Paste (OEP, Equex STM; Nova Chemical Sales Inc., Scituate, MA, USA). Final concentrations of glycerol and OEP in LEYGO medium were 2% and 0.5%, respectively. Spermatozoa were finally packed in 0.5-mL plastic straws (Minitüb Ibérica SL) and transferred to a programmable freezer (Icecube 14S-B; Minitüb Ibérica SL). The freezing program (SY-LAB software, version 1.0; Minitüb Ibérica SL) consisted of 313 seconds of cooling at the following rates: −6 °C/min from 5 °C to −5 °C (100 seconds), −39.82 °C/min from −5 °C to −80 °C (113 seconds), maintained for 30 seconds at −80 °C, and finally cooled at −60 °C/min from −80 °C to −150 °C (70 seconds). The straws containing the ejaculates were placed into liquid nitrogen (−196 °C) for storage.

When the samples were stored for a minimum of 5 days, four straws from each ejaculate were thawed, as recommended by Casas et al. [26]. The straws were immersed in a water bath at 37 °C for 20 seconds and the samples were mixed with three volumes of warmed Beltsville Thawing Solution [27], at a final dilution of 1:3 (v:v). Sperm quality assessment was carried out at Stage 2 and 3.

2.4. Sample preparation and protein extraction and quantification

From each ejaculate stored at 17 °C, an aliquot corresponding to 500×10^6 spermatozoa was taken before the cryopreservation protocol began. Spermatozoa were pelleted at 640 × g for 3 minutes at 17 °C, washed with 10 mL PBS, and centrifuged at 640 × g for 3 minutes at 17 °C, to wash the diluting media. Finally, the pellets were resuspended with 8 mL HAM F-10 1X (Gibco BRL, Life Technologies Ltd., Paisley, UK). Each sample was cleaned of contaminating cells using 50% Percoll solution (GE Healthcare, Uppsala, Sweden), and the sperm proteins were extracted using lysis buffer as performed in Martínez-Heredia et al. [28], adapted to boar sperm. The solubilized proteins were divided in aliquots and stored at −20 °C. To precipitate the protein fraction, samples were incubated in 80% cold acetone for 15 minutes at −20 °C, and centrifuged at 17,530 × g for 10 minutes at 4 °C. The Bradford method [29] was applied to determine the protein concentration of each sample in triplicate; results were confirmed using one-dimensional SDS-PAGE gel analysis (data not shown).

2.5. Two-dimensional fluorescence difference gel electrophoresis

CyDye DIGE Fluor minimal dye labeling protocol (GE Healthcare Life Sciences, Piscataway, NJ, USA) was applied as described in the manufacturer instructions. Briefly, 50 μg from samples belonging to extreme groups of freezability (10 GFE and 10 PFE, to achieve a stronger differentiation between the two groups) were randomized labeled with 400 pmol of Cy3 or Cy5. An internal standard, consisting of a mix of each individual experimental sample was labeled with Cy2. Samples were left on ice for 30 minutes in the dark and 1 μL of 10 mM lysine was then added to stop the labeling reaction. Finally, labeled samples were stored at −80 °C for subsequent analysis.

Fifty micrograms from each group of labeled samples, GFE, PFE, and internal standards, were randomly mixed with sample buffer containing 3.5 M urea (BioRad, Hercules, CA, USA), 1 M thiourea (Sigma Aldrich, Saint Louis, MO, USA), 1% CHAPS (BioRad) detergent, 1% immobilized pH gradient (IPG) buffer (Bio-Lyte 3-10 Buffer; BioRad) and 65 mM dithiothreitol (Sigma Aldrich). Six microliters of DeStreack was added in each mix and samples were loaded in 17-cm ReadyStrip IPG Strips linear pH 3 to 10 (BioRad) placed in the rehydration tray from the Protean IEF Cell (BioRad). After rehydration for 12 hours, samples in the IPG Strips were focused at 20 °C for 20 minutes at 250 V in a slow ramp, another slow ramp until 10,000 V for 150 minutes, a rapid ramp of 40,000 V/h at 10,000 V, and 10 hours of slow ramp to reach 500 V. The IPG strips were stored at −20 °C until the second dimension was performed.

IPG strips were equilibrated and SDS-PAGE was performed as described previously [28] and adapted to boar sperm. Briefly, strips were placed on the top of 12% acrylamide:bisacrylamide gels, previously polymerized in a Protean II xi multi-gel casting chamber (BioRad) using glasses with Silane solution (80% ethanol [v:v], 0.2% glacial acetic acid [v:v], and 0.01% of BindSilane [v:v] [PlusOne Bind-Silane, γ-methacryloxypropyltrimethoxysilane; GE Healthcare Life Sciences). The second dimension was performed on all the gels simultaneously using PROTEAN Plus DODECA Cell (BioRad) at 300 V for 6 hours.

Gel images were obtained using a Typhoon TRIO scanner (Amersham Biosciences Corp, Piscataway, NJ, USA) at a resolution of 100 μm and 550 V of exposure.

2.6. Protein spot analysis, excision, in-gel enzymatic digestion, and mass spectrometry identification

The most differentially expressed spots (N = 20), identified using the DeCyder 2D Differential Analysis Software 6.2 version (GE Healthcare Life Sciences) were excised robotically from three gels using the Ettan Spot Picker (GE Healthcare Life Sciences) and placed to a welled microplate to be digested with trypsin. For each digested spot, a total volume of 10 μL of tryptic peptide mixture was injected with a flow rate of 300 nL/min in a NanoLC (NanoEasy-PROXEON; Thermo Fisher Scientific, Walthman, MA, USA). A precolumn (EasyColumn-L2cm-I100μm-5μm-120A-C18 PROXEON; Thermo Fisher Scientific) and an analytical column (EasyColumn-L10cm-I75μm-3μm-120A-c18 PROXEON, Thermo Fisher Scientific) were used.

Mass spectrometry analysis was performed using an LTQ-Orbitrap Velos (Thermo Fisher Scientific) and MS/MS (tandem mass spectrometry) data acquisition was performed using Xcalibur 2.1 version software (Thermo Fisher Scientific) and submitted to the PIG UniProt-SwissProt release 2011-2 database. For the database search, a 0.8-Da of fragment mass tolerance was used. The criteria used to accept identifications included two peptides per protein and a false discovery rate of 1%.

2.7. Western blot analysis of acrosin-binding protein and triosephosphate isomerase

Seven randomly selected GFE and seven randomly selected PFE samples were used for validation using Western blot analysis. With this purpose, 20 μg of protein pellets from each sperm sample were resuspended with 5 μL of sample buffer with 0.5% of bromophenol blue [30] and stored at −20 °C until the beginning of the assay. Before electrophoresis, the samples were incubated for 5 minutes at 90 °C, cooled to 4 °C, and loaded into 0.75-mm SDS gels containing 12% acrylamide (wt/vol), with 4% acrylamide staking gel. Electrophoresis was performed according to standard procedures [31]. Proteins from the gels were transferred to nitrocellulose membranes for 60 minutes at 100 mA [32]. Membranes were rinsed for 5 minutes at RT and agitation with washing solution composed of TRIS-buffered saline containing 0.1% (v:v) Tween-20. Afterward, they were incubated with blocking solution (TRIS-buffered saline with 5% [wt/vol] powdered milk and 0.1% [v:v] Tween-20) overnight in agitation at 4 °C. Membranes were then incubated for 1 hour at RT with agitation with primary antibodies diluted in blocking solution at 1:1000 (v:v). The primary antibodies used were rabbit polyclonal anti-acrosin-binding protein (ACRBP) (R35877; Sigma Aldrich), rabbit polyclonal anti- triosephosphate isomerase (TPI)1 (QC22199; Sigma Aldrich) and mouse monoclonal anti-alpha-tubulin (05-829; Millipore Merck KGaA, Darmstadt, Germany). After cleaning membranes five times in washing solution, they were incubated with secondary antibody, either horseradish peroxidase conjugated polyclonal anti-rabbit donkey immunoglobulin (NA934; GE Healthcare) in the case of ACRBP and TPI assessment, or horseradish peroxidase conjugated polyclonal rabbit anti-mouse immunoglobulin (P0260; Dako, Glostrup, Denmark) in that of alpha tubulin. In both assessments, secondary antibodies were diluted 1:2000 (v:v) in blocking solution. ECL Plus Western Blotting Detection Reagent (GE Healthcare) was applied in each membrane following the manufacturer's instructions, and they were scanned with an LAS3000 imaging system (Fujifilm, Tokyo, Japan). Protein bands from scanned images were quantified using Quantity One software (version 4.6.2; BioRad). Values were expressed as the total signal intensity inside the boundary of a band measured in pixel intensity units (density $\times$ mm^2) avoiding background signal, considering the lowest intensity of a pixel as 0 (white). Alpha-tubulin was used as a protein control to normalize volume of protein expression.

2.8. Statistics

Data were analyzed using the statistical package SPSS for Windows (19.0 version; SPSS Inc., Chicago, IL, USA). Values from quality parameters are expressed as mean $\pm$ SEM and data in percentage (x) were previously transformed to arcsine square-root ($\sqrt{x/100}$) to match the parametric assumptions, previously checked using the Kolmogorov-Smirnov (normality) and Levene (variances homogeneity) tests.

First, 34 ejaculates were classified into GFE or PFE using a hierarchical cluster analysis including a complete linkage (furthest neighbor) clustering algorithm with data from sperm progressive motility and sperm viability in Stages 2 and 3, following the procedure described by Casas et al. [6]. The reliability of such classification was previously tested comparing the clusters obtained with those obtained on all sperm quality parameters in Stages 2 and 3 [6].

Data from protein spots were analyzed using the Differential In-Gel Analysis module of the software DeCyder 6.0 version and their volume was normalized dividing each spot volume for the corresponding volume from Cy2 spot images. Spot intensity comparison between the two groups (GFE and PFE) was performed using the Biological Variation Analysis module. Briefly, protein spots considered significantly differentially expressed were determined if the average spot intensity was greater than 1.3-fold and P value using Student *t* test was also less than 0.05.

In the case of the data coming from Western blot assessment, normalized band volumes (volume of each band/corresponding volume of alpha-tubulin) in GFE and PFE (seven replicates each) were compared using a *t* test for independent samples. In addition, ejaculate freezability (i.e., GFE or PFE) was correlated with normalized band volumes (volume of each band/corresponding volume of alpha-tubulin) from Western blot analyses in both freezability markers (i.e., ACRBP or TPI), using Pearson's correlation.

In all the statistical analyses, the significance level was set at $P < 0.05$.

3. Results

3.1. Classification of frozen-thawed ejaculates

Twenty-six ejaculates, from 30, satisfied the minimal values of sperm quality before cryopreservation with no significant differences between the two groups of samples

(sperm without morphoanomalies [%]: GFE, 90.86 ± 3.47; PFE, 92.10 ± 3.68; total motile sperm [%]: GFE, 96.56 ± 3.43; PFE, 92.14 ± 6.26; viable spermatozoa [%]: GFE, 93.84 ± 3.23; PFE, 91.83 ± 2.73; and nondamaged acrosome in the ORT [%]: GFE, 94.13 ± 4.54; PFE, 93.20 ± 4.78). After freeze-thawing, the previous ejaculates that fulfilled the minimal values for being included in our study were classified into two clusters. These clusters were obtained by running a hierarchical cluster analysis for dissimilarities that used the values of sperm progressive motility and sperm viability at Stages 2 and 3. The reliability of such classification was confirmed with the clusters obtained when all the sperm quality parameters were used. In both cases, the results were the same and the ejaculates were classified into the same groups.

From the 26 ejaculates used in this study, 13 were classified as GFE and the other 13 belonged to the PFE group. As expected, mean values of sperm viability and sperm progressive motility, studied in both stages after thawing (Stage 2 and 3), differed between GFE and PFE, so that the former presented a higher percentage of viable and progressive motility of spermatozoa than the latter (Fig. 1).

3.2. Proteomic analysis

3.2.1. Two-dimensional fluorescence difference gel electrophoresis and protein identification

Two-dimensional difference gel electrophoresis was carried out with sperm proteins extracted from samples in Stage 1 that showed a high number of protein spots detected along the pH and molecular weight range of the gel (Fig. 2). A total of 1577 protein spots were resolved and detected in gel images. Of these 1577 protein spots, 28 presented a significantly ($P < 0.05$) different normalized spot volume between GFE and PFE (Fig. 2). The 20 spots most significantly different between the two groups were analyzed using LC-MS/MS (liquid chromatography followed by tandem mass spectrometry). Protein identification reported that each spot identified corresponded to more than one single protein. Moreover, on some occasions, the same protein was identified in more than one spot.

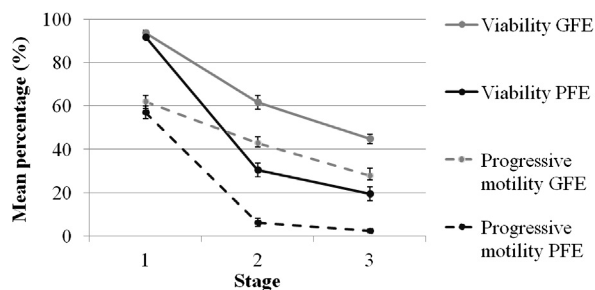

Fig. 1. Comparison of sperm viability and sperm progressive motility between GFE and PFE. Data on mean ± SEM viability and progressive motility percentages of boar sperm in three stages of the experimental design: Stage 1 (refrigerated semen, 24 hours at 17 °C after collection), Stage 2 (30 minutes after thawing), and Stage 3 (240 minutes after thawing), comparing the two clusters corresponding to: GFE (N = 13) and PFE (N = 13). GFE, good freezability ejaculates; PFE, poor freezability ejaculates.

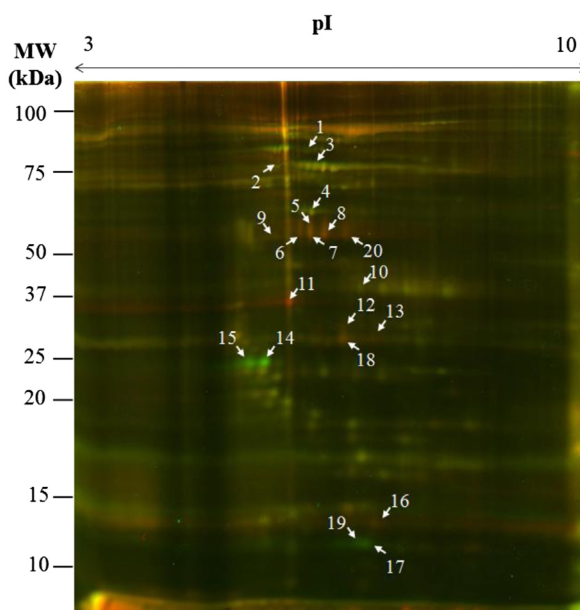

Fig. 2. Representative protein pattern of boar sperm proteins in two-dimensional difference gel electrophoresis. Statistically significant differences between GFE and PFE were observed in 28 protein spots (marked with an arrow and numerated). Protein spots with the most significant different expression comparing the two groups, numerated from one to 20, were then analyzed using mass spectrometry. GFE, good freezability ejaculates; MW, molecular weight; PFE, poor freezability ejaculates; pI, isoelectric points.

3.2.2. Validation using Western blot analysis

From all the proteins identified, two of them, ACRBP and TPI, presented the highest differences between GFE and PFE, and were selected to be studied using Western blot analysis. These proteins were selected for further validation because they were identified in most of the protein spots (spots 2, 4–6, 7, 9–13, and 16–20 in the case of ACRBP, and 10–13 in the case of TPI; Fig. 2) and also because they presented the largest number of peptides matched in the spots in which they were identified.

Western blot analysis results for the two selected proteins were normalized using the protein alpha-tubulin as an internal standard (Fig. 3). Significant differences were observed between GFE and PFE in the normalized mean band volumes for ACRBP and TPI, thus confirming the 2D-DIGE results derived from the initial stage. Acrosin-binding protein content was significantly lower in PFE than in GFE samples in Stage 1 ($P < 0.05$), whereas the content of TPI was significantly lower in GFE ($P < 0.05$) than in PFE samples (Fig. 3).

The strength of the association between the freezability capacity of a given ejaculate and the protein normalized volume of these two proteins was also studied. A significant positive correlation ($r = 0.82$; $P < 0.05$) was detected between ejaculate freezability (described by sperm viability and progressive motility, as previously mentioned) and normalized ACRBP band volume. In contrast, the correlation between freezability and normalized band volume of TPI was also significant ($P < 0.05$) but negative ($r = 0.59$).

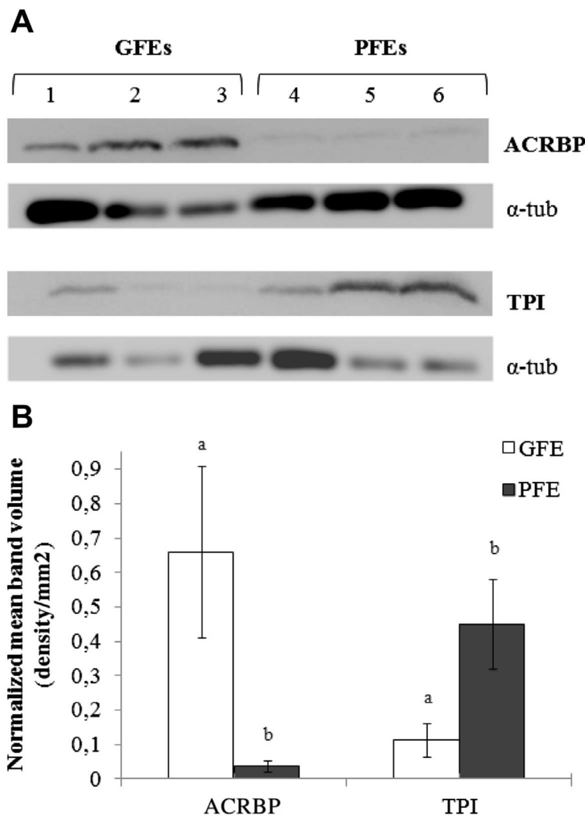

Fig. 3. Comparison of ACRBP and TPI expression pattern between GFE and PFE in Stage 1. On the left (A), Western blot images showing representative protein patterns in triplicate for GFE and PFE of ACRBP and TPI. Alpha-tubulin was used as an internal standard to normalize the intensity of the studied protein bands, expressed before normalization as pixel intensity units/mm^2. On the right (B), the graphic shows normalized band volumes for ACRBP and TPI in boar sperm. Each column indicates the mean normalized value of protein expression from seven replicates, the bar being the standard error. Protein patterns with different letters (a and b) represent mean band volumes significantly different between GFE and PFE groups ($P < 0.05$). α-tub, alpha-tubulin; ACRBP, acrosin-binding protein; GFE, good freezability ejaculate; PFE, poor freezability ejaculate; TPI, triosephosphate isomerase.

4. Discussion

The main aim of this study was to find new freezability markers of boar semen before performing the freeze-thawing process (i.e., in refrigerated semen). After establishing GFE and PFE groups according to viability of sperm after thawing, and motility of frozen-thawed ejaculates, we first carried out a comparative protein profiling of GFE and PFE proteome in refrigerated spermatozoa, using the 2D-DIGE technique. In this approach we identified 20 different spots in both ejaculate groups, and some of them corresponded to more than one single protein. In addition, in some cases, a given protein was identified in more than one spot. These results suggest that the proteins identified in a large number of spots corresponding with a wide range of isoelectric points and molecular weights, are very abundant proteins subjected to different post-translational modifications. Accordingly, differential protein processing in yeast total proteome extracts showed that proteins expressed in high quantity compete for localization in the gel with lower-abundance proteins [33]. This way, the overall number of features visible using MS on a 2-D gel was increased greatly, in the case of high-abundance proteins, producing more than one spot per protein and comigrating spots, suggesting that the 2D-DIGE technique might not suitable for the global and complete detection of proteins expressed by the cell. However, in the current study, this approach has led us to distinguish the major proteome differences between GFE and PFE. For this reason, we suggest that peptides presenting significantly different amounts in GFE and PFE might have an important role in sperm cell function and survival after freeze-thawing. In addition, the role of 2-D electrophoresis in the identification of proteins that might act as markers in mammalian sperm has been largely reported and used in the past decade [34–38], and significant differences in the amounts of 17 separate proteins have been detected between asthenozoospermic and normozoospermic human ejaculates [15]. Therefore, this technique also makes it easier to determine, in the case of boar sperm freezability, the important peptides that differ between GFE and PFE samples.

After this initial 2D-DIGE approach, the proteins which, before freeze-thawing, were identified as presenting the highest differences between GFE and PFE groups and were thus suggested as potential freezability markers, were further validated using unidimensional electrophoresis and Western blot analysis. These two proteins were ACRBP, which was confirmed to be present in a higher amount in GFE than in PFE samples, and TPI, which was present in a higher amount in PFE than in GFE samples. In our study, the reliability of these two freezability markers was also verified using Pearson correlation analyses using protein amounts from Western blot assessments and ejaculate freezability as variables, thereby showing that ACRBP and TPI amounts were, respectively, positively and negatively correlated with ejaculate freezability.

Acrosin binding protein, also known as SP32 and P32, is a calcium-dependent phosphoprotein related to boar sperm capacitation and specifically located in the acrosome [39,40]. It has been described, apart from in pig spermatozoa [41], in human [42], guinea pig [43], and mouse spermatozoa [38]. In the first report in which the function of this proacrosin binding protein was hypothesized, Baba et al. [44] suggested a role as a facilitator of proacrosin conversion to its mature form. Later, it was added that this protein might be the physiologically relevant substrate for the acrosin release from sperm [43]. Related to this, there is much evidence indicating that ACRBP is phosphorylated during boar sperm capacitation [39,40,45,46], conceding to the ACRBP a crucial role in the capacitation pathway and acrosin release from the acrosome. Moreover, it is important to bear in mind that ACRBP has the capacity to bind the proacrosin zymogen, thereby delaying its maturation [43]. Taken together, our findings suggest the higher amounts of ACRBP confer higher acrosome resistance to freeze-thawing procedures through a better regulation of the acrosome reaction. Indeed, we hypothesize that, considering that ACRBP is involved in acrosin pathway maturation, GFEs present a higher ability of retaining acrosin maturation, thus

avoiding GFEs to undergo a premature capacitation and denegerative acrosome exocytosis. This agrees with higher susceptibility of PFEs to cryocapacitation/capacitation-like changes when submitted to cryopreservation protocols [6]. Therefore, because PFEs present a lower content of ACRBP and low regulatory capacity of the acrosome reaction, they show a diminished life span when submitted to freezing-thawing protocols, because of premature acrosome reaction that is disastrous for sperm's fertilizing ability.

With regard to TPI, it is an enzyme from the glycolytic pathway present in higher amounts in PFE than in GFE. This enzyme leads the conversion of dyhydroxyacethone phosphate to glyceraldheyde phosphate and is a clue in sperm metabolism. Bone et al. [47] have demonstrated that the inhibition of this enzyme using ornidazole in rat spermatozoa blocks their capacitation through the inhibition of their hyperactivation. In our case, higher amounts of TPI in PFE could have predisposed the sperm to an early capacitation after freeze-thawing that would finally result in cell death and also compromise the fertilizing ability. Furthermore, Siva et al. [48], in human sperm, have shown that asthenozoospermic samples present higher levels of TPI than normospermic samples. This fact, in agreement with our findings, suggest that ejaculates with low sperm motility parameters (like PFE samples after freeze-thawing and asthenozoospermic samples) present a high content of TPI, conferring to this protein a great potential as a marker of low semen quality.

Until now, several efforts have been made to identify reliable markers of boar sperm freezing capacity. Promising results were suggested when boar sperm motility subpopulations were studied before freeze-thawing [10], and when specific motility parameters were investigated at the cooling step (5 °C) [6]. Furthermore, testing boar sperm proteomes to find molecular freezability markers in individual ejaculates has been reported to be a good choice because HSP90AA1 amounts have been demonstrated to be different between GFE and PFE samples [13]. Because of this previous work, the present study was designed following the same approach, such as predicting the freezability of a given boar ejaculate is suitable when assessing the content of specific protein markers. It is important to note that the present investigation represent an important forward step in development of new tests that allow detecting variations at the molecular level in boar ejaculates with different freezability. This procedure could be used to predict the freezability of a given ejaculate before the freeze-thawing protocol takes place, thereby avoiding unnecessary costs involved in cryopreservation practices.

4.1. Conclusions

In this investigation, we have demonstrated that the capacity of a boar sperm ejaculate to withstand cryopreservation protocols can be predicted by analyzing their ACRBP and TPI contents. This represents an advantage for boar sperm cryopreservation, because GFE and PFE can be selected before starting freeze-thawing protocols and thus avoid unnecessary costs for the industry.

Acknowledgments

This research was supported by project RZ 2011-00001-00-00 of the Spanish Ministry of Science and Innovation to S.B., and by a Predoctoral fellowship granted to I.V. from the University of Girona, the project BFU 2009-07118, fondos FEDER, to R.O., and a Predoctoral fellowship to J.C. from the University of Barcelona.

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Paper III

Comparative analysis of boar seminal plasma proteome from different freezability ejaculates and identification of Fibronectin 1 as sperm freezability marker

Ingrid Vilagran, Marc Yeste, Sílvia Sancho, Judit Castillo, Rafael Oliva, Sergi Bonet

Andrology, **2015**; 3: 345-356
DOI: 10.1111/andr.12009

ANDROLOGY

ISSN: 2047-2919

ORIGINAL ARTICLE

Correspondence:
Ingrid Vilagran, Biotechnology of Animal and Human Reproduction (TechnoSperm), Department of Biology, Institute of Food and Agricultural Technology, Scientific and Technological Park, University of Girona, Jaume Casademont building, E-door, C/ Pic de Peguera 15, E-17003 Girona, Spain.
E-mail: ingrid.vilagran@udg.edu

Keywords:
two-dimensional difference gel electrophoresis, boar sperm, cryopreservation, fibronectin-1, glutathione peroxidase 5

Received: 21-Jul-2014
Revised: 14-Dec-2014
Accepted: 17-Dec-2014

doi: 10.1111/andr.12009

Comparative analysis of boar seminal plasma proteome from different freezability ejaculates and identification of Fibronectin 1 as sperm freezability marker

[1]I. Vilagran, [2]M. Yeste, [1]S. Sancho, [3]J. Castillo, [3]R. Oliva and [1]S. Bonet

[1]Department of Biology, Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, Girona, Spain, [2]Nuffield Department of Obstetrics & Gynaecology, University of Oxford, Level 3, Women's Centre, John Radcliffe Hospital, Oxford, Headington, UK, and [3]Faculty of Medicine, Human Genetics Research Group, IDIBAPS, University of Barcelona, Barcelona, and Biochemistry and Molecular Genetics Service, Clinic Hospital, Barcelona, Spain

SUMMARY

Variation in boar sperm freezability (i.e. capacity to withstand cryopreservation) between ejaculates is a limitation largely reported in the literature. Prediction of sperm freezability and classification of boar ejaculates into good (GFEs) and poor freezability ejaculates (PFEs) before cryopreservation takes place may increase the use of frozen-thawed spermatozoa. While markers of boar sperm freezability have been found from sperm cell extracts, little attention has been paid to seminal plasma. On this basis, the present study compared the fresh seminal plasma proteome of 9 GFEs and 9 PFEs through two-dimensional difference gel electrophoresis (2D-DIGE) and liquid chromatography mass spectrometry (LC-MS/MS). The ejaculates were previously classified as GFE or PFE upon their sperm viability and progressive motility assessments at 30 and 240 min post thawing. From a total of 51 spots, four were found to significantly ($p < 0.05$) differ between GFEs and PFEs, and two were identified as fibronectin-1 (FN1) and glutathione peroxidase 5 (GPX5). These two potential markers were further studied by western blot and correlation analysis between protein relative abundances in fresh seminal plasma and regression factors from principal component analyses (PCA) run using post-thawing sperm quality parameters. Results confirmed that FN1 is a reliable marker of boar sperm freezability, because GFEs presented significantly ($p < 0.05$) higher FN1-amounts than PFEs and FN1 was found to be correlated with the first PCA component at 240 min post thawing. In contrast, GPX5 was not validated as a boar sperm freezability marker. We can thus conclude that levels of FN1 in fresh seminal plasma from boar semen may be used as a sperm freezability marker, thereby facilitating the use of frozen-thawed boar spermatozoa.

INTRODUCTION

Cryopreserved boar spermatozoa have many advantages such as genetic material preservation, control of the transmission of pathogens and better breeding programmes, amongst others (reviewed by Bailey *et al.*, 2008). However, their limitations in artificial insemination (AI) outcomes require further investigation to improve boar sperm cryopreservation protocols (Johnson *et al.*, 2000). In addition, boar spermatozoa present a high variability in their ability to withstand cryopreservation (i.e. freezability), not only among boars (Holt *et al.*, 2005) but also between ejaculates from a given boar (Roca *et al.*, 2006). This has led to classify the ejaculates into good (GFEs) and poor freezability ejaculates (PFEs) (Casas *et al.*, 2009) and it is one of the reasons that explains why frozen-thawed spermatozoa does not have a widespread use in swine industry. The cause of this individual variation remains an open question and has been attributed to genomic (Thurston *et al.*, 2002) and proteomic variations (Casas *et al.*, 2010; Vilagran *et al.*, 2013, 2014). However, while most of these previous studies have been focused on the sperm cell, little attention has been paid to the relationship between seminal plasma and boar sperm freezability.

Seminal plasma, the fluid that together with mammalian spermatozoa forms the semen, is a complex mixture of secretions originated from testes, epididymis and male accessory sexual

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glands (Sancho & Vilagran, 2013). There are growing evidences that seminal plasma plays critical roles on sperm fertilizing ability (Maxwell & Johnson, 1999; Novak *et al.*, 2010) and exerts important effects on female reproductive physiology (Rozeboom *et al.*, 2000; Okazaki *et al.*, 2012; Castillo-Martín & Yeste, 2013). So far, great efforts have been made to unveil the biological effects of seminal plasma factors (mainly proteins) on sperm function and cryotolerance. Results from studies that examined the effect of adding seminal plasma before sperm cryopreservation or after thawing are variable and conflicting (Caballero *et al.*, 2012; Okazaki & Shimada, 2012). These divergences have been attributed to differences in the protein composition of seminal plasma (Caballero *et al.*, 2004; Maxwell *et al.*, 2007). Differences in seminal plasma composition have also been related to individual differences in boar sperm freezability (Roca *et al.*, 2006). Indeed, supplementing freezing extenders with seminal plasma from boars with good sperm freezability has been reported to improve the sperm ability to withstand freeze–thawing (Hernández *et al.*, 2007). In bulls (Jobim *et al.*, 2004), stallions (Zahn *et al.*, 2005; Jobim *et al.*, 2011) and buffalos (Asadpour *et al.*, 2007), seminal plasma protein profiles have been related to sperm freezability. However, to the best of our knowledge, no seminal plasma proteins identified in boar ejaculates, before cryopreservation, have been used to predict boar sperm freezability.

Studying proteins from complex biological sources requires powerful proteomic approaches (Dietrich *et al.*, 2014), and different techniques have been used to identify seminal plasma and sperm proteins in higher vertebrates (Ramm *et al.*, 2009; Upadhyay *et al.*, 2013). Because of its identification capacity and resolution, two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) coupled to liquid chromatography mass spectrometry (LC-MS/MS) has been able to identify novel protein markers for environmental effects and disease factors involved in men fertility (Oliva *et al.*, 2008, 2010). Sperm proteome using 2D-DIGE has been investigated in humans (Liao *et al.*, 2009; Frapsauce *et al.*, 2014), bulls (D'Amours *et al.*, 2010), rats (Rolland *et al.*, 2007), boars (Vilagran *et al.*, 2013) and mice (Jockusch *et al.*, 2014). Research on seminal plasma through 2D-DIGE has also been conducted in carps (Dietrich *et al.*, 2014) and humans (Yamakawa *et al.*, 2007; Davalieva *et al.*, 2012), but studies using boar seminal plasma are yet to be conducted.

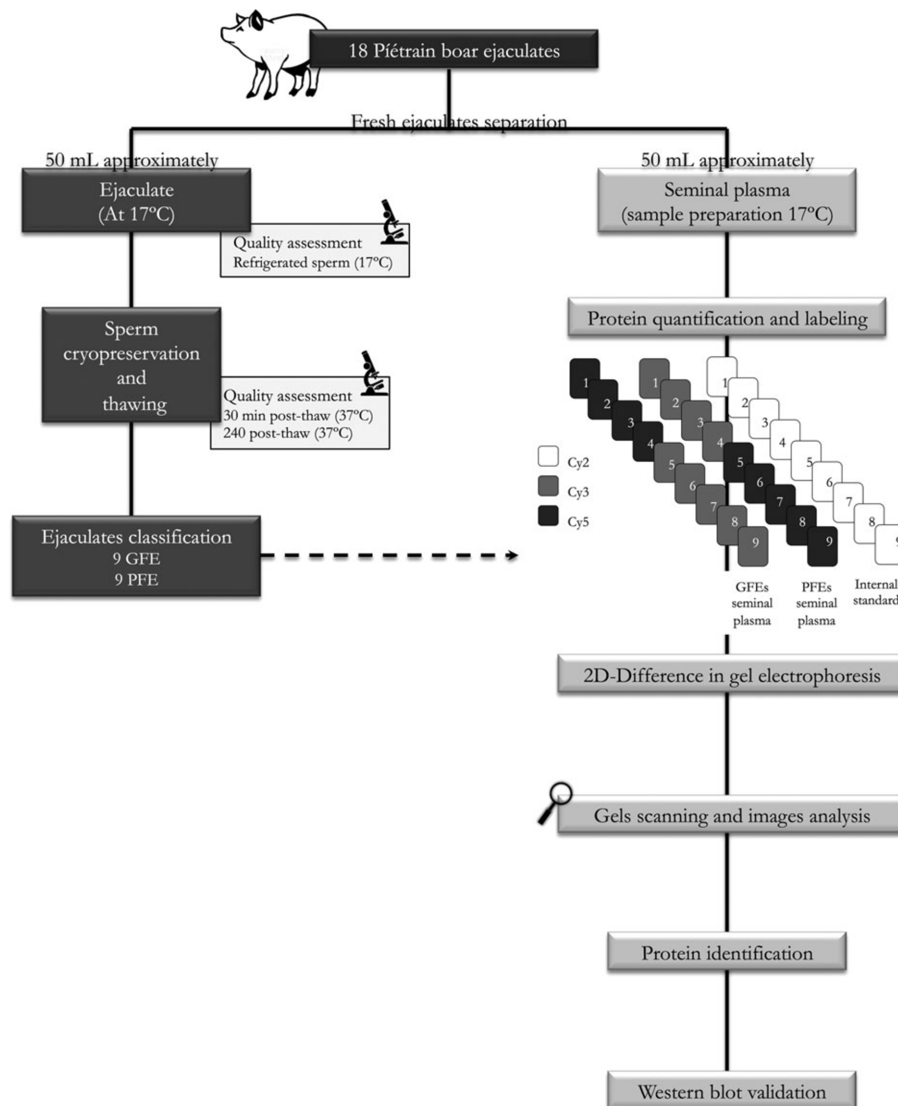

Figure 1 Experimental design conducted in this study. Eighteen Piétrain boar ejaculates rich fraction were split into two aliquots. One was intended for cryopreservation purposes and allowed classifying the samples into GFE and PFE groups based on 30 and 240 min post-thawing assessments. The other aliquot served for seminal plasma proteome analysis through 2D-DIGE and western blot, and took into account the freezability of each ejaculate. Once gels were analysed, protein identification of the potential freezability markers was made by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). The proteomic results were finally validated through western blotting.

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On the basis of the aforementioned, the aim of this study was to compare the proteome of boar seminal plasma from GFEs and PFEs through 2D-DIGE technique to find protein markers of boar sperm freezability. Results were confirmed by western blot assessments.

MATERIAL AND METHODS

Animals and preparation of seminal plasma samples

Semen from 18 healthy adult boars was collected using the gloved hand method, following the EU Directive 2010/63/EU for animal experiments. All boars were from Piétrain breed, and they were stalled in commercial herds (Selecció Batallé S.A., Girona, Spain). The animals were fed according to the standard protocols, and water was provided *ad libitium*. The semen collection rhythm was twice a week and one single ejaculate per male was used in this study.

After collection, the spermatozoa-rich fraction of each ejaculate (80–100 mL) was filtered through gauze and subsequently divided into two aliquots of equal volume. The first one was used for seminal plasma separation from spermatozoa through centrifugation at 640 × *g* and 17 °C for 5 min. Supernatants were subsequently filtered through 0.2 μm Whatman filters (GE Healthcare, Piscataway, NJ, USA) using a syringe and re-centrifuged at 10 000 × *g* and 4 °C for 15 min. Seminal plasma preparations were then examined using phase microscopy to ensure no spermatozoa remained. Clean seminal plasma samples were then stored at −80 °C to keep unaltered their fresh proteomic profile and immediately transported to our laboratory in dry ice. These aliquots referred as fresh seminal plasma samples were then used to identify potential cryopreservation markers.

The other spermatozoa-rich fraction aliquot was extended with a commercial long-term extender free from bovine serum albumin at 1 : 2 ratio (v:v, Vitasem LD; Magapor S.L., Zaragoza, Spain). Extended semen was then packed in bags, cooled to 17 °C and finally transported to our laboratory in an insulated container at the same temperature. Upon arrival, sperm quality was evaluated and samples were kept at 17 °C up to the next day, when they were cryopreserved (Fig. 1).

Samples classification into GFEs and PFEs

To classify seminal plasma samples into two groups (GFEs vs. PFEs), spermatozoa were cryopreserved and thawed and sperm quality assessments were carried out at three different points: refrigerated semen at 17 °C, frozen–thawed spermatozoa at 30 min post thawing and frozen–thawed spermatozoa at 240 min post thawing (Fig. 1).

Sperm quality assessments were made as described previously (Vilagran *et al.*, 2014). Briefly, at the first point (refrigerated semen at 17 °C), ejaculates were evaluated to verify if the minimum quality standards for sperm cryopreservation were fulfilled (80% morphologically normal spermatozoa, 80% total motile spermatozoa, 80% viable spermatozoa and 80% acrosome-intact spermatozoa (Casas *et al.*, 2009). After 30 and 240 min post thawing, boar sperm quality was evaluated on the basis of sperm motility, membrane integrity and osmotic resistance test. These two time points were chosen because this is the insemination-to-ovulation interval recommended for cryopreserved doses (Johnson *et al.*, 2000; Roca *et al.*, 2003).

Assessment of sperm quality

Morphology analysis was performed after fixation of spermatozoa at room temperature for 5 min. With this purpose, 0.5 mL of each sperm sample was mixed with 0.5 mL of pre-warmed phosphate-buffered saline solution containing 2% formaldehyde (1 : 1, v : v). Three replicates of 100 spermatozoa each were assessed placing 5 μL of each seminal sample on a slide mounted with a coverslip. These replicates were observed under a phase-contrast microscope (Olympus BX41, Hamburg, Germany) at 200× magnification (Olympus 20X 0.40 PLAN objective, positive phase-contrast field) and sperm cells were classified as (i) morphologically normal, (ii) morphologically abnormal with proximal or distal cytoplasmatic droplets, or (iii) aberrant (coiled tails, tails folded at the connecting piece, at the intermediate piece or at the Jensen's ring) (Bonet *et al.*, 2012).

Sperm motility assessment was carried out utilising a commercial computer assisted sperm analysis (CASA) system, placing 20 μL of each sperm sample in a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). CASA analysis was made using the aforementioned microscope (Olympus BX41), connected to a computer equipped with the Sperm Class Analyser software (SCA5, Microptic SL, Barcelona, Spain), at 100× magnification (Olympus 10X 0.30 PLAN objective lens; negative phase-contrast field). Sperm motility parameters obtained were those described by Yeste *et al.*, (2008). After evaluating three replicates per sample (a minimum of 1000 spermatozoa was counted per replicate), the corresponding mean ± standard error of the mean (SEM) was calculated.

Sperm viability was assessed as described previously (Garner & Johnson, 1995). Briefly, sperm samples were incubated with 100 nM of SYBR-14 (LIVE/DEAD Sperm Viability Kit, SYBR14/PI, Molecular Probes, Eugene, OR, USA) at 38 °C for 10 min. Labelled samples were then incubated at 38 °C for 5 min with 10 μM of propidium iodide (PI). Three replicates of 100 spermatozoa each were evaluated per sample using an epifluorescence microscope (Zeiss AxioImager Z1, Karl Zeiss AG, Göttingen, Germany) at 400× magnification. The corresponding mean ± SEM was subsequently calculated.

Osmotic resistance test (ORT) evaluated the acrosome integrity using a modified protocol from Pérez-Llano *et al.* (1998); (Pérez-Llano *et al.*, 1998). Two aliquots from each sperm samples of 0.5 mL were incubated at 37 °C for 15 min, one with 1.5 mL isotonic solution (3.2%, w/v, sodium citrate in distilled water at pH 7.4 and 304 ± 8 mOsm/Kg of osmotic pressure) and the other with 1.5 mL hypotonic solution (1%, w/v, sodium citrate in distilled water at pH 7.4 and 100 ± 3 mOsm/Kg of osmotic pressure). Samples were subsequently stained with the lectin from *Arachis hypogaea* (peanut agglutinin, PNA) conjugated with fluorescein isothiocyanate (FITC; final concentration of 2.5 μg/mL) and PI (final concentration of 10 μM) at 37 °C for 10 min, following Nagy *et al.* (2003). Samples were observed under an epifluorescence microscope (Zeiss AxioImager Z1, Zeiss) at a magnification of 400X and three replicates, counting 100 spermatozoa each, were performed per aliquot and sample. Finally, the percentages of acrosome-intact (PNA-FITC$^-$) and acrosome-damaged spermatozoa (PNA-FITC$^+$) were determined per sample as the mean between values in isotonic and hypotonic solutions (Casas *et al.*, 2009).

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Cryopreservation and thawing of sperm samples

Sperm cryopreservation of the 18 ejaculates, which all were above the aforementioned sperm quality thresholds, were frozen following the procedure described in Vilagran *et al.* (2014). From each seminal sample stored at 17 °C, an aliquot was taken and sperm quality parameters evaluated as described below, while the remaining volume was centrifuged at 640 × *g* and 17 °C for 5 min. Soft sperm pellets were subsequently diluted to 1.5×10^9 spermatozoa/mL, using a Makler counting chamber, in a freezing medium (LEY) containing lactose (80%, v:v; 310 mM) and egg yolk (20%, v:v). Sperm samples were cooled down to 5 °C for 120 min (cooling ramp 0.1 °C/min) in a programmable liquid nitrogen freezer (IceCube 14S-B, Minitüb; Tiefenbach, Germany and SY-LAB software v 1.0, Neupurkersdorf, Austria) and they were re-diluted in a second freezing medium (LEYGO) containing LEY supplemented with 6% glycerol and 1.5% Orvus ES Paste (Equex STM, Nova Chemical Sales Inc,; Scituate, MA, USA) to a final concentration of 1×10^9 spermatozoa/mL. Sperm samples were then packed in 0.5-mL straws (Minitüb) using a semiautomatic filling engine (SFS 133, Minitüb) and a freezing ramp was applied utilising the aforementioned programmable freezer (IceCube 14S-B), as described by Casas *et al.* (2009). The cooling ramp consisted of: −6 °C/min from 5 to −5 °C (100 s), −39.82 °C/min from −5 to −80 °C (113 s); 0 °C/min at −80 °C (30 s); and finally −60°C/min from −80 to −150 °C (70 s). Finally, straws were immersed in liquid nitrogen (−196 °C) where they were stored for at least 5 days.

Thawing was carried out following Casas *et al.*, (2012). Briefly, four straws per ejaculate were plunged in a water bath at 37 °C for 20 sec and sperm samples were diluted with three volumes of pre-warmed (37 °C) Beltsville Thawing Solution (BTS) (Pursel *et al.*, 1973) to a final dilution rate of 1/4. Sperm quality parameters of those frozen–thawed sperm samples were assessed after 30 min and 240 min of incubation at 37 °C.

Protein quantification and two-dimensional fluorescence difference gel electrophoresis

Protein concentration in each seminal plasma sample from fresh ejaculates (Fig. 1) was assessed per triplicate through the Bradford method (Bradford, 1976) using Quick Start™ Bradford Protein Assay (BioRad, Hercules, CA, USA). Quantification results were confirmed using one-dimensional SDS-PAGE (Polyacrilamyde Gel Electrophoresis) gel analysis, 5 μg of total protein from each seminal plasma sample being loaded onto 12% acrylamide : bisacrylamide gels. After electrophoretic separation, gels were stained with Coomassie Blue and the density of each lane was compared to the others. No significant differences ($p > 0.05$) between lanes were observed (data not shown).

Two-dimensional fluorescence difference gel electrophoresis was performed following Vilagran *et al.* (2013) and a set of preliminary experiments were carried out to optimize the protocol and thus obtain a reasonable spots resolution. Briefly, 50 μg of seminal plasma proteins from samples belonging to different freezability groups (9 GFEs and 9 PFEs, see *Sample classification into GFEs and PFEs groups and sperm quality assessments,* in Results section) were labelled with 400 pmol of Cy3 and Cy5, following the CyDye DIGE Fluor minimal dye labelling protocol from manufacturer's instructions (GE Healthcare). To ensure that dye binding showed no bias, four randomly selected seminal plasma samples from GFEs were labelled with Cy3, while the other five were labelled with Cy5. This procedure was repeated for the nine PFEs (Fig. 1). Cy2 was used to label an internal standard that contained 31 μg of each individual sample. After 30 min on ice in dark conditions, 1 μL of 10 mM lysine was added to quench the labelling reaction. Quenched labelled samples were stored at −80 °C until they were analysed.

One GFE-labelled sample (Cy3/Cy5), one PFE-labelled sample (Cy3/Cy5) and one aliquot of 50 μg of Cy2-pooled internal standard were randomly mixed (Fig. 1). Sample buffer (3.5 M Urea, BioRad; 1 M thiourea, Sigma Aldrich, Saint Louis, MO, USA; 1% CHAPS detergent, BioRad; 1% immobilized pH gradient (IPG) buffer, Bio-Lyte 3–10 Buffer, BioRad; 65 mM dithiothreitol, Sigma Aldrich) was then added up to a final volume of 400 μL. After 6 μL of DeStreack (GE Healthcare) were added to each mixture, samples were loaded onto 24-cm 3–10 non-linear IPG strips (ReadyStrip, BioRad) and placed in the rehydration tray from a Protean IEF Cell (BioRad). Samples were then focused at room temperature using the following programme: 50 V for 24 h (active rehydration), 50 V for 30 min (slow ramp), 250 V for 30 min (slow ramp), 500 V for 1 h (slow ramp), 1000 V for 1 h (slow ramp), 4000 V for 2 h (slow ramp), 8000 V for 2 h (rapid ramp), from 8000 to 80 000 V (slow ramp), 1000 V for 5 min (slow ramp) and 100 V for 24 h. When IPG strips reached 100 V, they were stored at −20°C until the second dimension was carried out.

For the second dimension, strips were thawed and equilibrated, prior to perform SDS-PAGE as described previously (Vilagran *et al.*, 2013). Second dimension was conducted in all gels simultaneously using PROTEAN Plus DODECA Cell (BioRad) at 300 V for 6 h, each strip being placed on the top of 12% acrylamide : bisacrylamide gels. Spot images from each gel ($n = 9$) were obtained using a Typhoon TRIO scanner (Amersham Biosciences Corp, Piscataway, NJ, USA) at 550 V of exposure and 100 μm of resolution.

Image analysis and protein identification

Gel images were analysed using DeCyder 2D-Differential Analysis Software (6.2 v, GE Healthcare). Differential expressed spots were excised robotically from three different gels (replicates) to ensure enough protein amounts, using the Ettan Spot Picker (GE Healthcare) and they were digested individually with 10 μL of tryptic peptide mixture injected at a flow rate of 400 nL/min in a NanoLC (NanoEasy-PROXEON; Thermo Fisher Scientific, Walthman, MA USA). A precolumn (EasyColumn-L2 cm-ID100 μm-5 μm-120A-C18-PROXEON, Thermo Fisher Scientific) and an analytical column (EasyColumn-L10 cm-ID75 μm-3 μm-120A-C18-PROXEON, Thermo Fixer Scientific) were used.

Protein identification was made through liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a LTQ-VELOS-Orbitrap (Thermo Fisher Scientific). Acquisition of tandem mass spectrometry data was carried out using Xcalibur software (2.1 v., Thermo Fisher Scientific), prior to submission to PIG UniProt-SwissProt release 2013-05 database. For searching in the database, 0.8-Da of fragment mass tolerance was used. A minimum of two peptides per protein was set to accept identifications as valid, while a false discovery rate was also set at 1%.

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Western blotting

Protein seminal plasma samples analysed by western blotting also came from fresh ejaculates and were aliquots from the same samples evaluated through 2D-DIGE analysis (Fig. 1). Three replicates from each sample (n = 18, 9 GFEs and 9 PFEs) were analysed following Vilagran *et al.* (2014). Briefly, 15 micrograms of each protein sample were solubilized with 10 μL of Laemmli buffer 1X consisting of 65 mM Tris (Serva, Heidelberg, Germany), 10.5% of glycerol (Panreac, Castellar del Vallès, Spain), 2.15% of SDS (Serva), 2.5% of beta-mercaptoethanol (BioRad) and traces of bromophenol blue (Panreac) and then stored at −20°C until the beginning of the analysis. Samples were thawed, boiled at 90 °C for 5 min, cooled down on ice and loaded into 1-mm SDS-PAGE. Stacking gels contained 4% acrylamide (Bio-Rad), while separating gels contained either 8% acrylamide in the case of FN1 evaluation, or 15% acrylamide in the case of GPX5 analysis. After gel separation according to the standard procedures (Gallagher, 2006), proteins were transferred from the gel to polyvinyl fluoride membranes (Immobilion-P, Millipore, Darmstadt, Germany) at 120 mA for 120 min. Membranes were washed with washing solution (TBS1X-Tween20) at room temperature for 5 min with agitation, and blocked with blocking solution made up of TBS1X-Tween20 with 5% of bovine serum albumin (BSA; Roche Diagnostics, Basel, Switzerland). Blocking conditions were agitation at 4 °C overnight. After cleaning the membranes thrice in washing solution at room temperature and agitation for 5 min, primary antibodies were incubated in agitation at room temperature for 1 h. Primary antibodies solutions consisted of: anti-FN1 polyclonal rabbit antibody (ref.F3648, Sigma-Aldrich) diluted 1 : 1000 (v : v) in blocking solution and anti-GPX5 polyclonal rabbit antibody (ref.18731-1-AP, Proteintech Europe, Chicago, IL, USA) diluted 1 : 200 (v : v) in blocking solution. Subsequently, membranes were washed five times with washing solution (TBS1X-Tween20) in agitation for 5 min, prior to incubate them with secondary antibodies at room temperature and agitation for 1 h. Solutions for secondary antibodies consisted of: goat anti-rabbit polyclonal horseradish peroxidase (HRP)-conjugated antibody (ref.P0161, Dako, Glostrup, Denmark) diluted in blocking solution 1 : 20 000 (v : v) for FN1 analysis and 1 : 2000 (v : v) for GPX5 assessments. After cleaning the membranes five times with washing solution for 5 min, chemiluminescent reaction was developed according to the manufacturer's instructions using Immobilon Western Chemiluminescent HRP Substrate (Millipore). Protein band images from the membranes were acquired through G:BOX Chemi XL 1.4 scanner (SynGene, Frederick, MT, USA). Protein amounts from each sample were determined using Quantity One software package (v. 4.6.2, BioRad) and expressed as adjusted density (sum of pixel intensities inside the volume boundary × area of a single pixel minus the back ground volume, in density per square millimetre). Protein band densities were normalised for within-sample comparisons using Coomassie staining following the protocol described by Welinder and Ekblad (Welinder & Ekblad, 2011). With this purpose, membranes were washed twice with washing solution in agitation for 5 min and stained with 0.1% Coomassie Brilliant Blue R-250 (BioRad) in a 1 : 1 (v : v) methanol–water solution for 1 min. Stained membranes were then distained for 20 min in a 1 : 5 : 4 (v : v : v) acetic acid–ethanol–water solution and washed with miliQ water until membranes were cleaned. Finally, membranes were air-dried, scanned using the aforementioned scanner and the density for each complete lane was analysed. Relative amounts of FN1 and GPX5 in seminal plasma before starting cryopreservation protocols were obtained through the ratio-specific band densities : complete lane density.

Statistical analyses

Data were analysed using Microsoft Excel (Microsoft Office 2003 Professional Edition; Microsoft Corp., Redmond, WA, USA) and statistical package SPSS for Windows (ver. 21.0 SPSS Inc., Chicago, IL, USA). In all the statistical analyses, minimal significance level was set at $p < 0.05$.

Classification of ejaculates into GFEs and PFEs

Values from sperm quality parameters were expressed as mean ± SEM. Normality and variance homogeneity were first checked through Shapiro–Wilk and Levene tests. When required, data in percentages (x) were transformed to arcsine square-root values ($\sqrt{x}/100$) to accomplish the parametric assumptions.

Eighteen ejaculates included in this study were classified into GFE or PFE as described previously in Casas *et al.* (2009). Data from sperm viability (SYBR14$^+$/PI$^-$, %) and progressive motility (PMOT, %) assessed at 240 min post thawing were used for a hierarchical cluster analysis for dissimilarities including a complete linkage (furthest neighbour) cluster algorithm. This classification was subsequently confirmed by comparing all sperm quality parameters evaluated in this study (i.e. not only sperm progressive motility and viability but also all other sperm motility parameters and ORT) between GFEs and PFEs. This comparison was made through a repeated measures analysis of variance (ANOVA; between-subjects factor: freezability group, within-subjects factors: cryopreservation step, i.e.refrigerated semen at 17 °C, frozen–thawed spermatozoa at 30 min post thawing, and frozen–thawed spermatozoa at 240 min post thawing).

Protein spots and bands analyses

Protein spots data were analysed using the Differential In-Gel Analysis module of DeCyder software (v.6.0, GE Healthcare) and their density was normalised dividing each spot volume by the corresponding spot volume from Cy2 images. Comparison between GFEs and PFEs spots density was analysed through a t-test for independent samples using the Biological Variation Analysis module from the aforementioned software. Protein spots were considered significantly different when the average spot intensity from one sample group was greater than 1.3-fold and p value was lower than 0.05.

In the case of western blot analysis, normalised band densities (density from each band divided by density from the total lane) were calculated for every replicate (each sample was evaluated in triplicate) and ejaculate, prior to calculating the corresponding mean ± SEM. These means per ejaculate were then used to run a t-test for independent samples, where GFEs and PFEs were compared. In all cases, normality and homogeneity of variances were checked before.

Principal component analyses

To test the reliability of FN1 and GPX5 as cryopreservation markers, factorial analyses were run and linear regression models were evaluated. In the first case, data from all sperm quality

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Table 1 Sperm quality parameters, assessed in fresh extended spermatozoa (17 °C) and in post-thawed spermatozoa incubated for 30 min and 240 min at 37 °C, in GFE vs. PFE

	Quality variable GFE	Freezability group PFE
Fresh extended spermatozoa (17 °C)		
Morphology (normal spermatozoa, %)	88.5 ± 4.1	91.2 ± 1.6
Total motility (TMOT, %)	95.2 ± 1.5	92.6 ± 2.1
Progressive motility (TMOT, %)	59.6 ± 2.6	55.7 ± 2.7
Curvilinear velocity (VCL, μm/s)	43.8 ± 2.1	39.5 ± 2.4
Straight line velocity (VSL, μm/s)	23.3 ± 2.2	28.1 ± 1.2
Average path velocity (VAP, μm/s)	41.2 ± 1.6	39.7 ± 1.4
Straightness (STR, %)	66.2 ± 1.2	71.3 ± 2.1
Motility parameter wobble (WOB, %)	78.7 ± 1.7	74.6 ± 2.6
Amplitude of lateral head displacement (ALH, μm)	2.3 ± 0.1	2.0 ± 0.1
Beat cross frequency (BCF, Hz)	6.8 ± 0.2	6.9 ± 0.1
Linearity (LIN, %)	56.3 ± 2.0	53.0 ± 3.3
Viability ($SYBR14^+/PI^-$, %)	93.5 ± 2.2	92.1 ± 2.4
ORT ($PNA\text{-}FITC^-$, %)	92.7 ± 2.6	90.3 ± 2.5
Post-thaw spermatozoa (30 min at 37 °C)		
Total motility (TMOT, %)	73.8 ± 4.2	20.3 ± 1.5***
Progressive motility (TMOT, %)	40.1 ± 3.0	7.2 ± 1.5***
Curvilinear velocity (VCL, μm/s)	34.5 ± 1.2	21.8 ± 0.9***
Straight line velocity (VSL, μm/s)	18.9 ± 1.0	8.9 ± 1.0***
Average path velocity (VAP, μm/s)	25.7 ± 1.0	13.8 ± 1.0***
Straightness (STR, %)	72.9 ± 1.6	62.7 ± 3.1*
Motility parameter wobble (WOB, %)	74.6 ± 1.2	62.6 ± 2.8**
Amplitude of lateral head displacement (ALH, μm)	1.9 ± 0.0	3.4 ± 0.1**
Beat cross frequency (BCF, Hz)	6.8 ± 0.1	4.6 ± 0.5**
Linearity (LIN, %)	54.6 ± 2.0	40.1 ± 3.6**
Viability ($SYBR14^+/PI^-$, %)	58.3 ± 4.5	30.7 ± 3.6**
ORT ($PNA\text{-}FITC^-$, %)	70.3 ± 3.4	58.7 ± 2.9**
Post-thaw spermatozoa (240 min at 37 °C)		
Total motility (TMOT, %)	60.2 ± 3.7	10.8 ± 1.2***
Progressive motility (TMOT, %)	25.4 ± 2.1	1.8 ± 0.4***
Curvilinear velocity (VCL, μm/s)	41.7 ± 1.3	21.3 ± 0.7***
Straight line velocity (VSL, μm/s)	15.8 ± 0.7	6.9 ± 0.6***
Average path velocity (VAP, μm/s)	21.5 ± 0.9	12.1 ± 0.6***
Straightness (STR, %)	73.4 ± 1.5	56.2 ± 2.2***
Motility parameter wobble (WOB, %)	74.9 ± 1.2	56.7 ± 1.2***
Amplitude of lateral head displacement (ALH, μm)	1.7 ± 0.1	2.1 ± 0.1*
Beat cross frequency (BCF, Hz)	6.1 ± 0.3	3.7 ± 0.5**
Linearity (LIN, %)	55.1 ± 2.0	32.1 ± 1.9***
Viability ($SYBR14^+/PI^-$, %)	39.9 ± 2.5	21.0 ± 1.8***
ORT ($PNA\text{-}FITC^-$, %)	59.2 ± 2.2	48.1 ± 2.2***

Sperm quality parameters values, coming from 9 GFEs and 9 PFEs, are represented as mean ± SEM. Values with superscripts are significantly different between GFEs and PFEs in the same time-point assessment (i.e. fresh extended spermatozoa at 17 °C, post-thawed spermatozoa at 30 min at 37 °C and post-thawed spermatozoa at 240 min at 37 °C). Symbol representation: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

parameters assessed at 30 min post thawing and at 240 min post thawing were sorted into some components extracted by principal component analysis (PCA). The obtained data matrix was rotated using the Varimax procedure with Kaiser normalization. Variables from the linear combination of j variables (z) in each component y_i were only selected when they presented a square factor loading (a_{ij}^2) higher than 0.3 with its respective component and lower than 0.1 with respect to the other components in the rotated matrix. After PCA, the resulting regression factors for each component were used as dependent variables in multiple regression analyses (Yeste *et al.*, 2014).

Linear regression models for ejaculate freezability prediction

All sperm parameters and regression factors from PCA coming from assessments at 30 and 240 min post thawing were correlated with FN1 and GPX5 normalised band densities using Pearson correlation. Furthermore, and to evaluate whether FN1 and GPX5 normalised band densities in fresh seminal plasma could predict the quality of frozen–thawed spermatozoa at 30 and 240 min post thawing, a series of multiple regression models were worked out. The procedure followed, the forward stepwise model, was that previously described by Yeste and colleagues (Yeste *et al.*, 2010). Regression equations were optimised to increase the determination coefficient (r^2). The regression factors from PCA at 30 and 240 min post thawing were used as dependent variables and normalised band densities of FN1 and GPX5 were used as independent variables. The significance level for introducing each parameter in the multiple regression models was 10% and the significance level for the models was set at $p < 0.05$.

RESULTS

Sample classification into GFE and PFE groups and sperm quality assessments

Eighteen individual boar ejaculates which satisfied the minimal values for being cryopreserved were classified into two groups according to their sperm viability ($SYBR14^+/PI^-$, %) and progressive motility (%) assessed at 30 and 240 min after thawing. This classification into two clusters resulted in 9 GFEs and 9 PFEs. Such classification was compared to that obtained when all the sperm quality parameters assessed at 30 and 240 min post thawing were included in the cluster analysis, and resulted in the same ejaculates classification (data not shown).

Table 1 shows sperm quality parameters assessed in this study in GFEs and PFEs. No significant ($p > 0.05$) differences between GFE and PFE were observed in any of the quality parameters analysed before starting cryopreservation procedures (i.e. fresh semen, extended in BTS at 17 °C). However, when post-thawing sperm quality parameters assessed in the two groups were compared, sperm viability, motility and osmotic resistance of spermatozoa were significantly ($p < 0.05$) higher in GFE than in PFE. The extent of these differences was higher at 240 min than at 30 min post thawing.

Comparison of seminal plasma proteome between GFEs and PFEs using 2D-DIGE

In order to ascertain which proteins were differentially expressed between GFEs and PFEs seminal plasma, and thus identify potential freezability markers, it was performed a 2D-DIGE analysis in seminal plasma samples from fresh ejaculates (17 °C). A total of nine gels were evaluated, and a similar spot pattern was found (Fig. 2A). In all these gels, most of the spots were located at an isoelectric point (pI) range of approximately 5–8. In contrast, the molecular weight distribution was much broader.

From gel analyses, 51 spots were detected in seminal plasma samples, and four spots were found to significantly ($p < 0.05$) differ between GFEs and PFEs (Fig. 2A). Two of these spots, which were present in all gels analysed and showed a lower p-value, were selected for protein identification to obtain the most reliable potential markers of boar sperm cryopreservation (spots number: 1 and 2, Fig. 2B). Although protein identification in both cases reported more than one single protein per spot, the results were filtered through the pI, molecular weight and score obtained from the identified peptides (see Table S1). This led to

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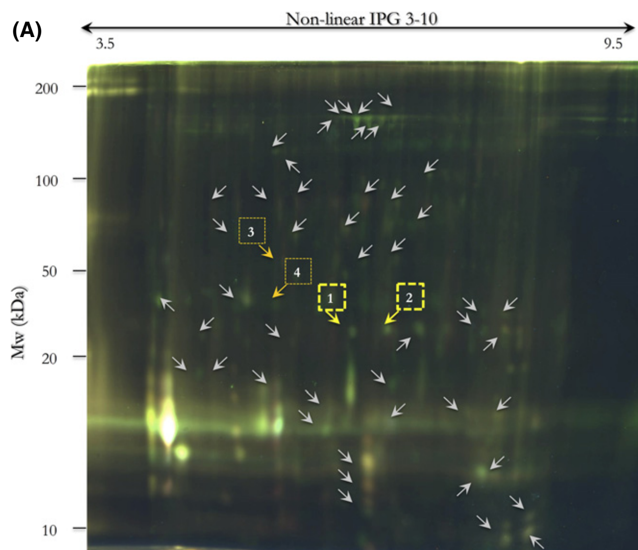

(B)

Spot number	Appearance	*t*-test	Significance	Identification
1	27 (27)	1.75	0.015	**GPX5**
2	27 (27)	2.11	0.038	**FN1**
3	12 (27)	1.71	0.043	-
4	15 (27)	1.77	0.041	-

Figure 2 (A) Representative pattern of gel spots in GFE (Cy3, green spots) and PFE (Cy5, red spots) after 2D-DIGE analysis of boar seminal plasma proteome. Arrows indicate protein spots assessed ($n = 51$), whereas numbers (1–4) are spots that significantly differ between GFEs and PFEs. (B) Table shows the spots appearance in all gels analysed (3 images per gel, $n = 27$), the *t*-test value and its significance. Spots 1 and 2 were further identified as FN1 and GPX5.

determine that GPX5 and FN1 corresponded to spots 1 and 2 respectively (Fig. 2B). Amino acidic fragments identified from these two proteins are shown in Table 2 and specific fragments identified for FN1 are shown in Figure S2.

Western blotting validation of FN1 and GPX5

To validate the proteins identified through 2D-DIGE analysis, western blot assessments were carried out. Figure 3 shows representative protein band patterns from western blots from FN1 and GPX5.

Levels of FN1 content in seminal plasma were significantly ($p < 0.05$) higher in fresh samples from GFE than in those from PFE (Fig. 3). In contrast, GPX5 content did not significantly differ between GFE and PFE ($p > 0.05$; Fig. 3).

Ejaculate freezability prediction through multiple regression analyses

First of all, principal component analyses from all sperm quality parameters assessed at 30 min and 240 min post thawing were carried out to correlate them with normalised band densities of FN1 and GPX5, thus testing their reliability as cryopreservation markers. Tables 3 and 4 present the results of principal components extracted from sperm quality parameters analysed at 30 min and 240 min post thawing respectively. In the case of assessment at 30 min post thawing, a total of two components were extracted. Together, the two components explained a total of 87.8% of variance, the first component containing most of the

Table 2 Amino acidic fragments identified by mass spectrometry in spots 1 and 2 which correspond to GPX5 and FN1 proteins respectively

Spot number	Protein	Amino acid sequence identified
1	GPX5	FLVGPDGVPVMR
		FLVGPDGVPVmR
		NSEILLGLK
		SDIMEYLK
2	FN1	QDGHLWcSTTSNYEQDQK
		ITYGETGGNSPVQEFTVPGSK
		YSFcTDHTVLVQTR
		TEIDKPSQMQVTDVQDNSISVR
		WcGTTQNYDADQK
		TYLGSALVcTcYGGSR
		EESPPLVGQQSTVSDVPR
		FGFcPmAAHEEIcTTNEGVMYR
		VPGTSASATLTGLTR
		GGNSNGALcHFPFLYNNR
		FGFcPmAAHEEIcTTNEGVmYR
		FGFcPMAAHEEIcTTNEGVMYR
		GFNcESKPEPEETcFDK
		TFYScTTEGR
		GDSPASSKPVSIDYR

parameters describing sperm integrity and function and explaining 72.2% of variance (Table 3).

Principal component analyses from assessments at 240 min post thawing also resulted in two components and explained 87.0% of total variance (Table 4). The first component, similar to that observed for PCA at 30 min post thawing, included most of the parameters describing sperm integrity and function and explained 75.4% of variance (Table 4).

Regression factors from all extracted components were subsequently correlated with normalised band densities of FN1 and GPX5 assessed in fresh seminal plasma (17 °C). A positive and significant ($p < 0.05$) correlation was only seen when first PCA component from 240 min post-thawing assessment and FN1 normalised band densities were used. Thus, only in that case a regression equation was worked out using FN1 amount in fresh seminal plasma as independent variable (x), and the first component of PCA at 240 min post thawing as dependent variable (y) (Table 5). Contrarily, there was no significant correlation ($p > 0.05$) between any PCA extracted components at 30 min post-thawing assessment and FN1 amounts. Levels of GPX5 in seminal plasma were not found to be correlated ($p > 0.05$) with PCA components from either 30 or 240 min postthawing assessments.

DISCUSSION

In order to provide new insights into proteins that may serve as markers for boar sperm freezability, we carried out a comparative proteome analysis of boar seminal plasma samples coming from 9 GFEs and 9 PFEs using 2D-DIGE technique. It is worth noting that this was the first time that seminal plasma proteome from boars was evaluated using 2D-DIGE. The application of this technology combined with LC-MS/MS allowed us to detect 51 protein spots and to identify two proteins as fibronectin 1 (FN1) and glutathione peroxidase 5 (GPX5). Fibronectin 1 was further validated as cryopreservation markers through western blot technique.

First of all, we separated the spermatozoa-rich fraction from 18 ejaculates coming from different boars into two aliquots. One

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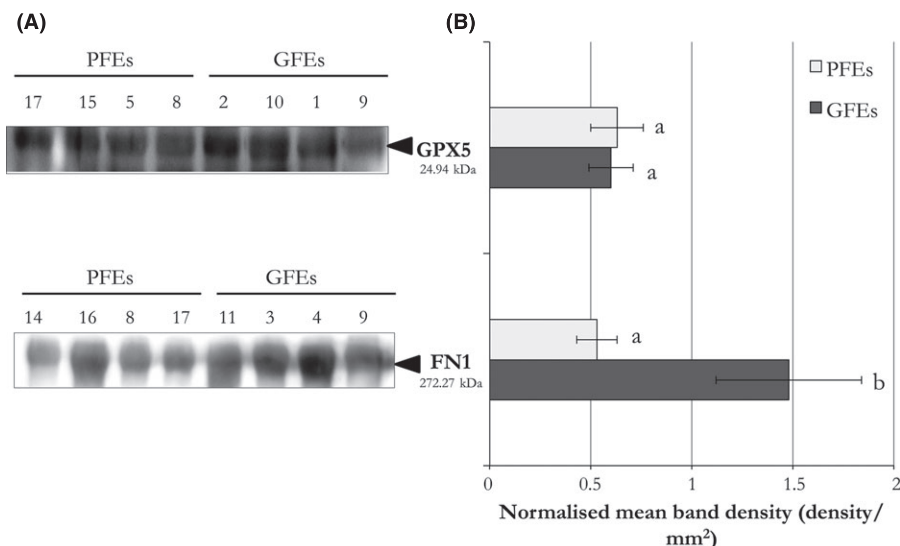

Figure 3 (A) Representative band patterns from western blot analysis of FN1 and GPX5 in seminal plasma from GFEs and PFEs. Numbers identify each different ejaculate assessed ($n = 18$, per triplicate) and their sample group (GFE vs. PFE). (B) This figure shows (as mean ± SEM) band density of FN1 and GPX5 in GFEs ($n = 9$) and PFEs ($n = 9$) after normalisation through Coomassie staining. Different letters (*a*, *b*) on the bars indicate significant differences ($p < 0.05$) between GFEs and PFEs.

Table 3 Principal component analysis of all sperm quality parameters assessed at 30 min post-thawing, incubating spermatozoa at 37 °C

Component	Variance	Combinations of variables	a_{ij}	a_{ij}^2
1	72.2%	VAP (μm/s)	0.99	0.98
		Progressive motility (PMOT, %)	0.98	0.96
		VCL (μm/s)	0.98	0.96
		Total motility (TMOT, %)	0.97	0.94
		VSL (μm/s)	0.97	0.94
		Viable spermatozoa (SYBR-14$^+$/PI$^-$, %)	0.89	0.79
	15.6%	WOB (%)	0.85	0.72
		BCF (Hz)	0.82	0.67
		Intact acrosome (ORT, PNA-FITC$^-$, %)	0.82	0.67
		LIN (%)	0.79	0.62
		STR (%)	0.74	0.55
2		ALH (μm)	0.95	0.90
Total	87.8%			

Variance is represented in percentage (%) corresponded to its explanation by each component and total variance. Factorial coefficients from each variable (a_{ij}) and its two square values (a_{ij}^2) were calculated.

Table 4 Principal component analysis of all sperm quality parameters assessed at 240 min post-thawing, incubating spermatozoa at 37 °C

Component	Variance	Combinations of variables	a_{ij}	a_{ij}^2
1	75.4%	VAP (μm/s)	0.97	0.94
		VSL (μm/s)	0.96	0.92
		VCL (μm/s)	0.94	0.88
		Total motility (TMOT, %)	0.93	0.86
		Progressive motility (PMOT, %)	0.90	0.81
		WOB (%)	0.88	0.77
	11.6%	LIN (%)	0.86	0.74
		STR (%)	0.84	0.71
		Viable spermatozoa (SYBR-14$^+$/PI$^-$, %)	0.82	0.67
		Intact acrosome (ORT, PNA-FITC$^-$, %)	0.79	0.62
		BCF (Hz)	0.76	0.58
2		ALH (μm)	−0.78	0.61
Total	87.0%			

Variance is represented in percentage (%) corresponded to its explanation by each component and total variance. Factorial coefficients from each variable (a_{ij}) and its two square values (a_{ij}^2) were calculated.

was intended for sperm cryopreservation and thawing to classify the ejaculates as GFEs or PFEs, and the other was used for evaluating seminal plasma proteins (Fig. 1). Since protein components in boar seminal plasma may be affected by ejaculate handling and sample preparation (Jonáková *et al.*, 2007), spermatozoa-rich fractions were immediately separated into two aliquots because this allowed us to deal with the following three critical points. First, we avoided degradation of seminal plasma proteins, which are known to play a critical role while allowing mammalian spermatozoa to better resist to cold shock (Guthrie & Welch, 2005; Muiño-Blanco *et al.*, 2008). Second, hydrolytic enzymes from damaged spermatozoa as well as seminal plasma

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Table 5 Regression equations between principal component (1) from PCA analysis with all quality parameters assessed at 30 min and 240 min post thawing (y) and seminal plasma proteins amounts (FN1 and GPX5, x) determined in fresh (17 °C)

		Regression equation	R^2	R	p value model
FN1	30 min	$y = 0.32$ (FN1)-0.33	0.11	0.32	0.152
	240 min	$y = 0.43$ (FN1)-0.39	0.43	0.65	0.005*
GPX5	30 min	$y = -1.13$ (GPX5)-0.69	0.16	0.40	0.104
	240 min	$y = -0.82$ (GPX)$+0.51$	0.08	0.29	0.235

*$p < 0.05$.

enzymes could provoke a degradation of other seminal plasma proteins (Jonáková *et al.*, 2007). Finally, in the first aliquot, boar spermatozoa together with seminal plasma were diluted and stored for 24 h at 17 °C. A previous holding time of 24 h at 17 °C is routinely spent before boar spermatozoa are cryopreserved, as this appears to provide sperm cells with a better resistance to cold shock (Casas & Althouse, 2013; Yeste *et al.*, 2014).

While our method of choice was 2D-DIGE coupled to LC-MS/MS, boar seminal plasma is a complex protein mixture that was difficult to be evaluated through this technique. For this reason, a series of preliminary experiments were conducted to obtain an acceptable resolution of the spots and an appropriate protein identification throughput. In our case, when seminal plasma proteins from GFEs were compared with those from PFEs through 2D-DIGE, four significant different spots were found in almost but not all gels evaluated. Thus, from these four spots, two were always seen to differ between GFE and PFE that is why they were the selected ones for further identification. In addition, the highest significant difference between GFEs and PFEs was also seen for these two spots which corresponded to glutathione peroxidase 5 (GPX5) and fibronectin 1 (FN1). As in other studies, we validated data from these potential freezability markers through western blotting (Chen *et al.*, 2013; Vilagran *et al.*, 2013; Tan *et al.*, 2014). This validation was a key point of this study and confirmed that FN1 is a good marker of boar sperm freezability, as 2D-DIGE coupled to LC-MS/MS had indicated. In contrast, our western blot results indicated that GPX5 amounts in seminal plasma did not differ between GFEs and PFEs. These results were different from those coming from 2D-DIGE analysis which suggested GPX5 as a putative freezability marker. However, when complex protein mix samples are screened through a 2D gel and some proteins are at high quantity, they compete for localization with lower abundance proteins, thereby increasing the overall number of features visible using LC-MS/MS (Gygi *et al.*, 2000; Vilagran *et al.*, 2013). Thus, while this study demonstrates that comparative proteomic analysis using 2D-DIGE is a powerful tool to reveal freezability markers in boar seminal plasma, the markers identified through this technique need further validation using western blot in a step that cannot be ignored. Furthermore, it is important to carry out this validation step since, as it can be observed, the molecular weight of FN1 in 2D-DIGE gels does not coincide with that observed in Western Blots. Indeed, while the molecular weight of this marker in western Blots concurred with its theoretical molecular weight, the weight found in 2D-DIGE gels was lower. This could be explained by the degradation of FN1 during 2D-DIGE processing, since physical and environmental laboratory modifications have been suggested to affect 2D-gel matrix and heterogenous post-translational modifications (Seo & Lee, 2004). For this reason, spot profiles in a 2D gel can differ from what expected from theoretical molecular weights and pI values.

As stated, one of the most interesting findings of our study is the amounts of FN1 in fresh seminal plasma (i.e. before starting cryopreservation) in GFEs were significantly higher than in PFEs. Since this was confirmed by correlation analyses, as FN1 protein amounts in fresh seminal plasma (assessed by western blot) were found to be significantly correlated with the first component of PCA analysis from 240 min post-thawing assessments, we suggest that FN1 is a reliable and predictive seminal plasma marker of boar sperm freezability. Fibronectin 1 is a dimeric glycoprotein made up of two high-molecular monomers (~250 kDa) and has been found in vertebrates in two different types: soluble plasma fibronectin (pFN) and cellular fibronectin (cFN) (Pankov, 2002). Soluble fibronectin has been found in human seminal plasma and has been related with total sperm motility and spermatozoa-fertilizing ability (Wennemuth *et al.*, 2001). In boar seminal plasma, FN1 has also been identified (Druart *et al.*, 2013; González-Cadavid *et al.*, 2014) and described as one of the most abundant proteins in the seminal plasma of this species (Druart *et al.*, 2013). Amounts of this protein in boar seminal plasma have also been correlated with midpiece and tail sperm defects (González-Cadavid *et al.*, 2014). In this study, we have shown, for the first time, that FN1 amount in seminal plasma may be also used as a freezability marker. Therefore, interesting prospective studies are warranted to consolidate FN1 as a freezability marker using another set of GFEs and PFEs. This should confirm its reliability as freezability marker in a double-blind manner. Apart from studying the reliability of FN1 as a freezability marker, as conducted in this study, future studies could also evaluate its suitability as an 'in vivo' fertility marker. In that case, reproductive performance rather than ability to withstand freeze–thawing would be evaluated.

Glutathione peroxidase 5 is an unusual member of the mammalian GPX family that plays an important role as selenium-independent free radical scavenger within epididymis (Grignard *et al.*, 2005; Chabory *et al.*, 2010). Since polyunsatured fatty acids, present in sperm cell membranes, are particularly vulnerable to peroxidative damage, antioxidants scavengers (like GPX5) are critical players in the protection of mammalian spermatozoa (Storey, 1997; Taylor *et al.*, 2013). Apart from this function, Novak *et al.* (2010) found GPX5 present in seminal plasma is positively correlated with farrowing rates. Despite all the aforementioned, we observed that GPX5 was not able to predict boar ejaculate freezability, as no significant differences in GPX5 content were seen when seminal plasma samples from GFEs and PFEs were compared. In addition, GPX5 levels were neither correlated with PCA components from 30 to 240 min post-thawing assessments. These results may not be surprising, since it has been described that variation of reactive oxygen species (ROS) levels during boar cryopreservation protocol are marginal (Kim *et al.*, 2011). Furthermore, Yeste *et al.* (2013) demonstrated that ROS levels did not differ between GFEs and PFEs, either before or after freeze–thawing. Moreover, GPX activity determined in boar seminal samples from GFEs and PFEs appears not to be related to sperm freezability (Hernández *et al.*, 2007).

In conclusion, 2D-DIGE has been carried out for the first time using boar seminal plasma proteome to find reliable markers of boar sperm freezability. Our results demonstrate that seminal

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plasma FN1 is a reliable marker of boar sperm freezability, since GFEs present significantly higher amounts of this protein than PFEs. Because FN1 has been correlated with sperm function and has been seen as a reliable marker for boar sperm freezability, further research about the mechanisms by which FN1 provides the sperm cells with higher resistance to freeze–thawing is warranted.

ACKNOWLEDGEMENTS

This research was supported by Grant Id. RZ 2011-00001-00-00, Spanish Ministry of Science and Innovation, and by a PhD studentship granted to Ingrid Vilagran from University of Girona. Proteomic analyses were carried out at the Proteomic Unit of the Scientific and Technological Centres, University of Barcelona (CCiTUB), a member of the ProteoRed network (http://www.proteored.org/).

AUTHOR'S CONTRIBUTION

I.V. contributed to the design of the study, did the laboratory work, interpreted the data and wrote the manuscript. M. Y. designed the research, analysed and interpreted the data, critically revised the manuscript and approved the final version of the manuscript. J.C. and R.O. contributed to conduct the 2D-DIGE analysis, make a critical revision of the manuscript and approved the final version of the manuscript. S.S and S.B. approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Scatter plot of normalised band volumes of fibronectin-1 (FN1) from western blot analysis correlated with first component (1) of Principal Component Analysis (PCA) from all sperm quality parameters analysed at 240 min post-thawing.

Figure S2. Primary sequence of fibronectin-1 (FN1), where the 15 peptide fragments identified using LC-MS/MS are highlighted. Consecutive fragments are highlighted in two different colours to indicate different events identified.

Table S1. Proteins identified in spots 1 and 2. Proteins were ordered by: molecular weight (MW), isoelectric point (pI, comparing these values with the location of the spot in the gel) and protein Score (calculated by the Xcalibur software, taking into account the peptide-spectrum matches (PSMs), peptides identified and if them were only coming from the protein (Unique peptides)).

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Supporting information

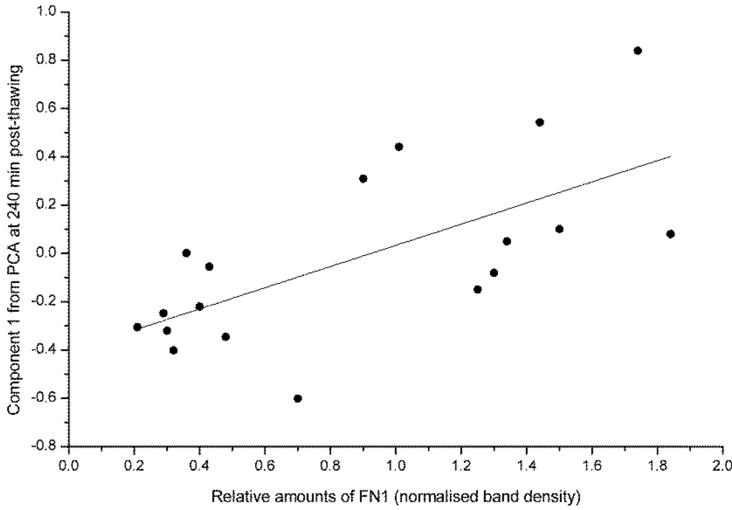

Figure S1. Scatter plot of normalised band volumes of fibronectin-1 (FN1) from western blot analysis correlated with first component (1) of Principal Component Analysis (PCA) from all sperm quality parameters analysed at 240 min post-thawing

Supplementary Data Figure 2

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>tr|F1SS24|F1SS24_PIG Uncharacterized protein OS=Sus scrofa GN=FN1
PE=4 SV=1
MLGGPGPGLLLLAVLSLGTTVPSTGASKSKRQAQQIVQPQSPLVDSQRKPGCYDNGKHYQ
INQQWERTYLGSALVCTCYGGSRGFNCESKPEPEETCFDKYTGNTYRVGDTYERPKDSMI
WDCTCIGAGRGRISCTIANRCHEGGQSYKIGDTWRRPHETGGYMLECVCLGNGKGEWTCK
PIAEKCFDHAAGGTSYVVGETWEKPYQGWMMVDCTCLGEGSGRITCTSRNRCNDQDTRTS
YRIGDTWSKKDNRGNLLQCICTGNGRGEWKCERHTSLQTTSAGSGSFTDVRTAIYQPQPH
PQPAPYGHCVTDSGVVYSVGMQWLKTQGNKQMLCTCLGNGVSCQETAVTQTYGGNSNGEP
CVLPFTYNGRTFYSCTTEGRQDGHLWCSTTSNYEQDQKYSFCTDHTVLVQTRGGNSNGAL
CHFPFLYNNRNYTDCTSEGRRDNMKWCGTTQNYDADQKFGFCPMAAHEEICTTNEGVMYR
IGDQWDKQHDMGHMMRCTCVGNGRGEWTCVAYSQLRDQCIVDDITYNVNDTFHKRHEEGH
MLNCTCFGQGRGRWKCDPVDQCQDSETRTFYQIGDSWEKYVHGVRYQCYCYGRGIGEWHC
QPLQTYPGTTGPVQVIITETPSQPNSHPIQWNAPEPSHISKYILRWKPKNSPNRWKEATI
PGHLNSYTIKGLRPGVVYEGQLISVQHYGHREVTRFDFTTTSTSSAVTSNTVVGETTPFS
PVVATSESVTEITASSFVVSWVSASDTVSGFRVEYELSEEGDEPQYLDLPSTATSVNIPD
LLPGRKYIVNVYQISEEGEQSLILSTSQTTAPDAPPDPTVDQVDDTSIVVRWSRPQAPIT
GYRIVYSPSVEGSSTELNLPETANSVTLSDLQPGVQYNITIYAVEENQESTPVFIQQETT
GVPRPDKVPPPKDLQFVEVTDVKVTIMWTPPESPVTGYRVDVIPVNLPGEHGQRLPISRN
TFAEVTGLSPGVTYHFKVFAVNQGRESKPLTAQQTTKLDAPTNLQFINETDSTVMVTWTP
PRARIAGYRLTVGLTRGGQPKQYNVGPSASQYLLRNLQPGSEYAVTLVAVKGNQQSPRAT
GVFTTLQPVGSIPPYNTEVTETTIVITWTPAPRIGFKLGVRPSQGGEAPREVTSDSGSIV
VSGLTPGVEYVYTISVLRDGQERDTPIVKKVVTPLSPPTNLHLEANPDTGVLTVSWERST
TPDITGYRITTTPTNGQQGYSLEEVVHADQSSCTFENLSPGLEYNVSVYTVKNDKESVPI
SDTIIPEVPQLTDLSFVDITDSSIGLRWTPINSSTIIGYRITVVAAGEGIPIFEDFADSS
VGYYTVTGLEPGIDYDISVITLINGGESAPTTLTQQTAVPPPTDLRFTNVGPDTIRVTWA
PPPSIELTNFLVRYSPVKNEEDVAELSISPSDNAVVLTNLLPGTEYLVSVSSVYEQHESI
PLRGRQKTGLDSPTGIDFSDITANSFTVYWIAPRATITGYKIRHHPEHMGGRPREDRVPP
SRNSITLTNLIPGVEYVVSIVAVNGREESPPLVGQQSTVSDVPRDLQVIATTPTSLLISW
DAPAVTVRYYRITYGETGGNSPVQEFTVPGSKSTATISGLKPGVDYTITVYAVTGRGDSP
ASSKPVSIDYRTEIDKPSQMQVTDVQDNSISVRWLPSSSHVTGYRVTTTPKNGSGPSKTK
TVGPDQTEMTIEGLQPTVEYVVSVYAQNQNGESQPLVQTAVTNIDRPKGLAFTDVDVDSI
KIAWESPQGQVSRYRVTYSSPEDGIHELFPAPDGEEDTAELQGLRPGSEYTVSVVALHDD
MESQPLIGTQSTAIPAPTNLKFTQVTPTSLTAQWTAPNVQLTGYRVRVTPKEKTGPMKEI
NLAPDSSSVVVSGLMVATKYEVSIYALKDTLTSRPAQGVVTTLENVSPPRRARVTDATET
TITISWRTKTETITGFQVDAVPANGQTPIQRTIKPDVRSYTITGLQPGTDYKIYLYTLND
NARSSPVVIDASTAIDAPSNLRFLATTPNSLLVSWQPPRAKITGYIIKYEKPGSPPREVV
PRPRPGVTEATITGLEPATEYTIQVIALKNNQKSEPLIGRKRTDELPQLVTLPHPNLHGP
EILDVPSTVQKTPFVTKPGYDTGNGIQLPGTSGQQPSLGQQMIFEEHGFRRTTPPTTATP
VRHRPGPYTPNVNEEIQVGHVPRGDVDHHLYPHVLGLNPNASTGQEALSQTTISWTPFQE
SSEYIISCHPVGIDEEPLQFRVPGTSASATLTGLTRGATYNIIVEALKDQKRHKIREEVV
TVGNSVDQGLSQPTDDSCFDPYTVSHYAIGEEWERLSESGFKLSCQCLGFGSGHFRCDSS
KWCHDNGVNYKIGEKWDRQGENGQMMSCTCLGNGKGEFKCDPHEATCYDDGKTYHVGEQW
QKEYLGAICSCTCFGGQRGWRCDNCRRPGAELGPEGSTGHSYNQYSQRYHQRTNTNVNCP
IECFMPLDVQADIEDSRE
```

Figure S2. Primary sequence of fibronectin-1 (FN1), where the 15 peptide fragments identified using LC-MS/MS are highlighted. Consecutive fragments are highlighted in two different colours to indicate different events identified.

º	UniProt Accession nº	Description	MW (kDa)	PSMs	Peptides	Unique peptides	pI	Score	Probability (%)
	O18994	Epididymal secretory glutathione peroxidase OS=Sus scrofa GN=GPX5 PE=1 SV=1 - [GPX5_PIG]	24.9	21	8	7	6.04	118.32	74
	F1RVZ8	Uncharacterized protein (Fragment) OS=Sus scrofa GN=LCN5 PE=4 SV=2 - [F1RVZ8_PIG]	12.1	5	4	4	9.26	62.03	10
	O97763	Epididymal secretory protein E1 OS=Sus scrofa GN=NPC2 PE=1 SV=1 - [NPC2_PIG]	16.3	3	3	3	6.87	34.36	4
	F1SGG3	Uncharacterized protein OS=Sus scrofa GN=KRT1 PE=3 SV=1 - [F1SGG3_PIG]	65.2	15	10	2	8.15	34.35	4
	F1SGG6	Uncharacterized protein (Fragment) OS=Sus scrofa GN=KRT5 PE=3 SV=2 - [F1SGG6_PIG]	63.4	10	8	3	9.20	23.95	3
	F1RI24	Uncharacterized protein OS=Sus scrofa GN=PSP-I PE=4 SV=2 - [F1RI24_PIG]	14.5	3	2	2	8.15	17.97	2
	F1RII4	Uncharacterized protein OS=Sus scrofa GN=PARK7 PE=4 SV=1 - [F1RII4_PIG]	14.4	2	2	2	8.27	7.89	2
	I3LVD5	Uncharacterized protein OS=Sus scrofa GN=ACTG1 PE=2 SV=1 - [I3LVD5_PIG]	41.8	8	3	2	5.48	5.35	1
	F1SS24	Uncharacterized protein OS=Sus scrofa GN=FN1 PE=4 SV=1 - [F1SS24_PIG]	272.27	11	8	5	8.15	103.55	60
	F1S0L1	Uncharacterized protein OS=Sus scrofa GN=KRT14 PE=3 SV=2 - [F1S0L1_PIG]	51.5	3	3	2	5.02	59.97	25
	I3LBF1	Inhibitor of carbonic anhydrase OS=Sus scrofa GN=ICA PE=4 SV=1 - [I3LBF1_PIG]	77.5	6	5	3	6.21	16.43	11
	I3LDS3	Uncharacterized protein OS=Sus scrofa GN=KRT10 PE=3	58.0	8	8	7	4.98	8.08	3

Table S1. Proteins identified in spots 1 and 2. Proteins were ordered by: molecular weight (MW), isoelectric point (pI, comparing these values with the location of the spot in the gel) and protein Score (calculated by the Xcalibur software, taking into account the peptide-spectrum matches (PSMs), peptides identified and if them were only coming from the protein (Unique peptides).

5

Discussion

Cryopreservation is currently the most efficient method for storing boar sperm for a long period of time (Watson 2000; Johnson *et al.* 2000; Mazur *et al.* 2008; Yeste 2015). Although the use of frozen-thawed boar sperm has several advantages (Bailey *et al.* 2008), this technique is not much used commercially because of its lower reproductive performance compared to fresh/extended semen (Johnson *et al.* 2000; Mazur *et al.* 2008; Rodríguez-Gil & Estrada 2013). Furthermore, not all boar ejaculates present the same ability to withstand freeze-thawing protocols, but rather a high variability between breeds (Park & Yi 2002; Waterhouse *et al.* 2006), individuals (Thurston *et al.* 2002; Holt *et al.* 2005), ejaculates (Casas *et al.* 2009; Yeste *et al.* 2013) and even fractions of the same ejaculate (Peña *et al.* 2006) has been reported.

In order to minimize the negative impact of high freezability variability on the use of cryopreserved doses, several efforts have been made to predict ejaculate freezability before cryopreservation protocol takes place. Some attempts have tried to find a relationship between conventional sperm quality parameters analyzed in fresh/extended boar sperm and sperm survival after thawing. However, several studies have concluded that conventional sperm analyses are not useful for predicting boar sperm freezability (Hernández *et al.* 2006; Casas *et al.* 2009). This has been confirmed in the three papers that make the core of this Dissertation (**PAPER I**, **PAPER II** and **PAPER III**), since in all cases sperm parameters in refrigerated semen at 17ºC failed to predict the sperm freezability. Therefore, sperm viability and motility in GFE and PFE were seen to differ after but not before cryopreservation took place.

In this context, it is of paramount importance seeking new markers, different from conventional parameters, capable to predict boar sperm freezability. Thurston and colleagues using amplified restriction fragment length polymorphism technique reported that GFE and PFE present genetic differences (Thurston *et al.* 2002). Following this, protein differences between GFE and PFE were also evaluated and some markers, such as, heat-shock protein 90 (HSP90AA1) were identified (Casas *et al.* 2010). However, there could be other markers, so that the present work sought to find other potential markers of boar sperm freezability comparing GFE and PFE. To achieve this goal, the same first step was followed in **PAPER I**, **PAPER II** and **PAPER III**: classification of ejaculates into GFE and PFE. First, ejaculate samples were split up into two different aliquots. One aliquot was used to evaluate sperm quality and to identify potential freezability markers prior to freeze-thawing, whereas the other was intended for cryopreservation procedures and allowed identifying GFE and PFE. It is important to notice that because of methodological constraints in the case of the 2D-DIGE studies (**PAPER II and PAPER III**), identifying GFE and PFE was a mandatory previous step, otherwise differential protein staining with fluorescent dyes could not have been applied.

Ejaculates were classified following the procedure described by Casas and colleagues (Casas *et al.* 2009). This method clusters, through hierarchical analysis, the ejaculates on the basis of sperm viability and progressive motility at 30 min and 240 min post-thawing. As a result, a dendogram is constructed. It is worth mentioning that

the work by Casas (2010) demonstrated that dendograms obtained when only sperm viability and progressive motility are taken into account match with those obtained when all sperm quality parameters are used (Casas 2010). According to this, the three studies (**PAPER I**, **PAPER II** and **PAPER III**) clearly settled this method of classification since the clusters obtained were the same (i.e. when both only viability and sperm motility were used and when all quality parameters assessed).

In the present Dissertation, two different approaches were conducted to identify boar sperm freezability markers. The first one, which resulted in **PAPER I,** was the same as that performed by (Casas 2010), whereas the second, which resulted in **PAPERS II** and **III**, consisted of using 2D-DIGE. With regard to the first one, it is widely understood that cryopreservation induces changes in membrane permeability and DNA integrity, and osmotic and thermal stress, and cells try to mediate a response against these changes (Yeste 2015). Thus, it seems reasonable to suggest that proteins participating in the response to freeze-thawing-linked events could be used markers of boar sperm freezability (Casas et al. 2009; Casas et al. 2010). For this reason, and after a broad search in the literature, two candidate proteins: small heat-shock protein 10 (ODF1/HSPB10) and voltage-dependent anion channel 2 (VDAC2) were tested (**PAPER I**). Ejaculates were classified into PFE and GFE based upon their sperm quality at post-thawing, and the amounts of these two proteins were evaluated in fresh samples and compared through Western Blot. The ability of these proteins to predict boar ejaculate freezability was examined through linear regression models.

On the one hand, ODF1/HSPB10 was chosen because belongs to the family of small heat-shock proteins (sHSPs) (Fontaine *et al.* 2003). Thus, since other HSPs, such as HSP90AA1 (Casas *et al.* 2010), have been found to be related to sperm cryotolerance, it could be that ODF1/HSPB10 also exerted a role when sperm are exposed to stressful conditions, such as freeze-thawing. Moreover, ODF1/HSPB10 has been revealed as the major protein of outer dense fibres in the tail of mammalian sperm. Therefore, this protein plays a crucial role in the maintenance of elastic structures and protects sperm tail from shear forces (Fawcett 1975; Baltz *et al.* 1990; Lindemann CB. 1996). According to this, mice with homozygous deletion for this protein present acephalic sperm, and those heterozygous are subfertile (Yang *et al.* 2012). In humans, ODF1/HSPB10 was described as marker for sperm motility and expression deficiencies have been related to impaired sperm development and male infertility (Haidl *et al.* 1991; Zarsky *et al.* 2003; Chen *et al.* 2009; Yang *et al.* 2012; Yang *et al.* 2014). These evidences suggest that apart from its role as chaperone, alterations in ODF1/HSPB10 during cryopreservation procedures could result in an impairment of its structural function and thus affect the fertilizing ability of frozen-thawed boar sperm. All these features make this protein a freezability marker candidate.

On the other hand, VDAC2 was described in bull (Hinsch *et al.* 2004), buffalo (Mohanarao & Atreja 2012), mouse (Arcelay *et al.* 2008) and human (Ficarro *et al.* 2003) spermatozoa but not yet in boar sperm. This protein is a pore-forming, ion channel protein involved in the regulation of membrane permeability and sperm motility in

other species (Blachly-Dyson & Forte 2001; Hinsch *et al.* 2004; Shoshan-Barmatz *et al.* 2010; Petit *et al.* 2013). Therefore, this protein was also an excellent freezability marker candidate since the transport of water, ions and solutes across plasma membrane is crucial during freeze-thawing procedures.

Our data demonstrated that while VDAC2 amounts determined by Western Blot were significantly and positively correlated with sperm quality parameters at 240 min post-thawing, ODF1/HSPB10 amounts were not correlated with any parameter. Therefore, although the present Dissertation had hypothesized that ODF1/HSBP10 could be involved in boar sperm freezability, **PAPER I** discarded this possibility. In contrast, VDAC2 was confirmed as a marker for boar sperm freezability. First of all, VDAC2 amounts were significantly higher in GFE than in PFE. In addition, a regression equation was worked out using VDAC2 amounts in extended semen as independent variable and sperm quality parameters at 240 min post-thawing as a dependent variable. It is worth noting that VDAC2 amounts failed to predict sperm quality evaluated at 30 min post-thawing. To explain these differences between 30 and 240 min post-thawing, it should be borne in mind that previous studies have demonstrated that differences between GFE and PFE are more consistent at 240 min than at 30 min post-thawing (Casas et al. 2009; Yeste et al. 2013; Yeste et al. 2014).

VDAC2 has been found to be involved in spermatogenesis, sperm maturation, motility and fertilisation (Liu *et al.* 2010). Although no previous study has evaluated the role of this protein in cryopreservation of mammalian species, different elements may contribute to explain the findings reported herein First of all, this protein forms a channel structure in the lipid bilayer which mediates the transport of ions and small molecules such as: Na^{+}, Ca^{2+}, Cl^{-}, HCO_3^{-}, ATP, glutamate etc. (Shoshan-Barmatz *et al.* 2010). This function has been demonstrated in bull spermatozoa, where co-incubation with anti-VDAC2 antibodies results in a time- and dose-dependent reduction of ion transport (Triphan *et al.* 2008). Therefore, it is reasonable to hypothesize that VDAC2 is involved in the ion fluxes that result from the osmotic-changes that occur during freeze-thawing (Watson 2000). It should be kept in mind that capacitation-like changes triggered by cold shock during cryopreservation are initiated with lipid and protein membrane rearrangements. This compromises the selective permeability of plasma membrane provoking a rapid entrance of ions and water, and activating Ca^{2+}-dependent events that ultimately affect the sperm lifespan (Green & Watson 2001; Leahy & Gadella 2011). Taking all above into account, it appears that higher amounts of VDAC2 in GFE samples could protect sperm from these great changes in membrane fluidity through a better regulation of ion transmembrane flux (Shoshan-Barmatz *et al.* 2010). This would ultimately prevent/reduce the damaging effects that cold shock clearly inflicts on PFE during freeze-thawing.

VDAC2 has also been related to sperm motility. In human, and despite the role of mRNA in the function of mature sperm being still under debate, VDAC2-mRNA was demonstrated to be a marker for idiopathic asthenozoospermia since infertile patients had significantly lower content of VDAC2-mRNA than normozoospermic

individuals (Xu X, Wang Y, Yu Z, Chen J, Guo M, Gui YT 2009; Liu *et al.* 2010). As aforementioned, VDAC2 has been related with regulation of sperm motility in bulls (Hinsch *et al.* 2004), and sperm capacitation in mouse (Arcelay *et al.* 2008; Baker *et al.* 2010), human (Ficarro *et al.* 2003) and buffalo spermatozoa (Mohanarao & Atreja 2012). Therefore, one could suggest that because of the lower content of VDAC2 in PFE than in GFE, the latter could present higher sperm motility following post-thawing due to a better regulation by VDAC2. The mechanism by which VDAC2 could play this role in frozen-thawed sperm is yet to be revealed, but previous studies in bull semen and somatic cells suggested that VDAC2-mediated effect is related to ATP transport through its pore (Hinsch *et al.* 2004; Shoshan-Barmatz *et al.* 2010). More research is thus warranted on the function of this protein in fresh, extended and frozen-thawed boar semen.

With regard to the second approach conducted in the present Dissertation (**PAPER II** and **PAPER III**), the non-cryopreserved aliquot was used to compare the seminal proteome of GFE and PFE through 2D-DIGE, upon classification of boar ejaculates. In the case of **PAPER II**, 13 GFE and 13 PFE were compared and up to twenty different spots were identified in both groups of ejaculates. These spots were further analyzed using liquid chromatography coupled to double tandem mass spectrometry (LC-MS/MS) for protein identification. This proceeding has been largely used in the identification of sperm proteins from other mammalian species (Baker *et al.* 2005; Rolland *et al.* 2007; Martínez-Heredia *et al.* 2008; Liao *et al.* 2009; Poland *et al.* 2011; Tardif *et al.* 2012). It should be mentioned that in **PAPER II**, some spots accumulated more than a single protein and that a given protein was found in more than one spot. This handicap in the use of 2D-DIGE technique was already reported by Gygi and colleagues (Gygi *et al.* 2000). These authors observed that when the whole yeast proteome was analysed, highly-expressed competed with lower-abundance proteins for localization in the 2D-gel, giving a deviated result when protein-spot identification took place. These difficulties were also experienced by other authors. For example, Van den Bergh and colleagues encountered the same problem when striate cortices of kitten and adult cats were analyzed (Van den Bergh *et al.* 2003). In the case of the present Dissertation, this obstacle was also found in **PAPER III**, when boar seminal plasma proteome was compared between GFE and PFE through 2D-DIGE. Therefore, a further validation step through another proteomic technique, such as Western Blot, is required following 2D-DIGE. For this reason, 2D-DIGE technique allowed distinguishing the major proteome differences at sperm (**PAPER II**) and seminal plasma (**PAPER III**) levels between GFE and PFE, so that it facilitated the tedious work of comparing potential markers of boar sperm freezability one-by-one. Those proteins that presented the highest difference in terms of abundance between the two groups (i.e. GFE and PFE) in the 2D-DIGE approach were further tested through Western Blot.

In **PAPER II**, the two candidate proteins that were identified using 2D-DIGE were acrosin-binding protein (ACRBP) and triosephosphate isomerase (TPI). These two markers were further validated through Western Blot. Following Western Blot and using Pearson correlation, ACRBP amounts were found to be significantly and

positively correlated with ejaculate freezability. On the contrary, there was a significant but negative correlation between TPI amounts and freezing capacity. Therefore, the main outcome of **PAPER II** was that ACRBP and TPI can be used as markers of boar sperm freezability before starting the cryopreservation procedure. Each protein will be individually discussed in the following paragraphs.

Acrosin-binding protein (ACRBP, P32 or SP32) is a calcium-dependent phosphoprotein located in sperm acrosome and has been described in pig (Polakoski & Parrish 1977), human (Whitehurst *et al.* 2010), guinea pig (Baba *et al.* 1994) and mouse spermatozoa (Tardif *et al.* 2012). In the eighties, Baba and collaborators suggested that ACRBP played a role as a facilitator for proacrosin conversion to its mature form (Baba *et al.* 1989). Some years later, the same group demonstrated that this protein has the capacity to bind proacrosin zymogen and delay its activation to acrosin. Furthermore, they hypothesized that ACRBP might be a physiologically relevant substrate in the acrosin maturation pathway and further release (Baba *et al.* 1994). These findings have been confirmed recently since ACRBP has been proven as the key regulator of autocatalytic conversion of proacrosin into its mature form and as an important regulator of proteolytic processing events during acrosomal matrix disassembly in mouse and pig sperm (Foster 2013; Kanemori *et al.* 2013). In addition, ACRBP has also been demonstrated to be phosphorylated during boar sperm capacitation (Tardif *et al.* 2001; Dubé *et al.* 2003; Dubé *et al.* 2005; Bailey *et al.* 2005; Sun *et al.* 2013). All these evidences show that this protein exerts important roles in several complex processes, such as packaging of acrosomal matrix proteins and their further release during acrosomal exocytosis. Moreover, it is important to know that the ACRBP role is modulated via phosphorylation of its tyrosine residues.

In this Dissertation **(PAPER II)**, amounts of ACRBP in PFE were found to be significantly lower than in GFE. Therefore, and taking all the above into account, it could be suggested that PFE, due to their low content of ACRBP, present a low regulatory capacity of acrosome reaction. Thus, premature/degenerative acrosome exocytosis is more likely to occur in response to freeze-thawing protocols, which ultimately results in a diminished life span. Accordingly, it has been demonstrated that PFE present higher susceptibility to cryocapacitation/capacitation-like changes than GFE when submitted to cryopreservation protocols (Casas *et al.* 2009). In this context, the higher amounts of ACRBP in GFE seem to imply a higher ability to maintain the proacrosin/acrosin system intact, thereby avoiding premature capacitation and degenerative acrosome exocytosis. Further research is required to confirm the hypothesized role for ACRBP in boar cryopreserved sperm and for determining by which precise mechanism this protein is related to sperm cryotolerance.

With regard to triosephosphate isomerase (TPI), it is widely understood that is an enzyme of the glycolytic pathway that allows conversion of dyhydroxyacethone phosphate into glyceraldheyde-3-phosphate. Thus, this protein is a clue enzyme in sperm metabolism. In addition, TPI has also been found to be involved in sperm capacitation, since blocking of TPI in rat spermatozoa using ornidazole inhibits sperm

hyperactivation and capacitation (Bone *et al.* 2001). In **PAPER II**, PFE were reported to present higher amounts of TPI than GFE. Following Bone and colleagues (Bone *et al.* 2001), one could hypothesise that higher amounts of TPI in PFE predispose sperm to premature capacitation or contribute to destabilise their plasma membrane, which may ultimately result in cell death. In agreement with this hypothetic mechanism, Chen and colleagues recently determined that amounts of this enzyme are higher in frozen-thawed than in fresh sperm. These authors also suggested that the increment of TPI levels in frozen-thawed sperm could be due to an increased demand of energy during the hyperactivation of premature capacitated spermatozoa (Chen *et al.* 2014). This supports the idea the higher amounts of TPI in PFE could favour membrane destabilisation and hyperactivation following freeze-thawing, decreasing the lifespan of these samples. However, again, this hypothesis has to be further validated, elucidating the mechanism by which TPI is related to boar sperm freezability.

It is worth mentioning that TPI has been described as a marker in human sperm, the human asthenozoospermic samples presenting higher levels of TPI than the normozoospermic ones (Siva *et al.* 2010). This matches with the results described herein since PFE not only presented higher TPI amounts but also lower sperm motility at post-thawing. Finally, it has recently been found that TPI is a predictive biomarker for litter size in pigs, since its amounts are negatively correlated to the number of piglets born (Kwon *et al.* 2015). Compiled together, all these data not only give a global overview about the significance of this protein for sperm function but also warrants further research horizons. In the case of pigs, TPI is not only a freezability marker but could also predict the fertilizing ability.

The purpose of **PAPER III** was to find protein markers of boar sperm freezability in seminal plasma from GFE and PFE. Related to this, it is important to note that little attention has thus far been paid to the relationship between seminal plasma and boar sperm freezability. However, there are multiple evidences suggesting that this complex mixture of secretions may exert an important role upon survival of boar sperm after freeze-thawing. In this regard, it is worth noting that studies that examined the effect of adding seminal plasma before sperm cryopreservation or after freeze-thawing reported variable and conflicting results (Caballero *et al.* 2012; Okazaki & Shimada 2012), which was attributed to differences in seminal plasma protein composition (Caballero *et al.* 2004; Maxwell *et al.* 2007). Moreover, differences in seminal plasma composition have been related to individual differences in boar sperm freezability (Roca *et al.* 2006) and supplementing freezing extenders with seminal plasma from boars with good sperm freezability has been reported to improve the sperm ability to withstand freeze-thawing (Hernández *et al.* 2007). In spite of this, **PAPER III** represented the first time that seminal plasma proteins were attempted to be used to predict boar sperm freezability. By contrast, similar approaches had been undertaken in bulls (Jobim *et al.* 2004), stallions (Zahn *et al.* 2005; Jobim *et al.* 2011) and buffalos (Asadpour *et al.* 2007).

Due to its high throughput and admittedly encouraging results from **PAPER II**, 2D-DIGE technique was used and allowed detecting up to 51 protein spots in seminal plasma samples that differed between GFE and PFE. From these 51 spots, two putative freezability markers were identified: Fibronectin 1 (FN1) and glutathione peroxidise 5 (GPX5). It is important to note that of the followed procedure was the same as that followed in **PAPER I** and **PAPER II.** This involved that sperm-rich fractions were separated into two aliquots. One aliquot was intended for cryopreservation and further classified as GFE or PFE, whereas the other served to evaluate seminal plasma proteins in fresh semen. In this particular case, it is noticeable that sperm-rich fractions were immediately separated before dilution into these two aliquots, since protein components in seminal plasma could be affected by ejaculate handling and sample preparation (Jonáková *et al.* 2007). Thus, this procedure allowed dealing with the following three critical points: 1) It avoided degradation of seminal plasma proteins coating/adhering the sperm surface, which are known to play a critical role for sperm resistance to cold-shock (Guthrie & Welch 2005; Muiño-Blanco *et al.* 2008); 2) It avoided enzymatic hydrolysis of seminal plasma proteins by enzymes released from damaged spermatozoa and/or enzymes present in seminal plasma (Jonáková *et al.* 2007); and 3) It allowed the aliquot intended for sperm cryopreservation to be extended and kept in contact with seminal plasma, since extended seminal doses that are ultimately cryopreserved present better resistance to cold shock when stored with diluted seminal plasma for 24 h at 17ºC (Casas & Althouse 2013; Marc Yeste et al. 2014).

Overall, 51 protein spots were found to differ between GFE and PFE when all gels were evaluated. However, out of these 51, there were only four spots that always differed in all gels. Since differences in the amounts of two out of these four protein spots were the highest, they were selected for further identification using LC-MS/MS. These two proteins were FN1 and GPX5. As it has been discussed above and according to other studies (Chen *et al.* 2013; Tan *et al.* 2014), these two proteins were validated through Western Blotting. Results confirmed that FN1 was a good marker of boar sperm freezability, as 2D-DIGE coupled to LC-MS/MS indicated. On the contrary, GPX5 amounts determined through Western Blotting did not differ between GFE and PFE, as 2D-DIGE indicated. As aforementioned, this fact could be due to localization competition of highly expressed proteins in 2D-gels (Gygi *et al.* 2000; Van den Bergh *et al.* 2003). Thus, from these data, it could be concluded that comparative proteome analysis of seminal plasma using 2D-DIGE is a powerful tool to reveal freezability markers in boars; however, again, further validation through Western Blot is a mandatory step that should not be ignored. Related to the importance of this validation, it is important to note that molecular weight of FN1 in 2D-DIGE gels did not coincide with that observed in Western Blots, that of the 2D-DIGE being lower than that of Western Blot and in this latter coinciding with the theoretical one. This difference between molecular weights could be due to degradation of FN1 during 2D-DIGE processing, since physical and environmental laboratory modifications have been demonstrated to affect 2D-gel matrix and heterogeneous post-translational

modifications and this may ultimately result in spot profiles differing from those expected following theoretical weights and pI values (Seo & Lee 2004).

Apart from these handicaps, FN1 was determined as a reliable and predictive seminal plasma marker for boar sperm freezability, since GFE presented significantly higher amounts of this protein than PFE, and FN1-amounts in fresh seminal plasma were significantly correlated with sperm quality parameters at 240 min post-thawing. Fibronectin 1 is a dimeric glycoprotein found in vertebrates in two different types: soluble plasma fibronectin (pFN) and cellular fibronectin (cFN) (Pankov 2002). The soluble form has been identified in human seminal plasma and has been related to total sperm motility and fertilizing ability (Wennemuth *et al.* 2001). In pigs, FN1 has also been found in seminal plasma. In this case, FN1 is one of the most abundant proteins (Druart *et al.* 2013; González-Cadavida *et al.* 2014) and its amounts are correlated with defects in mitochondrial and principal tail pieces (González-Cadavida *et al.* 2014). Therefore, one could suggest that higher amounts of FN1 in seminal plasma of GFEs could exert protective roles upon sperm during cryopreservation, thereby maintaining its motility post-thawing. Nevertheless, interesting prospective studies are warranted to study by which mechanism FN1 is related to boar sperm cyrosurvival.

With regard to GPX5, this protein plays an important role as a selenium–independent, free radical scavenger within epididymis (Grignard *et al.* 2005; Chabory *et al.* 2010). Thus, it protects mammalian spermatozoa from peroxidation, since polyunsatured fatty acids in sperm plasmalemma are particularly vulnerable to that damage (Storey 1997; Taylor *et al.* 2013). Furthermore, Novak and colleagues found that seminal plasma GPX5 was positively correlated with farrowing rates (Novak *et al.* 2010). Despite these encouraging evidences and the outcome of 2D-DIGE approach, in the present dissertation GPX5 was found not to be able to predict boar sperm freezability, since no differences between GFE and PFE were observed through Western Blotting. In addition, there was no significant correlation between GPX5 levels in fresh seminal plasma and sperm quality parameters evaluated either at 30 or at 240 min post-thawing. In order to explain these results, one should bear in mind that increases in intracellular ROS levels following boar sperm cryopreservation are marginal and they do not differ between GFE and PFE, either before or after freeze-thawing (Kim *et al.* 2011; Yeste *et al.* 2013). Furthermore, GPX5 activity does not differ between GFE and PFE post-thawing (Hernández *et al.* 2007). Thus, it is not surprising that GPX5 levels in boar seminal plasma failed to predict sperm quality at post-thawing.

Taking all above into consideration, it can be concluded that the present Dissertation has found that three sperm proteins: acrosin-binding protein (ACRBP), triose-phosphate isomerise (TPI) and voltage-dependent anion channel 2 (VDAC2), and one seminal plasma protein: fibronectin 1 (FN1), are reliable markers for boar sperm freezability when evaluated in fresh/extended semen. Therefore, these four proteins are able to predict boar sperm capacity to withstand cryopreservation before freeze-thawing protocols take place.

Since effective boar semen cryopreservation may ultimately enhance herd productivity, increase biosafety measures, encourage international exchange, facilitate sex-selection technology and facilitate gene banking, research is warranted to counteract detrimental effects of cryopreservation inflicts upon boar sperm. This may get a widespread use of this advantageous technology. While, so far, investigations have mainly been focused upon empirical factors, such as diluents composition, type and concentration of cryoprotective agents, rates of dilution and cooling etc., they have admittedly failed to identify other important issues related to sperm cryobiology, such as freezability markers and proteins involved in cryopreservation-linked stress. For these reasons, studies that provide new insights into molecules involved in sperm freezability are of great interest, as this ultimately sheds light on mechanisms and pathways involved in boar sperm cryotolerance. Furthermore, these findings may help to develop new tests to detect sperm freezability capacity at molecular level and thus avoid cryopreserving PFE. If this was the case, swine industry might wish to reconsider its interest for cryopreserved doses. In fact, despite the niche for boar cryopreserved sperm being narrow, due to the current success of long-term sperm storage in liquid state (Yeste 2015), some particular cases (e.g. gene banking) may benefit from the freezability markers reported herein.

6

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1. VDAC2 content in extended boar ejaculates is significantly higher in good than in poor freezability ejaculates. Therefore, VDAC2 is a marker for boar sperm freezability, as predicts the sperm ability to withstand freeze-thawing protocols before these procedures take place **(PAPER I)**.

2. The amounts of ODF1 in boar sperm before cryopreservation do not differ between GFE and PFE. Therefore, ODF1 is discarded as a freezability marker for boar sperm **(PAPER I)**.

3. Two dimensional differential gel electrophoresis (2D-DIGE) is able to distinguish the major differences of sperm proteome between GFE and PFE. Hence, it is a suitable method to screen putative freezability markers for boar sperm **(PAPER II** and **PAPER III)**.

4. In spite of the aforementioned, boar sperm freezability markers identified through 2D-DIGE technique need further validation using Western Blot. This step cannot be ignored, since artefacts linked to 2D-gels may lead to false positive results **(PAPER II** and **PAPER III)**.

5. Following 2D-DIGE and further Western Blot validation, ACRBP has emerged as a sperm freezability marker, the ACRBP content in extended boar semen being significantly higher in GFE than in PFE **(PAPER II)**.

6. Levels of TPI in extended boar semen are significantly lower in GFE than in PFE. Thus, the semen from boars with high TPI content should not be used for cryopreservation procedures **(PAPER II)**.

7. Not only sperm proteins but also those of seminal plasma may be good markers for boar sperm freezability. The present Dissertation has demonstrated, for the first time, that 2D-DIGE can also screen the boar seminal plasma proteome of GFE and PFE **(PAPER III)**.

8. As found by 2D-DIGE and validated by Western Blot, FN1 in boar seminal plasma is a good marker of sperm freezability, since its content in GFE is significantly higher than in PFE **(PAPER III)**.

9. In contrast, GPX5 in fresh seminal plasma has not been validated as a freezability marker, despite preliminary results obtained from 2D-DIGE **(PAPER III)**.

7

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