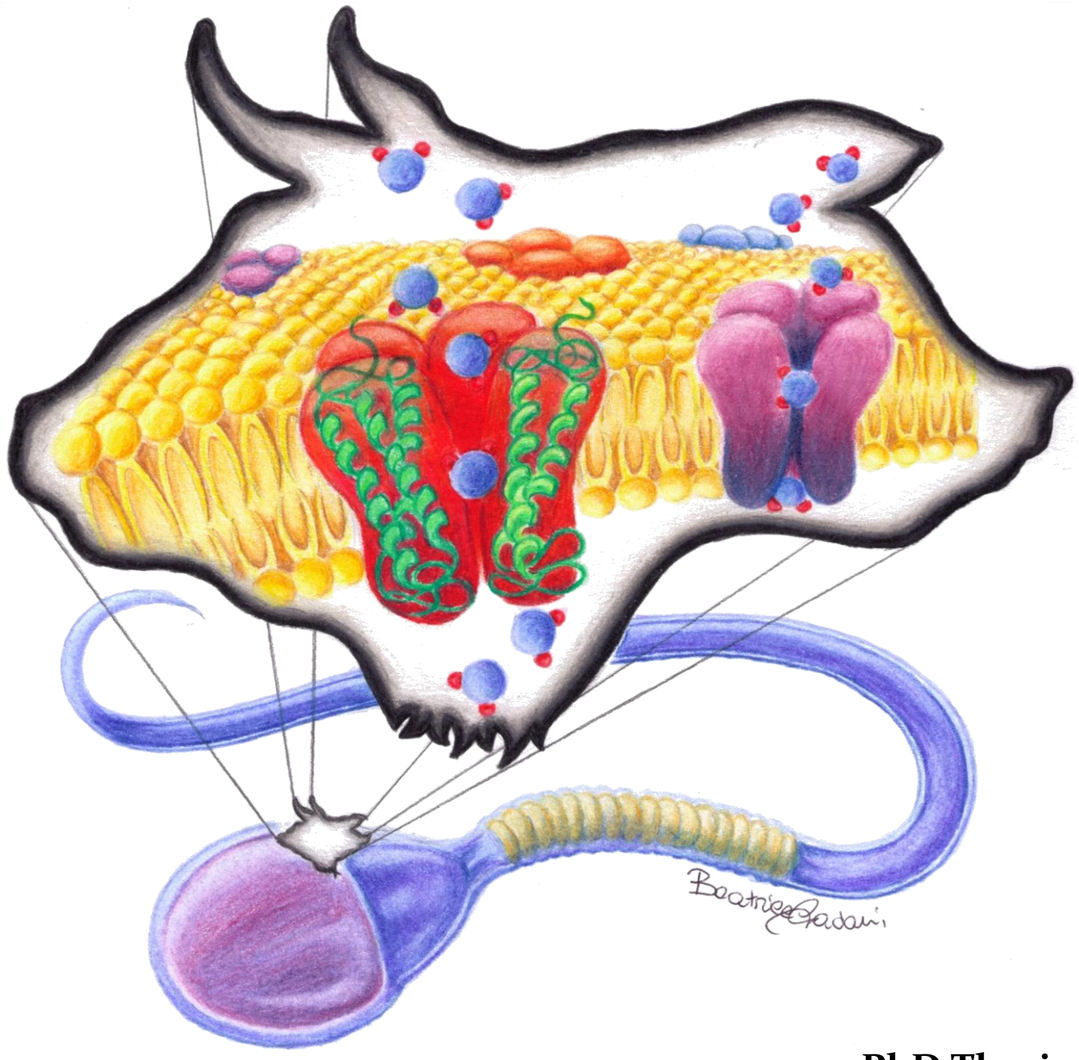
AQUAPORINS IN BOAR AND BULL SPERMATOZOA: IDENTIFICATION AND FUNCTIONAL IMPLICATIONS

Noelia Prieto Martínez

Universitat de Girona



PhD Thesis

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Doctoral Programme in Technology

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CERTIFIQUEM:

Que la tesi titulada **"Aquaporins in boar and bull spermatozoa: identification and functional implications"**, presentada per Noelia Prieto Martínez per optar al grau de Doctor per la Universitat de Girona, s'ha realitzat sota la nostra direcció i, considerant-la acabada i complint els requisits per poder optar a la Menció Internacional, autoritzem la seva presentació perquè sigui jutjada per la Comissió corresponent.

I, perquè així consti als efectes oportuns, signem aquest document.

Dr. Marc Yeste Oliveras Dr. Sergi Bonet Marull

Girona, 25 de novembre del 2016.

A tots els que m'heu acompanyat en aquest viatge i en especial,
a la meva família i al meu amor

Tutti i nostri sogni possono diventare realtà se abbiamo il coraggio di perseguirli

–Walt Disney--

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List of papers

This thesis is presented as compendium of five publications:

PAPER I

Prieto-Martínez N, Vilagran I, Morató R, Rodríguez-Gil JE, Yeste M & Bonet S. (2016) **Aquaporins 7 and 11 in boar spermatozoa: detection, localisation and relationship with sperm quality**. *Reproduction Fertility and Development* 28, 663-672.

PAPER II

Prieto-Martínez N, Morató R, Vilagran I, Rodríguez-Gil JE, Bonet S & Yeste M. (2015) **Aquaporins in boar spermatozoa. Part II: detection and localisation of Aquaglyceroporin 3**. *Reproduction Fertility and Development* (doi: 10.1071/RD15164).

PAPER III

Prieto-Martínez N, Vilagran I, Morató R, Rodríguez-Gil JE, Bonet S & Yeste M. (2016) **Relationship of aquaporins 3 (AQP3), 7 (AQP7) and 11 (AQP11) with boar sperm resilience to withstand freeze-thawing procedures**. *Submitted to Andrology*.

PAPER IV

Prieto-Martínez N, Morató R, Muiño R, Hidalgo CO, Rodríguez-Gil JE, Bonet S & Yeste M. **(**2016) **Aquaglyceroporins 3 and 7 in bull spermatozoa: identification, localisation and their relationship with sperm cryotolerance**. *Reproduction Fertility and Development* (doi: 10.1071/RD16077).

PAPER V

Morató R, Prieto-Martínez N, Muiño R, Hidalgo CO, Rodríguez-Gil JE, Bonet S & Yeste M. **(**2016) **Aquaporin 11 is related to cryotolerance and fertilising ability of frozen-thawed bull sperm**. *Submitted to Scientific Reports.*

Abbreviations

Abbreviations

ACRBP	Acrosin-binding protein
AEF	Alanine-Glutamic acid-Phenylalanine
AFLP	Amplified fragment length polymorphism
AI	Artificial Insemination
AKAP3	α-kinase anchor protein 3
ALH	Amplitude of lateral head displacement
ANOVA	Analysis of variance
AQP	Aquaporin
ar/R	Aromatic/arginine
aSFP	Acidic seminal fluid protein
ATP	Adenosine triphosphate
BCF	Beat cross frequency
β-HEX	N-acetyl-β-hexosaminidase
BSA	Bovine serum albumin
BSPA1/A2	Bovine seminal plasma A1/A2
BTS	Beltsville thawing solution
CASA	Computer assisted sperm analysis
CatSper1	Cation channel of sperm 1
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHIP28	Channel-forming integral protein, 28 KDa
CLSM	Confocal laser scanning microscope
CPA	Cryoprotectant
CPEB	Cytoplasmatic polyadenylation element binding protein
cRNA	Complementary ribonucleic acid
DAPI	4', 6-diamino-2-phenilindole
DMA	Dimethylacetamide
DMSO	Dimethylsufoxide
DNA	Deoxyribonucleic acid
DRMD	Detergent-resistant membrane domains
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EG	Ethylene glycol
EGTA	Ehylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EU	European Union
EUROSTAT	Statistical office, European Union
EV	Electronic volume

FAEGAS	Spanish Federation of Associations of Select Livestock
FAO	Food and Agriculture Organization
FITC	Fluorescein isothiocyanate
FN1	Fibronectin 1
FT	Frozen-Thawed
GFE	Good freezability ejaculates
GIP	glpF-like intrinsic protein
GLP	Aquaglyceroporin
GOT	Glutamic oxaloacetic transaminase
GPT	Glutamate pyruvate transaminase
HAL	Halothane gene
HES	Hydroxyethyl starch
HGT	Horizontal Gene Transfer
HIP	Hybrid intrinsic protein
HRP	Horseradish peroxidase
HSP90AA1	Heat-shock protein 90 alpha family class A member 1
IgG	Immunoglobulin-G
IMF	Intramuscular fat
INLAC	Dairy Interbranch Organization
ISAC	International Society for Advancement of Cytometry
IVF	In vitro fertilisation
LEY	Lactose-egg yolk
LEYGO	Lactose-egg yolk-glycerol-Orvus ES Paste®
LIN	Percentage of linearity
MAGRAMA	Ministry of Agriculture, Food and Environment
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MIP	Major intrinsic protein
mRNA	Messenger ribonucleic acid
M540	Meriocianine-540
NIP	NOD26-like intrinsic protein
NOD26	Nodulin26
NPA	Asparagine-Proline-Alanine
NPC	Asparagine-Proline-Cysteine
ODF2	Outer dense fibre protein
P1/P2	Protamine 1 and 2
PAGE	Polyacrylamide gel electrophoresis
PAR-1	Protease-activated receptor 1
PBS	Phosphate buffered saline solution
PC	Phosphatidylcholine
PCA	Principal component analysis

PCOS	Polycystic ovary syndrome
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PFE	Poor freezability ejaculates
PG	Propylene glycol
PGDS	Lipocaline-like prostaglandin D synthase
PGK2	Phosphoglycerate kinase 2 gene
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PI	Phosphatidylinositol
PKC	Protein kinase C
PMOT	Percentage of progressive motile spermatozoa
PMSF	Phenylmethane sulfonyl fluoride
PMT	Photomultiplier tube
PNA	*Arachis hypogaea* (peanut) agglutinin
PR	Penetration rate
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene fluoride
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SIP	Small intrinsic protein
siRNA	Small interfering ribonucleic acid
SM	Sphingomyelin
SOD1	Superoxide dismutase 1
SS	Side scatter
STR	Percentage of straightness
SYBR®	Commercial name of nucleic acids synthetic dyes ($C_{32}H_{37}N_4S$)
TBS	Tris-buffered saline
TIP	Tonoplast intrinsic protein
TMH	Transmembrane helices
TMOT	Percentage of total motile spermatozoa
TNP2	Nuclear transition protein 2
TPI	Triosephosphate isomerase
TRIS	Hydroxymethyl aminomethane
TRIS-HCl	Hydroxymethyl aminomethane hydrochloride
Tween 20	Polysorbate 20
VAP	Average path velocity

VCL	Curvilinear velocity
VDAC2	Voltage-Dependent Anion Channel 2
V2R	Vasopressin type 2 receptor
VSL	Straight-linear velocity
WOB	Percentage of wobble coefficient
XIP	X Intrinsic Protein
YO-PRO®-1	Commercial name of a non-permeant nuclear dye ($C_{24}H_{29}I_2N_3O$)
ZP	Zona pellucida

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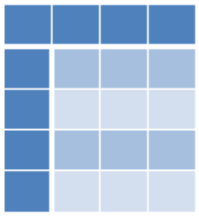

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Summary

Aquaporins (AQPs) are integral transmembrane, water-selective channels that enable high permeability fluxes of water and some other small solutes across plasma membranes. Mammalian spermatozoa are highly permeable to water, which is in agreement with the presence of these proteins. However, the presence, localisation and function on AQPs in mammalian sperm have been poorly studied, which contrasts with the deep knowledge and numerous studies on somatic cells. Against this background, the first objective of the present Dissertation was to identify three separate members of the AQP family, AQP3, AQP7 and AQP11 in the sperm of two major livestock species such as porcine and bovine by immunoblotting. The second objective was to evaluate the distribution and localisation of these proteins through immunocytochemistry and confocal laser microscopy. Finally, a third objective was to investigate the putative function of AQPs in ejaculated boar and bull spermatozoa, through evaluating different sperm functional parameters, cryopreservation procedures and fertilizing ability. Results from Western blot proved the presence of these three proteins in both livestock species. Regarding boar spermatozoa, blots showed the presence of one specific band for AQP3 (25 KDa), AQP7 (25 KDa) and AQP11 (50 KDa). In the case of bull spermatozoa, the same molecular weights were obtained, except for AQP3, where two specific bands were observed (30 and 60 kDa). With regard to the second aim, our immunocytochemical data indicated that these three proteins exhibited a homogeneous distribution and localisation in the ejaculated sperm of both species. In boars, two different AQP3-localisation patterns were identified: one was restricted to the mid-piece and the other involved the entire tail. In both cases, AQP3 was also present in the acrosomal region. In contrast, AQP3 was only found in the mid-piece of bull sperm. In relation to AQP7, the distribution of this protein was confined to the connecting-piece and partially to the mid-piece of boar sperm. In bull sperm, AQP7 was detected in both post-acrosomal region and mid-piece. Finally, AQP11 was found throughout the sperm head and tail in both species. Cryopreservation did not affect the localisation of these three AQPs, except for AQP7 in boars, which was mainly found at the sperm mid-piece and in the acrosomal region following freeze-thawing. Regarding the third objective, only relative AQP11-content was significantly and positively correlated with different sperm quality parameters in fresh boar spermatozoa. In relation with the possible

involvement of these three AQPs with sperm cryotolerance, relative AQP7-amounts in fresh semen were significantly higher in GFE than in PFE demonstrating that this protein is able to predict the ejaculate freezability in boar and bull sperm before cryopreservation procedures take place. In contrast, while AQP3 content was found to be involved in the cryotolerance of boar spermatozoa, AQP11 was only related to that of bull spermatozoa. Interestingly, bull fresh ejaculates with higher relative amounts of AQP11 also exhibited higher *in vitro* fertilizing ability at post-thawing. In conclusion, AQPs are present in boar and bull spermatozoa and play a crucial role in their ability to withstand freeze-thawing procedures. All these results contribute to increase our knowledge about the function of water channels on mammalian sperm physiology and cryopreservation and may also have practical applications for the selection of those ejaculates exhibiting higher sperm cryotolerance.

Resum

Les aquaporines són canals selectius transmembrana altament permeables i involucrats en el transport de molècules d'aigua i altres soluts a través de les membranes biològiques. L'espermatozoide madur de mamífer és una cèl·lula molt permeable a l'aigua fet que, en principi, fa pensar amb la presència d'aquestes proteïnes. Tanmateix, l'existència, localització i funció de les aquaporines en els espermatozoides de mamífer s'ha estudiat més aviat poc, la qual cosa contrasta amb l'elevat nombre d'estudis realitzats en cèl·lules somàtiques. Així doncs, el primer objectiu d'aquesta Tesi Doctoral va ser determinar la presència de les aquaporines AQP3, AQP7 i AQP11 en els espermatozoides de dues espècies d'interès productiu (porcina i bovina) mitjançant el mètode de Western blot. El segon objectiu va ser estudiar la distribució i localització d'aquestes tres proteïnes emprant mètodes immunocitoquímics i microscòpia òptica confocal de rastreig amb làser. Finalment, el tercer objectiu va ser establir la relació d'aquestes tres aquaporines amb la qualitat espermàtica i la criotolerància de les ejaculacions porcines i bovines. Els resultats de Western blot van demostrar la presència d'aquestes tres proteïnes en ambdues espècies animals. En el cas de l'espermatozoide porcí, es va identificar una única banda específica per a cadascuna de les aquaporines i amb un únic pes molecular, tal i com segueix: 25 KDa per l'AQP3 i l'AQP7 i 50 KDa per l'AQP11. Pel que fa a l'espermatozoide de toro, es van obtenir els mateixos resultats per l'AQP7 i l'AQP11, però no per l'AQP3 on es van identificar dues bandes específiques de 30 i 60 KDa. Quant al segon objectiu, les anàlisis immunocitoquímiques van mostrar una distribució i localització homogènies per les tres aquaporines en les ejaculacions d'ambdues espècies. En el cas de l'espermatozoide de porcí, es van observar dos patrons de distribució per l'AQP3: un restringit a la peça intermèdia i l'altre difús a tota la cua. En ambdós casos, també es va observar marcatge a la regió acrosòmica. En el cas de l'espermatozoide de toro, es va determinar que l'AQP3 es localitzava exclusivament a la peça intermèdia. Pel que fa a l'AQP7, es va trobar marcatge a la peça de connexió i, en menor mesura, a la peça intermèdia de l'espermatozoide porcí. En el cas de l'espermatozoide de toro, l'AQP7 es va localitzar a la peça intermèdia i a la regió post-acrosòmica. Finalment, es va determinar que l'AQP11 es trobava al cap i a la cua dels espermatozoide d'ambdues espècies. En aquesta Tesi també es van determinar els canvis de localització d'aquestes tres proteïnes

durant la criopreservació. No es van observar canvis substancials, llevat del cas de l'AQP7 a l'espermatozoide de porcí on s'observava un marcatge més intens a la peça intermèdia i a la regió acrosòmica després de la descongelació. Respecte al darrer objectiu, es va veure que el contingut relatiu d'AQP11, però no el de les altres dues aquaporines, estava correlacionat positivament i significativa amb diferents paràmetres de qualitat de l'esperma de porcí. D'altra banda, es va demostrar que la quantitat relativa d'AQP7 en els espermatozoides de porc i toro estava relacionada amb la congelabilitat de les ejaculacions, de tal manera que aquelles que, abans de la criopreservació, presentaven espermatozoides amb un major contingut relatiu d'AQP7 tenien una criotolerància superior. De manera similar, es va observar que l'AQP3 i l'AQP11 estaven relacionades amb la criotolerància dels espermatozoides de porcí i boví, respectivament. A més, les ejaculacions bovines fresques amb major quantitat relativa d'AQP11 no només van mostrar més tolerància a la criopreservació espermàtica sinó que també van exhibir una major capacitat fecundant *in vitro* després de la descongelació. Així doncs, es pot concloure que les aquaporines es troben als espermatozoides de porc i toro, on juguen un paper fonamental quant a la seva capacitat de resistència enfront als protocols de congelació i descongelació. Tots aquests resultats contribueixen a incrementar el nostre coneixement sobre el paper d'aquests canals d'aigua en la fisiologia i criopreservació dels espermatozoides, la qual cosa pot ser d'utilitat en la selecció de les ejaculacions de major congelabilitat.

Resumen

Las acuaporinas son canales selectivos transmembrana altamente permeables a las moléculas de agua y otros solutos, permitiendo su transporte a través de las membranas biológicas. El espermatozoide maduro de mamífero es una célula altamente permeable al agua, lo que hace pensar en la presencia de estas proteínas. No obstante, la existencia, localización y función de las acuaporinas en los espermatozoides de mamífero se ha estudiado más bien poco, lo que contrasta con el elevado número de estudios que se han realizado en células somáticas. Por ello, el primer objetivo de la presente Tesis Doctoral fue determinar la presencia de las acuaporinas AQP3, AQP7 y AQP11 en dos especies de interés productivo (el porcino y el vacuno) mediante el método de Western blot. El segundo objetivo fue estudiar la distribución y localización de estas tres acuaporinas mediante inmunocitoquímica y microscopia óptica láser confocal. Finalmente, el tercer objetivo fue establecer la relación de estas tres acuaporinas con la calidad espermática y la criotolerancia de los eyaculados de verraco y toro. Los resultados de Western blot probaron la presencia de estas tres proteínas en ambas especies animales. En el caso del espermatozoide porcino, las tres proteínas se presentaron como bandas específicas y únicas con unos pesos moleculares de 25 KDa en el caso de la AQP3 y la AQP7 y de 50 KDa en el caso de la AQP11. Para el espermatozoide de toro se obtuvieron resultados parecidos, exceptuando el caso de la AQP3 donde se identificaron dos bandas específicas de 30 y 60 KDa. En relación con el segundo objetivo, los resultados de los análisis inmunocitoquímicos mostraron una distribución y localización homogéneas para las tres acuaporinas en los eyaculados de ambas especies. En el caso del cerdo, se detectaron dos patrones de distribución para la AQP3. En el primero de ellos, el marcaje quedaba restringido a la pieza intermedia y en el segundo se difundía a lo largo de la cola del espermatozoide. En ambos casos, también se observó marcaje en la región acrosómica. En cambio, se observó un único patrón en el caso del espermatozoide de toro, localizándose la AQP3 en la pieza intermedia. En cuanto a la AQP7, se observó que esta proteína estaba fundamentalmente localizada en la pieza de conexión y, en menor medida, en la pieza intermedia del espermatozoide porcino. En lo que al toro se refiere, la AQP7 fue detectada en la pieza intermedia y en la región post-acrosómica. Finalmente, se determinó que la AQP11 se encontraba en la cabeza y cola de los espermatozoides de ambas especies. Cabe destacar que en esta Tesis Doctoral también

se observó que la criopreservación espermática no alteraba los patrones de localización de las acuaporinas, excepto en el caso de la AQP7 en el espermatozoide porcino la cual fue hallada en la pieza intermedia y en la región acrosómica después de su descongelación. En relación al último objetivo, se determinó que el contenido relativo de la AQP11 en el espermatozoide de verraco estaba positiva y significativamente correlacionado con diferentes parámetros de calidad del semen refrigerado. En lo que concierne a la relación de estas tres acuaporinas con la criotolerancia, se halló que el contenido relativo de la AQP7 en el semen fresco de ambas especies era significativamente mayor en los eyaculados de buena (GFE) que en los de mala congelabilidad (PFE). Asimismo, se observó que la AQP3 y la AQP11 estaban relacionadas con la criotolerancia de los espermatozoides de verraco y toro, respectivamente. Además, los eyaculados frescos de toro con mayor contenido de AQP11 no sólo mostraron una mejor tolerancia a la criopreservación sino que también exhibieron un mayor poder fecundante *in vitro* después de la descongelación. Así pues, se puede concluir que las acuaporinas se encuentran en los espermatozoides de verraco y toro, donde juegan un papel fundamental en cuanto a su capacidad de resistencia frente a los protocolos de congelación y descongelación. Todos estos resultados contribuyen a incrementar nuestro conocimiento acerca del papel de estos canales de agua en la fisiología y criopreservación de los espermatozoides, pudiendo tener así implicaciones prácticas en la selección de los eyaculados con mejor congelabilidad.

Riassunto

Le acquaporine (AQP) sono proteine canale transmembranarie selettive per le molecole d'acqua, che permettono un flusso veloce di quest'ultime e di altri piccoli soluti attraverso le membrane plasmatiche. Gli spermatozoi dei mammiferi sono altamente permeabili all'acqua proprio per la presenza di queste proteine. Nonostante ciò, la presenza, la localizzazione e la funzione delle AQP negli spermatozoi dei mammiferi è ancora un tema poco studiato, a differenza della profonda conoscenza e dei numerosi studi in merito alle cellule somatiche. Proprio per questo motivo, il primo obiettivo della presente tesi di dottorato è stata l'identificazione di tre membri distinti appartenenti a tale famiglia di proteine, ossia AQP3, AQP7 ed AQP11, in due specie di alto interesse produttivo quali il maiale ed il toro, attraverso la tecnica di *immunoblotting*. Il secondo obiettivo preposto è stata la successiva valutazione della loro distribuzione e localizzazione nella cellula spermatica attraverso l'utilizzo delle tecniche di immunocitochimica e di microscopia laser confocale. Terzo ed ultimo obiettivo è stato quello di studiare quale fosse il possibile ruolo di AQP3, AQP7 e AQP11 negli spermatozoi di maiale e toro attraverso la valutazione di diversi parametri di qualità spermatica, del processo di crioconservazione e della capacità fecondante del seme. I risultati di *western blot* hanno dimostrato la presenza di queste tre acquaporine in entrambe le specie animali. Nello spermatozoo di maiale, i dati hanno mostrato la presenza di una banda specifica per AQP3 (massa molecolare pari a 25 KDa), AQP7 (25 KDa) ed AQP11 (50 KDa). Nel caso del toro, sono state identificate le medesime masse molecolari, ad eccezione di AQP3 che ha invece presentato due bande specifiche pari a 30 e 60 KDa. In relazione al secondo obiettivo, i nostri dati d'immunocitochimica hanno indicato che queste tre AQP hanno una distribuzione ed una localizzazione omogenea nello sperma di entrambe le specie. Nel suino, per AQP3 sono stati identificati due diversi pattern di localizzazione: nel tratto intermedio e in tutta la lunghezza della coda degli spermatozoi; in entrambi i casi, AQP3 era anche presente nella regione acrosomiale. Al contrario, nel caso del toro, AQP3 è stata trovata solo nel tratto intermedio degli spermatozoi. Nel maiale la distribuzione di AQP7 è limitata al collo e solo parzialmente al tratto intermedio; nel toro, è stata invece localizzata nella regione post-acrosomiale e nel tratto intermedio. Per quanto riguarda AQP11 la distribuzione si è dimostrata essere a livello di testa e coda della cellula spermatica, ciò

in entrambe le specie studiate. È importante sottolineare che la crioconservazione del seme non ha mutato la localizzazione di queste proteine, ad eccezione della AQP7 che nel maiale, dopo il processo di congelamento-scongelamento, si è identificata nel tratto intermedio e nella regione acrosomiale. In merito al terzo obiettivo, solo il contenuto relativo di AQP11 è stato significativamente e positivamente correlato ai diversi parametri di qualità spermatica sul seme fresco di maiale. Per quanto riguarda la possibile correlazione di queste tre acquaporine con la criotolleranza, il contenuto relativo di AQP7 nel seme fresco di maiale e di toro è stato significativamente maggiore i quegli eiaculati aventi una maggiore criotolleranzza (GFE) rispetto a quelli in cui quest'ultima era inferiore (PFE), dimostrando che AQP7 è in grado di predire la congelabilità del seme prima del congelamento in entrambe le specie. Al contrario, il contenuto di AQP3 è si è mostrato correlato alla criotolleranza solamente nel maiale, e l'AQP11 unicamente nel toro. È interessante notare che gli eiaculati freschi di toro con una maggiore quantità relativa di AQP11 hanno evidenziato, allo scongelamento, una capacità fecondante *in vitro* più alta. Tutti questi risultati possono contribuire ad aumentare le nostre conoscenze sul ruolo dei canali acqua-selettivi nella fisiologia della cellula spermatica dei mammiferi e nella sua crioconservazione e ciò potrebbe avere anche applicazioni pratiche per quanto riguarda la scelta di eiaculati con una maggiore criotolleranza nel processo di crioconservazione del seme.

Thesis Outline

The present Thesis dissertation is divided into three sections and a final part for concluding remarks. It also provides a summary and its translation into Catalan, Spanish and Italian.

The **first section** is a general introduction about the current knowledge of aquaporins (AQPs) and also includes global overviews on pig, cattle and spermatology. The objectives of this Thesis are also stated at the end of this first section.

The **second section** contains the main contributions and specifically describes the results obtained. This section consists of five papers and all are organized in a similar way. A specific introduction covering the research related to the aims; the materials and methods; the results obtained; a specific discussion together with suggestions for future investigations; and the conclusions of the study.

- **Paper I** and **Paper II** were aimed at investigating the presence and localisation of three AQPs (AQP3, AQP7 and AQP11) in boar spermatozoa and their potential relationship between these three water channels and sperm quality in boar extended semen.

- **Paper III** was conducted to address whether the relative content of AQP3, AQP7 and AQP11 in boar spermatozoa differed between good (GFE) and poor (PFE) freezability ejaculates, and whether freeze-thawing procedures induced any change in the localisation of these three AQPs.

- **Paper IV** and **Paper V** were performed to identify and determine the precise localisation of AQP3, AQP7 and AQP11 in fresh and frozen-thawed bull spermatozoa and to evaluate the relationship between the relative abundance of these proteins and bull sperm cryotolerance. In addition, the relationship between relative AQP11-content in fresh bull semen and *in vitro* fertilization (IVF) outcomes of frozen-thawed sperm was also investigated.

The **third section** is a general discussion that deals with the results obtained in these five papers and suggests different scenarios for further research.

The last part of this Thesis dissertation, the **concluding remarks**, is a summary of the most important findings from the five papers of this Thesis.

Introduction

Introduction

1. Porcine and bovine market and the importance of reproductive biotechnologies in these sectors

The pig is a very important agro-economically mammal with a worldwide distribution. Consumers interest for pork meat in their daily meals is increasing. This makes the pig one of the main food resources for humans around the world (reviewed by Knox, 2014). Pigs have adapted to survive in a great diversity of environments. In addition, their efficiency has been related to their high fertility, short interval to maturity, short gestation period (about 113 days), high litter size and quick tendency to rebreed (reviewed by Knox, 2014). All these features make this animal as one of the most efficient livestock species for global food production.

Worldwide, the EU is the second largest producer of pork (20%) after China (50%). Spain is the fourth producer after China, USA and Germany (European Statistics; EUROSTAT, 2015; Ministry of Agriculture, Food and Environment; MAGRAMA, 2015). According to figures released for 2015, the pig sector in Spain represented 14% of the final agricultural production and 37% of the final livestock production. As shown in **Figure 1**, Catalonia was the main producing region, accounting for 42.29% of the total pig production in Spain, followed by Castile and Leon (13.9%).

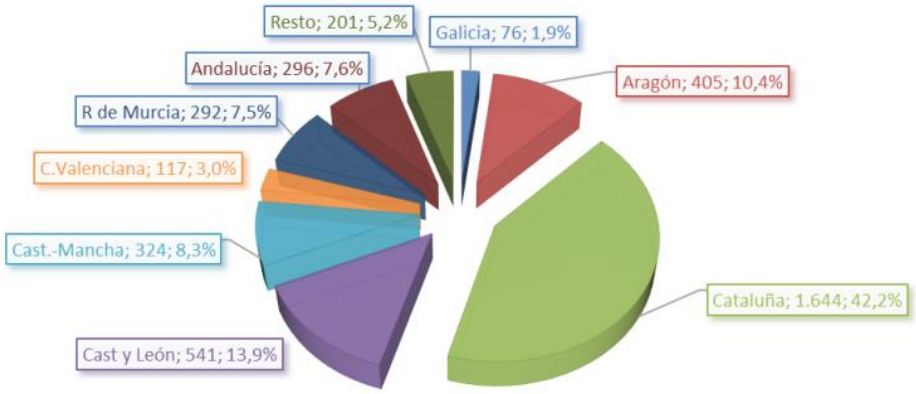

Figure 1. Regional distribution of pork production in Spain in 2015. Source: MAGRAMA, 2015.

Besides the importance of pig for agricultural purposes, it is also important as a model for biomedical studies. Taking into account that humans and pigs share many

similarities, the use of pig organs for therapeutic applications and xenotransplantation is an issue of great interest that merits further research (reviewed by Nieman & Rath, 2001 and Flisiokowska *et al*. 2013).

In Spain, bovine production has a dual purpose. For this reason, there are two production systems involving different breeds for beef and milk production. Regarding meat, and compared to the pig, beef has a longer production cycle, lower feed efficiency (conversion index) and higher value of individual animals. For this reason, prices in the beef sector are higher than those for the pig, which in turn explains the consumer preference for pork meat. In addition, all these reasons also make dairy animals more profitable than beef cattle (EUROSTAT, 2015). According to MAGRAMA, the beef sector in 2015 represented 6% of the final agricultural production in Spain. In terms of Spanish livestock production, beef cattle ranks at the fourth place after pig, dairy cattle and poultry, representing 16.49% of the final livestock production. When comparing the European countries, Spain ranked at the fifth place in beef cattle production in 2015, after France, Germany, United Kingdom and Ireland. By regions, the most important ones were Castile and Leon (21% of Spanish production) followed by Galicia (15%), Extremadura (13%), Catalonia (11%) and Andalusia (8%) (MAGRAMA, 2015; **Figure 2**).

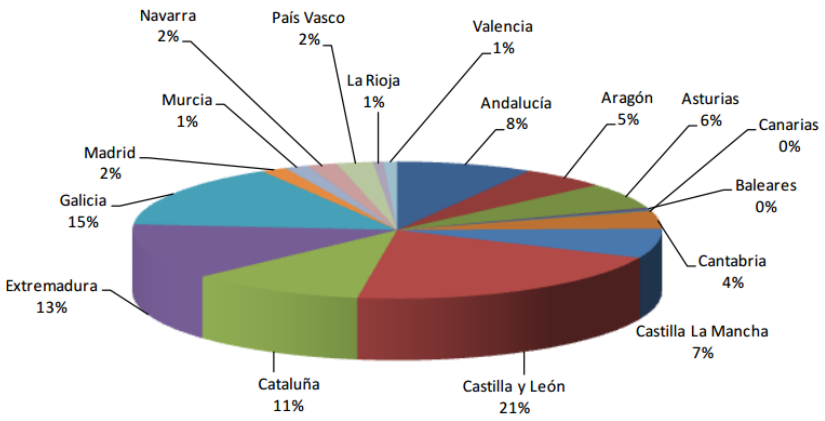

Figure 2. The distribution of the production of bovine in Spain by autonomous communities in 2015. Source: MAGRAMA, 2015.

On the other hand, most of the milk produced in Spain is from bovine origin. Concretely, 89.75% of the total milk produced in Spain during 2015 was from bovine origin, followed by that obtained from ovine (5.50%) and caprine species (4.75%; Dairy Interbranch Organization; INLAC, 2016). The dairy sector in Spain focuses on the production of milk, cheese, yoghurts, butter and other derivates. Following the quotas set by the European Commission (SIGLAC), 5,973,272 tonnes of milk were produced

in Spain between 1 April 2014 and 31 March 2015 (MAGRAMA, 2016). By regions, the major production was concentrated in the Cantabrian coast (56%), which comprises Galicia (38%), Asturias (10%) and Cantabria (8%), and in Castile and Leon (13%; MAGRAMA, 2016).

In the recent decades, the global livestock sector has changed rapidly in response to globalization and demand and consumption of animal-source food have much increased. Currently, livestock is one of the fastest-growing sectors in agriculture in developing countries (Thornton, 2010), as provides essential nutrients through meat, dairy and eggs as well as wool and leather (FAO, 2012). Related to this, reproductive biotechnologies in livestock have contributed to increase productivity, which can help to fight poverty and hunger, reduce the threats of diseases and ensure environmental sustainability in developing countries (FAO, 2010). The most applied biotechnology in the reproduction of farm animals is Artificial Insemination (AI) which in combination with sperm preservation enables the genetic improvement and dissemination of selected male germplasm (FAO, 2010). In effect, these technologies have been utilised in several species, particularly in dairy cattle in which the impact on genetic improvement and control of venereal diseases has been very high (reviewed by Foote, 2001). In other species, such as the pig, AI with fresh/extended semen is used in 95% of cases (reviewed by Riesenbeck, 2011 and Rodríguez-Gil & Estrada, 2013). Due to their high sensitivity to cold shock, the use of cryopreserved sperm in pigs is less often than in horses and cattle (reviewed by Curry, 2000 and Bailey *et al*. 2008). A main concern is the fact that pregnancy rates and litter sizes are reduced with cryopreserved boar sperm (reviewed by Knox, 2015). This topic has also gained further knowledge from the prediction of boar sperm freezability, another matter of concern that also contributes to explain the low use of frozen-thawed boar sperm for breeding purposes (see Section 5.1).

1.1 Piétrain pig breed

This swine breed was originated in Belgium, specifically at a town called Brabant. In morphologic terms, the breed is of medium size, white colour with black spots, robust and long body, and erected ears. Moreover, the extremities are shorter than in other breeds (MAGRAMA, 2011; **Figure 3**).

Figure 3. Phenotype characteristics of Piétrain boar. Source: FAO, 2012.

Piétrain breed is extremely heavy muscled and their meat carries a high proportion of lean to fat, which makes Piétrain males to be used as terminal boars. However, the breed presents a low growth and requires a high level of feeding to increase its weight (i.e. low conversion index; Spanish Federation of Associations of Select Livestock; FAEGAS, 2010). Moreover, Piétrain breed is characterized for low prolificacy rates in females (few piglets per sow and year), bad nursery performance and poor milk production (Quiniou *et al*. 2007).

On the other hand, it is worth mentioning that some Piétrain individuals carry a gene mutation which affects the quality of meat. Intramuscular fat (IMF) content in Piétrain breed is associated with a mutation (HAL^{n}) in the halothane genotype (HAL^{N}), which presents a recessive pattern. More than 80% of total Piétrain breed population are homozygous recessive ($HAL^{n/n}$) and this genotype is related to low quality meat, with features such as pale, soft and exudative (PSE) (Alves *et al*. 2014). This determines the use of Piétrain individuals for producing fresh meat like ham, sausages or mortadella rather than higher quality products such as cured ham, bacon or pork loin (Alves *et al*. 2014). For these reasons, the use of Piétrain purebred in pig production is relatively rare. Instead, this breed is more commonly used in crossbreeding programs with sows from other breeds as this improves carcass quality (Rybarczyk *et al*. 2011).

1.2 Holstein bovine breed

Holstein is a bovine breed originating from separate regions, North Holland, Friesland, Germany and Netherlands. Two breeds of cattle, black animals from Germany and white animals from Holland were crossed to create a new breed of cattle. This

crossbreed consists of large-size animals of white colour with black or red spotted markings (Holstein Association USA, Inc, 2016; **Figure 4**).

Figure 4. Phenotype characteristics of Holstein Bull. Source: Holstein Association USA, Inc, 2016.

Holstein cattle are known as the world's highest-production dairy animals. The breed is characterized by good durability, high productivity and high fat and protein levels in milk, making it a cost-effective and highly profitable livestock in farms around the world (Holstein Association USA, Inc, 2016). Moreover, the breed produces vigorous calves, distinguished by rapid growth, early maturity and easy care. Apart from this, these animals also contribute to the meat supply worldwide. Related with this, the Holstein breed produces a high quality beef with less external fat in comparison with other breeds such as Airshires, Brown Swiss, Guernseys and Jerseys (The SA Holstein Society, 2016).

2. Generalities of reproduction

2.1 Overview

There is a widespread variation in gamete structure, particularly on male gametes. Within this context, spermatozoa show a pronounced degree of variation both in shape and size among mammals as a result of evolution. Nevertheless, they all share a common structure and function (i.e. to fertilize ova) (reviewed by Gomendio & Roldan, 2008). Generally speaking, two major parts are distinguished in the mammalian ejaculated spermatozoon. The first comprises the head that carries the male genome (haploid nucleus) and the mechanisms for sperm-oocyte recognition, interaction and fusion (acrosome). The second includes the tail which contains the mitochondrial piece

and is involved in the intrinsic sperm motility following activation and hyperactivation (Briz & Fàbrega, 2013).

2.2 General structure of mammalian spermatozoa

The sperm head comprises the acrosome and the nucleus being surrounded by the plasma membrane (reviewed by Pesch & Bergmann, 2006).

The acrosome looks like a cap enveloping the anterior part of sperm head. It is a secretory vesicle that contains mucopolisaccharides, proteins, lipids and hydrolytic enzymes (hyaluronidase and acrosin) to digest the oocyte covers (Zona Pellucida; ZP) and possibly cumulus cells during fertilization. Although the acrosomal membrane is a continuous vesicle, the upper surface is called the outer acrosomal membrane and the lower surface facing the nucleus is called the inner acrosomal membrane (Briz & Fàbrega, 2013). In addition, the acrosome is divided into four morphological segments corresponding to three plasma membrane domains of the acrosomal region: apical, pre-equatorial and equatorial (Briz & Fàbrega, 2013). While the two first regions are exocytosed when spermatozoa pass through the cumulus cells or interact with the zona pellucida, the equatorial region is retained after acrosomal exocytosis (Manandhar & Toshimori, 2001).

The haploid nucleus constitutes the major part of the sperm head and contains the highly compact, hypercondensed chromatin with the transcriptionally inactive genetic information (reviewed by Pesch & Bergmann, 2006). The enormous compaction of sperm chromatin is due to the partially replacement of histones by protamines. These proteins are capable of forming stable disulphide bounds (S-S) that protect the sperm chromatin from damage. After fertilization, protamines are reduced by the reduced glutathione present in the oocyte cytoplasm, which reduces disulphide bonds (S-S) to sulfhydryl groups (-SH) allowing sperm head to decondense (Manandhar & Sutovsky, 2007).

The connecting piece or neck of the spermatozoon is a short linking segment located between the base of the head and the first mitochondrion of the mid-piece that plays a crucial role upon sperm-oocyte fusion as it harbors the proximal centriole (Briz & Fàbrega, 2013).

The tail is the longest part and impulses the spermatozoon with a helicoidal forward movement through the uterus and oviduct until meets and penetrates the oocyte (Manandhar & Sutovsky, 2007). It has a filamentous and cylindrical shape and can be

subdivided into three pieces: the mid-piece, presenting an axonematic structure covered with a mitochondrial sheath (diverse mitochondria helicoidally disposed around the axonema) that plays a major role during sperm capacitation; the principal piece, presenting an axonematic structure covered with a fibrous sheath; and the terminal piece, having a simple axonematic structure enclosed by the plasmalemma (Briz & Fàbrega, 2013). The mid-piece is connected with the principal piece by the Jensen's ring, a circumference of packed filamentous subunits attaching the two structures that avoids displacements of the mitochondrial sheath (Guan *et al.* 2009; Briz & Fàbrega 2013; **Figure 5**).

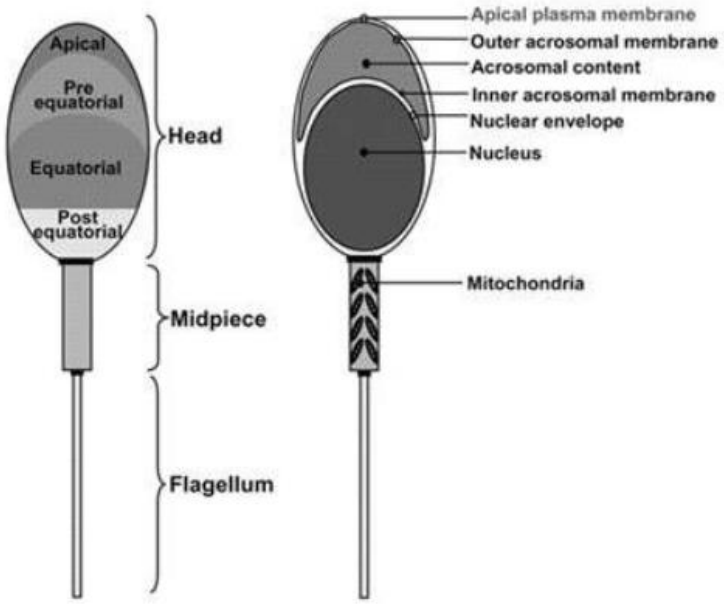

Figure 5. General structure of mammalian spermatozoa. Source: Modified from Brewis & Gadella, 2009.

2.3 Morphological differences between species

The principal mission of a mammalian sperm cell is the transmission of paternal genome to the next generation. To accomplish this goal, these cells are very specialized and have an accurate cellular design that is related with the evolutionary reproductive strategy of each species (Rodríguez-Gil, 2013). Despite spermatozoa showing a remarkable uniformity in their structure across mammalian species, there is a wide variation. Concretely, differences between species exist in the lengths and widths of spermatozoa and in the shapes of sperm heads (reviewed by Yániz *et al.* 2015). **Table 1** summarizes the dimensions of sperm from different species.

Table 1. Differences in sperm dimensions between different species (Pesch & Bergmann, 2006).

Species	Length (µm)	Head (µm)			Mid-piece (µm)		Principal piece (µm)	
		Length	Width	Shape	Length	Width	Length	Width
Man	50-60	3.4-4.6	1.5-2.8	Paddle shaped	3.5-5	0.6-0.8	44-50	0.4-0.5
Stallion	60	5	2.4	Paddle shaped	8	0.5	30	0.49
Boar	50-60	8.5	4.25	Paddle shaped	10	*	30	*
Bull	75-90	9.15	4.25	Paddle shaped	14.84	0.67	50	0.51
Sheep ram	70-80	8.2	4.25	Paddle shaped	14	0.8	45	0.5

Apart from these morphological differences, the composition of their plasma membrane and their resilience to withstand cryopreservation procedures are also diverse (See Section 5.1).

3. Cell membranes

3.1 Composition and structure of cellular membranes

Nowadays, the consensual model to describe the plasma membrane structure and function is called the fluid mosaic model. This theory was formulated by Singer and Nicolson in the 1970s (Singer & Nicolson, 1972; reviewed by Lombard, 2014). According to this model, mammalian cell membranes consist of a lipid bilayer composed primarily of phospholipids and cholesterol. The structure also contains integral and peripheral proteins embedded into the lipid bilayer and carbohydrates as minority compounds attached to either lipids (glycolipids) or proteins (glycoproteins) on the outer layer of the plasma membrane (Cooper, 2000; **Figure 6**). The 'mosaic' term refers to the mixture of lipids and proteins in the membrane and the 'fluid' term corresponds to the capacity of the movement of the components across this structure (reviewed by Lombard, 2014).

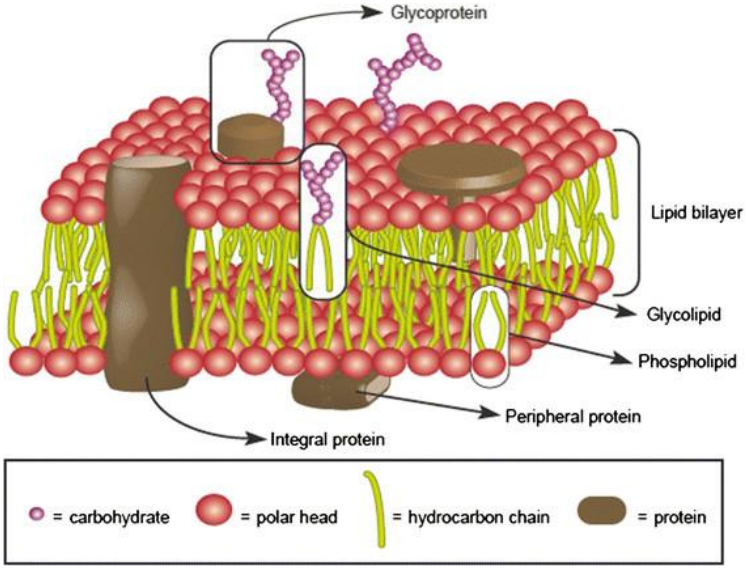

Figure 6. Schematic diagram of biological membranes structure. Source: Lombard, 2014.

The four major phospholipids are phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE) and sphingomyelin (SM). Their distribution is asymmetric. While the outer side of the plasma membrane mainly contains PC and SM, PE and PS are present in the inner layer. In addition, a fifth phospholipid called phosphatidylinositol (PI) is localized in the inner plasma membrane in minor quantities and plays a crucial in transduction signal pathways involving G-coupled proteins (Cooper, 2000; **Figure 7**).

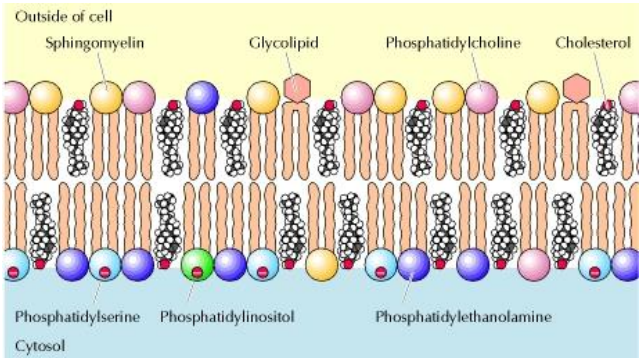

Figure 7. General structure of an animal cell membrane. Source: Cooper, 2000.

The interior of lipid bilayer is occupied by hydrophobic fatty acid chains. There are responsible for membrane impermeability to water-soluble molecules. Fatty acids may be saturated or unsaturated (with one or more double bounds). Unsaturated chains introduce kinks into the hydrocarbon structure which makes them difficult to pack into a regular structure. This strategy allows plasma membrane to remain fluid at low

temperatures (Alberts *et al*. 2002). With regard to cholesterol, this molecule plays an important role in determining membrane fluidity. It presents a hydrocarbon ring structure which interacts with fatty acid chains of phospholipids decreasing their mobility, which in turn makes this structure more rigid (Cooper, 2000). These features are of great importance in certain processes, such as sperm cryopreservation in which the fluidity of membrane at low temperatures determine their sensitiveness to freezing and thawing (See Section 5.1).

It has been observed that lipid molecules mix randomly across the lipid monolayer. The attractive forces between tails of neighbour fatty acids hold the adjacent molecules together in small micro domains known as lipid rafts. These domains are rich in sphingolipids, cholesterol and proteins (Alberts *et al*. 2002; **Figure 8**). Since lipid rafts in gametes contain proteins that regulate intracellular functions and cell signalling, these domains are important for sperm maturation, fertilization, and early embryogenesis (reviewed by Kawano *et al*. 2011).

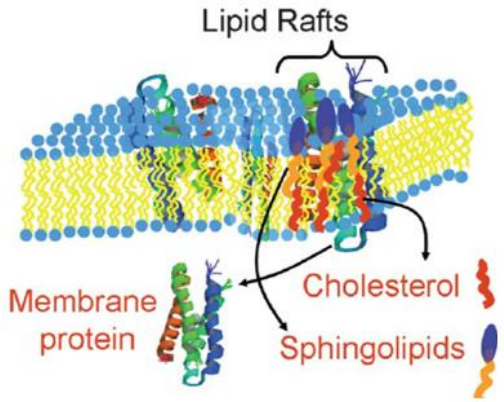

Figure 8. Lipid raft structure. Source: Kulkarni, 2012.

On the other hand, the protein fraction is the other major constituent of cell membranes. These proteins are involved in cell signalling and in the active and passive transports of ion/solutes (See Section 3.2). Finally, despite being a minority, carbohydrates also play an important paper in the cell recognition process (Alberts *et al*. 2002).

3.2 Cellular transport

The plasma membrane is a semi-permeable barrier between the cell and the extracellular environment (Lodish *et al*. 2000). In short, the semi-permeable nature of cell membrane determines which molecules can enter the cell. Small hydrophobic molecules and gases

such as carbon dioxide and oxygen cross membranes rapidly without any assistance. On the other hand, small polar molecules such as water and ethanol can also traverse membranes but at lower diffusion rates. Moreover, cell membranes limit the pass of charged molecules as ions and large molecules such as sugars or amino acids. The passage of these molecules requires a specific transport mediated by proteins (reviewed by Watson, 2015).

There are two mechanisms (passive and active) by which molecules can move across plasma membranes. The first mechanism, called passive transport, consists of the movement of substances across membranes in favour of their gradient of concentration and without requiring any energy (reviewed by Watson, 2015; **Figure 9**). There are two types of passive transport: simple and facilitated diffusion. Simple diffusion is a non-selective process that enables the transport of small hydrophobic molecules and gases that are able to freely diffuse across the phospholipid bilayer. In contrast, facilitated diffusion is mediated by transmembrane integral proteins as the transported ions and molecules, which are large, polar and charged, are not able to pass through the phospholipid bilayer. There are two classes of proteins involved in facilitated diffusion: carrier and channel proteins. Whereas carrier proteins allow the transport of specific molecules through conformational changes and are responsible for the transport of sugars, amino acids and nucleosides, channel proteins enable the transport of any molecule with appropriate size and charge, generally polar and charged substances, forming a hydrophilic passage (Cooper, 2000).

Active transport requires energy and occurs when substances are transported against concentration gradients (reviewed by Watson, 2015). There are three ways of driving active transport: coupled-carriers, ATP-driven pumps and light-driven pumps, which are mainly found in bacterial cells (Alberts *et al*. 2002).

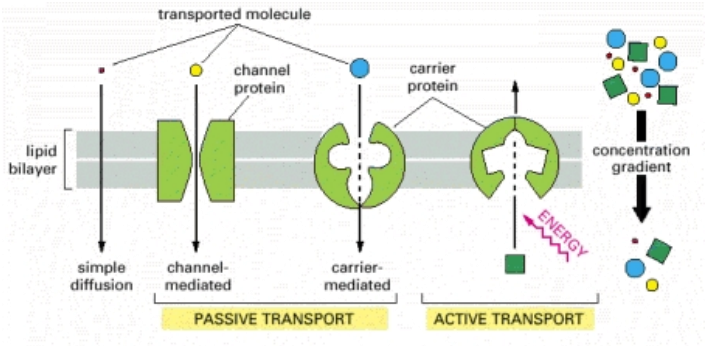

Figure 9. Diagram illustrating the main differences between active and passive transport

Source: Alberts *et al*. 2002.

4. Water transport and functional diversity of Aquaporins

4.1 The importance of water and their transport through lipid bilayer membranes

Water is the main component of cells and tissues and plays an essential role for all physiological and biochemical processes in live organisms (reviewed by Sales *et al.* 2013). Therefore, water can be described as the "solvent of life" since there is no life without water (reviewed by Agre, 2006). Cell survival requires the suitable concentration of water and solutes. It is crucial that the right substances enter cells (e.g. nutrients) and waste substances (e.g. toxins) are removed (reviewed by Watson, 2015). Biological membranes have an intrinsic water permeability that depends upon their lipid composition (reviewed by Törnroth-Horsefield *et al.* 2010). Due to the hydrophobic nature of lipid bilayer, water penetrates slowly this structure by simple diffusion (reviewed by Huang *et al.* 2006a and Matsuzaki *et al.* 2002). In fact, if water only passed through plasma membrane via simple diffusion, it would be hard to explain water permeability of some cells, such as red blood cells, renal tubular epithelial cells or gametes (reviewed by Huang *et al.* 2006a). For this reason, Sidel and Solomon (1957) early assumed that water should mainly flow through cell membranes by a passive transport mechanism other than simple diffusion **(Figure 10;** reviewed by Parisi *et al.* 2007). Nevertheless, it not was until the early nineties when Peter Agre and their colleagues discovered the Aquaporins (AQPs) (Preston *et al.* 1992).

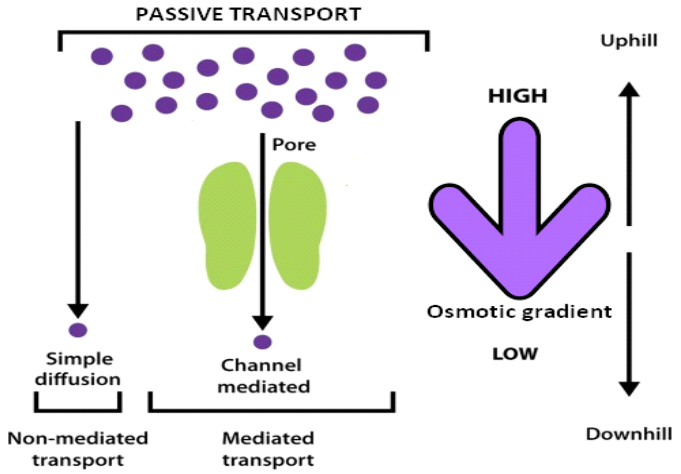

Figure 10. Schematic representation of the different types of passive transport. Source: modified from Watson, 2015.

4.2 Aquaporins: Water Channel Proteins

4.2.1 Discovery

The first water channel protein (Aquaporin) was discovered in 1992 by Professor Peter Agre **(Figure 11)**. The contribution of this researcher to the study of structural and functional properties of AQPs and their distribution across tissues made him to be awarded with the Nobel Prize in Chemistry in 2003. This award was shared with Professor Roderick MacKinnon, who focused his research upon the structure and function of ion channels, specifically those of potassium (K^+) (reviewed by Mackinnon, 2004).

Agre and his colleagues of Johns Hopkins University were trying to purify a 32-kDa protein related to the determination of Rh blood group when they found a polypeptide of lower weight (28 kDa) that was co-purified with the targeted protein. The structural analysis of this molecule indicated that it was an integral membrane protein with no relationship with the Rh protein. Interestingly, Agre and colleagues found that this polypeptide was identical to another one found in the kidney. The fact that red blood cells and kidney tubules are highly permeable to water led these researchers to suggest that this new protein of 28 kDa could be related with water transport (Preston *et al*. 1992).

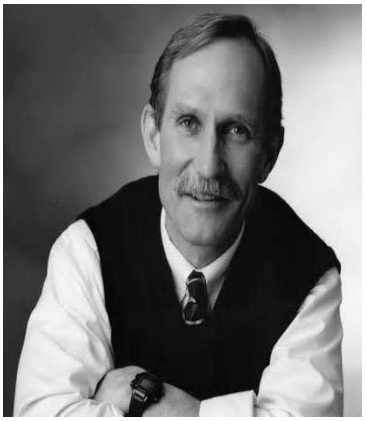

Figure 11. Prof Peter Agre. Source: Agre, 2004.

This hypothesis was later tested by Dr Gregory Preston, a Postdoctoral researcher working at Agre's Lab. Preston cloned the sequence of this new protein and microinjected a cRNA construct into *Xenopus laevis* oocytes which have very low water permeability. Control oocytes were injected with water alone. After microinjection, both control and treated oocytes were dropped into distilled water. While control oocytes fad

to swell due to their low water permeability, those microinjected with the cRNA encoding for the AQP exhibited higher water permeability and swollen and subsequently exploded when came into contact with distilled water **(Figure 12)**. These results confirmed the initial hypothesis and this protein, which was initially named as CHIP28 due to its molecular weight, was finally called AQP1 (Preston *et al*. 1992).

Figure 12. Discovery of AQP1. Functional demonstration of AQP1 as a water transporter in *Xenopus laevis* oocytes. Upper left: Control oocyte (injected with water) not expressing recombinant-AQP1 (left) and treated oocyte (injected with cRNA expressing AQP1 (right). Thirty seconds after coming into contact with distilled water, AQP1-injected oocytes began to swell by osmosis and they exploded after three minutes (bottom left image). Right: Postdoctoral Researcher Dr. Gregory Preston. Source: Agre, 2006.

4.2.2 Function

Aquaporins, from the Latin words *aqua*=water and *porus*=passage, are a family of highly conserved, integral transmembrane proteins that serve as selective water channels (Agre *et al*. 1993). This family of proteins belong to the superfamily of integral membrane channel proteins, known as major intrinsic proteins (MIP) (Marchler-Bauer *et al*. 2013). Aquaporins allow the passage of water and, in some cases, certain small uncharged solutes such as glycerol (Borgnia & Agre, 2001; Grayson *et al*. 2003), urea (Borgnia *et al*. 1999; Litman *et al*. 2009), ammonia (Saparov *et al*. 2007) and arsenite (Liu *et al*. 2002) across the membrane. This increases the transport rates of these molecules across the plasma membrane by 10-100 fold (reviewed by Agre *et al*. 2002). Aquaporins are considered as passive transporters, so that the driven force

for moving water molecules across channels is the osmotic gradient (reviewed by Perez Di Giorgio *et al.* 2014).

4.2.3 Classification and structure

Thus far, 13 members of the AQP family have been identified in mammalian cells (reviewed by Huang *et al.* 2006a). Although the presence of AQPs relies upon cell types and tissues, more than one isoform may be expressed in a given cell or tissue at the same time (reviewed by Matsuzaki *et al.* 2002 and Verkman, 2005; **Table 2)**.

Aquaporins are subdivided on the basis of their sequence similarity and substrate selectivity into three major groups (reviewed by Agre *et al.* 2002 and Ishibashi *et al.* 2009): orthodox AQPs, aquaglyceroporins (GLPs) and superaquaporins (reviewed by Sales *et al.* 2013). The first group is composed of seven members: AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8. They are water-selective channels, because they are permeable to water but not to small organic and inorganic ions/molecules (reviewed by Huang *et al.* 2006a). In spite of this, the anion conductance of AQP6 is involved in the transport chloride anion (Cl^-) at low pH (Yasui *et al.* 1999b).

The second group, also known as aquaglyceroporins, includes four members: AQP3, AQP7, AQP9 and AQP10, which are not only permeable to water but also to glycerol, urea and other small non-electrolytes (reviewed by Borgnia *et al.* 1999 and Agre *et al.* 2002). The basic difference between orthodox AQPs and GLPs is the pore size. In the case of orthodox AQPs, the pore measures about 2.8 Å whereas in GLPs measures 3.4 Å (reviewed by Sales *et al.* 2013).

Finally, the third group, also known as superaquaporins, includes AQP11 and AQP12, which present low homology to the other two groups (Murai-Hatano *et al.* 2008). They are permeable to water and AQP11 has been suggested to be a glycerol channel in human adipocytes (Madeira *et al.* 2014). Superaquaporins differ from orthodox AQPs and GLPs in the absence of the asparagine-proline-alanine (NPA) motif, as alanine is replaced by cysteine (NPC) (Gorelick *et al.* 2006). They are expressed inside the cell and they are concretely localized in the membrane of intracellular organelles rather than in the plasma membrane. Therefore, superAQPs are involved in the intracellular water transport and the regulation of organelle volume and intravesicular homeostasis (reviewed by Ishibashi, 2006; Nozaki *et al.* 2008 and Badaut *et al.* 2014). The derivate NPA boxes may cause this intracellular retention (reviewed by Ishibashi *et al.* 2009).

Table 2. Tissue distribution and roles of mammalian AQPs (Matsuzaki *et al*. 2002; Sales *et al.* 2013; Ricanek *et al.* 2015).

Aquaporin	Permeability characteristics	Localisation	Possible function
AQP0	Water (Low)	Lens fiber cell	Sustain lens transparency
AQP1	Water (High)	Erythrocyte Capillary endothelium Kidney proximal tubule, Henle's loop and vasa recta Corneal endothelium, iris and ciliary lens epithelia Epithelium of respiratory system Cholangiocyte	Water transport Water transport across vessel wall Urinary concentration Water movement in ocular tissues Osmoprotection Bile modification
AQP2	Water (High)	Kidney collecting duct	Urinary concentration
AQP3	Water (High) Glycerol (High) Urea (Moderate)	Kidney collecting duct Epithelium of urinary tract Epithelium of respiratory system Epithelium of digestive tract Epidermis of skin Corneal epithelium Conjunctival epithelium Meningeal cells	Urinary concertation Osmoprotection Osmoprotection Osmoprotection Osmoprotection Osmoprotection Osmoprotection Transfer of cerebrospinal fluid
AQP4	Water (High)	Kidney collecting duct Gastric parietal cell Epithelium of respiratory system Meningeal cell, ependymal cell Glial cell Retinal glia Skeletal muscle	Urinary concertation Gastric-acid secretion Osmoprotection Transfer of cerebrospinal fluid Regulation of brain volume Water movement in ocular tissues Muscle-volume regulation

AQP5	Water (High)	Salivary gland Lacrimal gland Sweat gland Alveolar type I pneumocyte Corneal epithelium	Salivary secretion Tear secretion Sweat secretion Water transfer between alveolar and air spaces Water movement in ocular tissues
AQP6	Water (Low)	Kidney collecting duct	Urinary concentration
AQP7	Water (High) Glycerol (High) Urea (High) Arsenite (High)	Kidney proximal tubule Adipose tissue	Urinary concentration Regulator of adipocyte metabolism/ Glycerol transport
AQP8	Water (High)	Kidney proximal tubule Epithelium of duodenum, jejunum and colon Bile canaliculus Pancreatic acinar cell Liver	Urinary concertation Water absorption and colonic fluid transport Bile secretion Secretion of pancreatic juice Unknown
AQP9	Water (High) Glycerol (High) Urea (High), Arsenite	Liver Leucocytes	Hepatocyte glycerol influx and urea efflux Regulation of leukocytes volume
AQP10	Water (Low) Glycerol (High) Urea (High)	Small intestine	Water absorption
AQP11	Water (High)	Kidney Liver	Water movement across intracellular membranes
AQP12	Water (High)	Pancreas	Water movement across intracellular membranes, digestive enzyme secretion and pancreas cell fluids

Structural studies of AQPs have revealed that they are assembled in the cell membrane as tetramers (reviewed by Agre, 2002; **Figure 13**). As each monomer in the tetramer has been demonstrated to be a functional unit (Preston *et al.* 1992; Shi *et al.* 1994), the tetrameric structure has been proposed to be necessary to stabilize the position of individual monomers (reviewed by Agre, 2006).

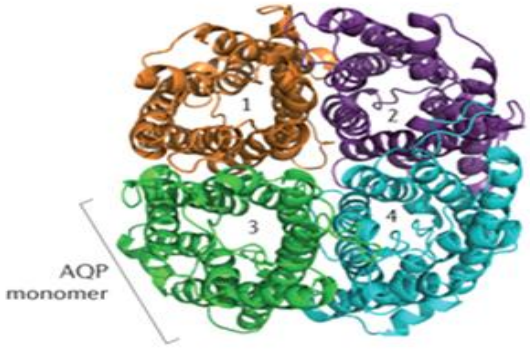

Figure 13. Tetrameric structure of AQPs with monomers labelled 1-4 (Verkman *et al.* 2014).

Each monomer consists of a single polypeptide chain of approximately 270 amino acids and spans the membrane six times with six hydrophobic, transmembrane α-helices (TMH1-6) (Maurel *et al.* 1993; Benga 2009). These helices are connected by three extracellular loops (loops A, C and E) and two intracellular loops (loops B and D) (reviewed by Fujiyoshi *et al.* 2002 and Kruse *et al.* 2006). Both the amino- and carboxyl-terminal ends are always located in the cytoplasm and show a significant sequence similarity suggesting an ancient gene duplication (Zardoya & Villalba, 2001; Quigley *et al* 2002). Loops B and E are hydrophobic and present a conserved domain composed by asparagine-proline-alanine (NPA) which is associated with substrate selectivity because play a critical function in charge and size obstacle (Zardoya & Villalba, 2001; Wallace & Roberts, 2004). Loops B and E overlap into the membrane forming a single transmembrane aqueous pathway (hourglass structural model). The center of the molecule is formed by oppositely juxtaposing two NPA motifs (reviewed by Echevarría & Zardoya, 2006). Both loops stay in touch through their proline residues and hydrophilic asparagine residues are vital for protein selectivity (reviewed by Agre & Kozono, 2003).

There is a second narrower pore constriction called aromatic/arginine (ar/R) which is composed of four residues, two from helices 2 (H2) and 5 (H5) and two from loop E (LE1 and the invariant R). This region is based on the presence of a conserved arginine residue in loop E and on the high prelevance of aromatic residues at H2. This constriction is involved in the rejection of large molecules and determines the rate of transport (Fu *et al.* 2000; Sui *et al.* 2001; Harries *et al.* 2004; Mitami-Ueno *et al.* 2011).

Finally, AQPs also have a third motif called AEF (Ala-Glu-Phe) that is located in TMH1. This motif is conserved in all family members but its function is still unknown (Zardoya & Villalba, 2001; Perez Di Giorgio *et al.* 2014; **Figure 14**).

The discrimination between water or glycerol molecules seems to be given by the P1-P5 motif (Froger *et al.* 1998). P1 is located in the terminal part of H3 and is occupied by a non-aromatic amino acid in the case of AQPs conducting water (also called orthodox AQPs) and by an aromatic residue in the case of GLPs. Moreover, P2 and P3 are located in loop E after the second NPA motif and are normally formed by a Ser-Ala pair in orthodox AQPs and by Asp-Arg or Asp-Lys residues in GLPs. All GLPs identified thus far contain an aspartic acid (Asp) residue at position P2. This contrasts with orthodox AQPs, which do not present aspartic acid at P2. In fact, the presence of aspartic acid at P2 expands the pore and makes it able to accept large molecules such as glycerol. This is the reason why GLPs but not orthodox AQPs are able to allow the passage of molecules other than water. Finally, P4 and P5 are located in H6. While they contain aromatic residues in orthodox AQPs, there is a proline followed by a non-aromatic residue in GLPs (Hub & de Groot, 2008).

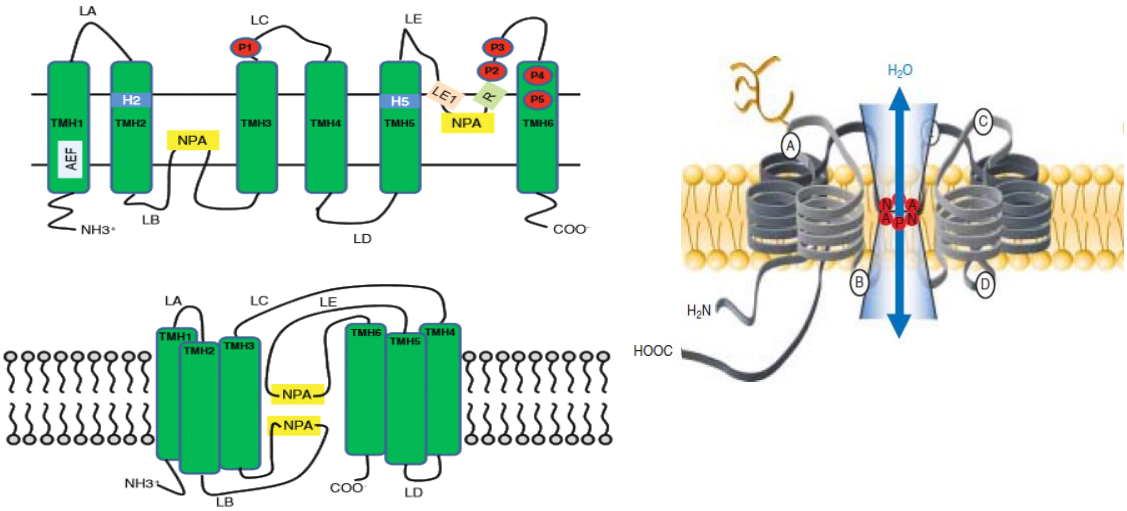

Figure 14. Left: Schematic representation of the classical structure of AQPs. An AQP monomer showing the six transmembrane helices (TMH1-6) connected by two intracellular (B and D) and three extracellular (A, C and E) loops. NPA motifs are shown in yellow, ar/R constriction are shown in blue, P1-P5 residue positions are shown in red and the AEF motif is shown in grey. Source: Perez Di Giorgio *et al.* 2014. Right: Hourglass structural model formed by the superposition of NPA motifs. Source: Echevarría & Zardoya, 2006.

4.2.4 Mechanism of water transport

A very fast water transport through AQPs is carried out from one side of the membrane to the other. The size of the pore is not large enough to accommodate more than a single water molecule along the channel (Tajkhorshid *et al.* 2002). Interestingly, NPA and ar/R regions have been proposed to exert a large influence on substrate specificity (Wallace & Roberts, 2004; Forrest & Bhave, 2007; **Figure 15**). In effect, the ar/R region, which is located at the extracellular side of the pore, serves as a selectivity filter for molecular transport acting as a size-exclusion barrier (Fu *et al.* 2000; Sui *et al.* 2001).

Within the channel, water molecules establish hydrogen bonds between them and with the amino acid residues constituting the walls of the channel (reviewed by Echevarría & Zardoya, 2006). Nevertheless, when the water molecules are getting closer to the narrowest part of the pore, the positive electrostatic charges of NPA motifs help to reorient the water molecules passing through the channel. This reorientation of water dipoles disrupts hydrogen-binding interactions between water molecules (reviewed by Forrest & Bhave, 2007). The oxygen atom forms a hydrogen bond with asparagine residues of NPA motifs. Concretely, water molecules break their hydrogen bonds between them and subsequently form two hydrogen bonds with Asn residues (reviewed by Echevarría & Zardoya, 2006).

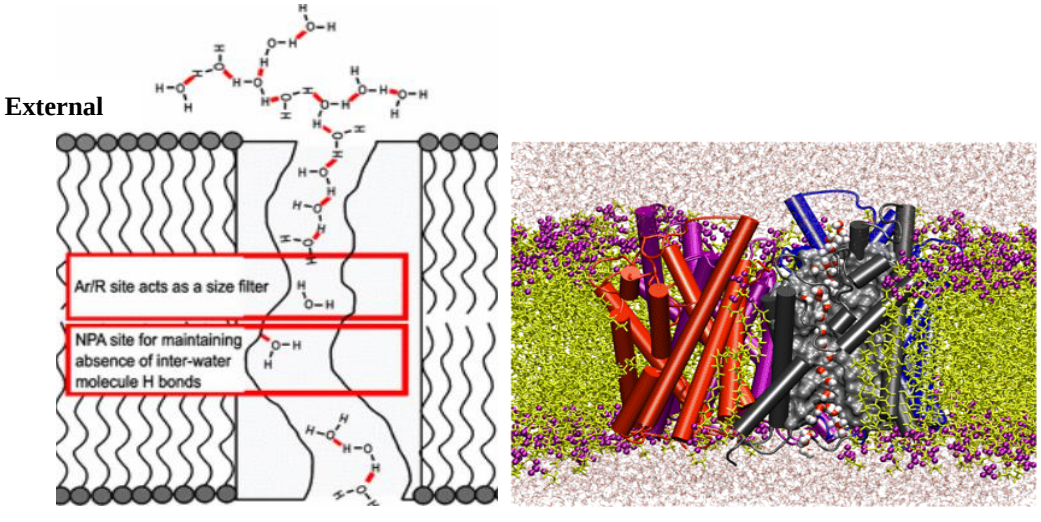

Figure 15. Left: Water transport mediated by AQPs in cell membranes. It should be noticed that NPA and ar/R motifs act as selectivity filters. Source: Forrest & Bhave, 2007. Right: A membrane-embedded model of an AQP tetramer. Water crosses the membrane through pores present in each AQP monomer following a strict single file. Source: Wang & Tajkhorshid, 2007.

4.2.5 Regulation

The study of how water transport through AQPs is regulated has received great attention from researchers due to its relevance for cell function (reviewed by Engel *et al*. 2000). Aquaporins are regulated post-translationally by phosphorylation of serine and threonine residues, which plays a vital role in channel-gating and trafficking of eukaryotic AQPs (reviewed by Törnroth-Horsefield *et al*. 2010). This phosphorylation is mediated by protein kinases activated through transduction pathways that involve G-coupled protein receptors. Moreover, changes in pH affect the AQPs conformation and modify their transport activity (Tournaire-Roux *et al.* 2003; Németh-Cahalan *et al.* 2004; Hedfalk *et al.* 2006). Finally, it should be noted that specific stimuli may lead AQPs to translocate and/or trigger up/down-regulation of AQP expression (reviewed by Gunnarson *et al.* 2004). Some examples about these regulation mechanisms are given below.

The expression of *AQP4*, which is found in astrocytes, is down-regulated by thrombin. Concretely, it has been hypothesised that thrombin binds to protease-activated receptor 1 (PAR-1), which is coupled to a G-protein that activates a PLC-mediated pathway. While the activation of this pathway seems to regulate *AQP4*-expression via MAPK/ERK, the exact mechanism remains unknown (Tang *et al*. 2007). On the other hand, a G-coupled receptor linked to adenylate cyclase regulates AQP5 by modulating its expression and by triggering its translocation to the plasma membrane (Yang *et al*. 2003).

The role of AQP2 in concentrating urine in kidneys is regulated by phosphorylation and translocation in response to vasopressin. In effect, vasopressin binds to vasopressin type-2 receptor (V2R), which is coupled to a G-protein that activates adenylate cyclase. The increase of cAMP levels activates protein kinase A (PKA), which phosphorylates Ser^{256} residue of AQP2. Such a phosphorylation ultimately results in redistribution of AQP2 from intracellular vesicles to plasma membrane (Bouley et al. 2006). AQP1 and AQP8 are also regulated by site-specific

phosphorylation. In both cases, phosphorylation induces the redistribution of these two cell AQPs to plasma membrane (Garcia *et al*. 2001; Conner *et al*. 2010).

Apart from recruiting AQPs to the plasma membrane, phosphorylation may also alter their permeability by inducing conformational changes. In effect, phosphorylation of Ser^{180} mediates AQP4-gating (Zelenina *et al*. 2002).

On the other hand, a few mammalian AQPs appear to be regulated by pH (reviewed by Engel *et al*. 2000). For example, AQP0 is exclusively found in the plasma membrane of vertebrate lens fiber cells, which are filled with crystalline proteins that maintain the transparency of the eye lens (reviewed by Törnroth-Horsefield *et al*. 2010). In this context, it is worth mentioning that specific mutations of AQP0 have been identified as a cause of cataracts (Francis *et al*. 2000; Geyer *et al*. 2006). AQP0 has a double function, as does not only regulate the water homeostasis of lens fibers but also forms tight-junctions between the membranes of lens fiber cells (Costello *et al.* 1989). The influence of pH on AQP0 is apparent from pH variations ranging between 6.5 and 7.5, as while AQP0 becomes activated at pH 6.5, this ability to transport water is reduced by three-fold at pH 7.5 (Németh-Cahalan *et al*. 2000; Németh-Cahalan *et al*. 2004). On the other hand, while AQP3, which is expressed in kidneys, airway epithelia and secretory glands, is permeable to both water and glycerol at neutral pH, the channel appears to close at pH< 6 (Zeuthen & Klaerke, 1999). Finally, AQP6, which resides in the acid-secreting cells of the renal collecting duct, undergoes a conformational change at pH< 5.5 through which the channel opens for selective permeation by water and chloride ions (Yasui *et al*. 1999a, 1999b).

Apart from this, regulation of AQPs is also related with their sensitivity to certain molecules. It is well stablished that heavy metals (mercury, cooper and nickel) can directly interact with AQPs thereby affecting their activity, as studies focused upon AQP1, AQP2 and AQP3 have demonstrated (Preston *et al.* 1993; Hasegawa *et al*. 1994; Zelenina *et al.* 2003; 2004). Indeed, mercurial compounds, such as $HgCl_2$, have been found to inhibit water transport in AQP1 and AQP2. In the case of AQP1, this inhibition effect occurs through Cys^{189} site, which is located close to the NPA motif in loop E (Preston *et al.* 1993; **Figure 16**). On the other hand, three amino acid residues (Trp^{128}, Ser^{152} and His^{241}) located in loops C and E have been identified as the cause of cooper (Cu) and nickel (Ni) sensitivity in AQP3 (Zelenina *et al.* 2003; 2004).

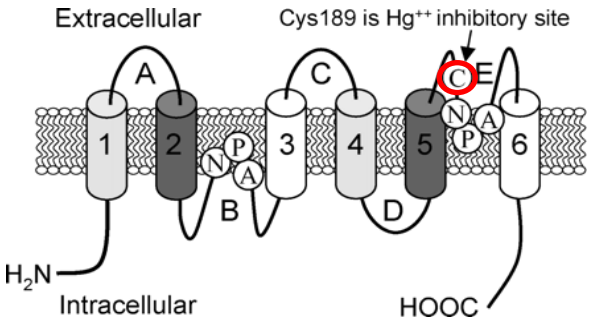

Figure 16. Site of mercurial inhibition in AQP1. It is localized at Cys^{189}, near to the NPA motif situated in loop E. Source: Huang *et al.* 2006a.

4.2.6 Evolution

AQPs are present in the three domains of life: Bacteria, Eukarya and Archaea (reviewed by Perez Di Giorgio *et al.* 2014). They show a sequence homology and share functional and structural similarities (reviewed by Engel *et al.* 2000). Different studies on the presence of AQPs in bacteria, fungi, plants and animals concur in that there are two major phylogenetic divisions: the water-selective type channels (AQPs) and the glycerol facilitators (GLPs) (Heymann & Engel, 1999). This dichotomy implies an early gene duplication event in Bacteria (Zardoya, 2005).

The earliest evolutionary studies of diverse subfamilies of aquaporins in bacteria recognized two AQPs: aqpZ and glpF in *Escherichia coli*, which appear to be the predecessor forms of orthodox AQPs and aquaglyceroporins, respectively (reviewed by Ishibashi *et al.* 2011). As commented in the previous sections, the size of the pore restricts the spectrum of permeating substrates. The first AQP had a larger pore which allowed the uptake of nutrients and release of waste products, which suggests that this first AQP was more likely a GLP. The loss of the conserved aspartic residue situated close to the second NPA box could have converted aquaglyceroporins into orthodox AQPs, which are more specialized in water transport (see Section 4.2.3). In the case of superaquaporins, they may have been derived from orthodox AQPs to bring about the intracellular water transport for animal cells. The absence of superaquaporins in lower organisms and plants suggests that they could have been originated from horizontal gene transfer (HGT) between cohabiting ancient bacteria (reviewed by Ishibashi *et al.* 2011).

It is also worth mentioning the absence of orthodox AQPs and GLPs in many archaea organisms, such as thermophilic archaea. This could be explained by the fact that they live near to submarine volcanoes where water channels may not be necessary because diffusion rates are very high at elevated temperatures. In spite of this, aqpM, an AQP permeable to both water and glycerol has been identified in the archea *Methanothermobacter marburgensis* (Lee *et al.* 2005). Intriguingly, while aqpM has been suggested to facilitate the movement of water across plasma membranes in response to osmotic gradients, it presents low permeability rates to water and glycerol so that their actual function in these organisms is yet to be addressed (Kozono *et al.* 2003).

Comparative studies of genomes between Bacteria and Eukarya suggest that a major fraction of genes in prokaryotic genomes have been acquired by HGT (reviewed by Koonin *et al.* 2001). Fixation and long-term persistence of HGT imply that the corresponding genes might confer a selective advantage onto the recipient organism (reviewed by Phillips, 2006). On the other hand, gene transfer in eukaryotic cells has mainly occurred via symbiotic or parasitic relationship with bacteria (Novichkov *et al.* 2004).

In the case of Eukarya, AQP diversification occurred in vertebrates and plants. The classification of mammalian aquaporins consists of thirteen members (AQP0-AQP12) grouped in three major subfamilies as described in Section 4.2.3. Regarding plants seven subfamilies have been established: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), *X* intrinsic proteins (XIPs), hybrid intrinsic proteins (HIPs), and GlpF-like intrinsic proteins (GIPs) (Johanson *et al.* 2001; Danielson & Johanson, 2008; Perez Di Giorgio *et al.* 2014).

In contrast to animals, plants only present orthodox AQPs. The absence of GLPs and superaquaporins could be explained by a functional conversion of orthodox AQPs into GLPs (NIPs). In addition, the presence of other intracellular AQPs (TIPs and SIPs) could have made intracellular superaquaporins redundant (reviewed by Perez Di Giorgio *et al.* 2014).

4.2.7 The importance of AQPs in the reproductive system

As aforementioned, AQPs are found in many mammalian tissues and organs. In particular, they are present in the reproductive system of both males and females (reviewed by Huang *et al*. 2006a; **Table 3**), which suggests that AQPs play an important role in mammalian reproduction (Thoroddsen *et al.* 2011). In effect, they are involved in the fluid movement across male and female reproductive tracts (reviewed by Huang *et al.* 2006a). For example, in the female reproductive tract, AQPs act regulating the transport of water within the mammalian uterus and their expression is in turn regulated by ovarian steroid-hormones (Jablonsky *et al*. 2003). Moreover, the presence of these proteins also contributes to the cervical dilatation during gestation (Anderson *et al.* 2006). AQPs also participate in the ova transport along the fallopian tube (or oviduct) by altering its luminal diameter and influence the production of the oviductal fluid, which provides the physiological medium for fertilization and early embryonic development (Gannon *et al*. 2000). Steroid hormones may regulate the expression of AQPs and thus control water transport into the oviduct lumen (reviewed by Leese *et al*. 2001). AQPs also take part in follicular development as their gonadotropin-influenced expression in granulosa cells not only ensures rapid transport of water transport but also that of other small neutral molecules (McConnell *et al.* 2002). On the other hand, AQPs participate in blastocyst formation, as different AQPs have been found in trophectoderm and play an important role in the water movement across the epithelium during the process of cavitation (reviewed by Watson & Barcroft, 2001). AQPs are also involved in embryo implantation, in a process that depends on estrogen stimulation (Richard *et al.* 2003). Finally, AQPs are expressed in chorioamniotic membranes and placenta where they play a vital role in the reabsorption of amniotic fluid (reviewed by Huang *et al*. 2006a).

Regarding the male, AQPs have a very important reabsorptive function throughout the reproductive tract. In addition, AQPs have been described in the plasma membrane of seminal vesicles and prostate, where play an important role in the secretion of fluids rich in nutrients which are required for sperm survival (reviewed by Huang *et al*. 2006a). Furthermore, not only do AQPs have an important role in both male and female reproductive tracts but also on the sperm adaptation before and after copulation. After ejaculation, sperm enter the female reproductive tract and are exposed

to a mild osmotic variation (reviewed by Cooper & Yeung, 2003). While this osmotic change is involved in the activation of sperm motility, it needs to be precisely regulated as, otherwise, it may lead sperm cells to swell or shrink thereby negatively affecting their integrity (reviewed by Cooper & Yeung, 2003). Therefore, AQPs have been proposed to be active players in the regulation of sperm volume (Yeung *et al*. 2006; Yeung *et al*. 2010).

Although a large number of studies have described the potential role of AQPs in the reproductive system, the identification, localisation and function of these proteins in spermatozoa has been studied less.

Table 3. Cellular localisation of mammalian AQP isoforms in female and male reproductive tracts (Huang *et al*. 2006a; Sales *et al*. 2013).

Aquaporin	Major tissue distribution
AQP0	Testis
AQP1	Vagina, ovary, oviduct, uterus, placenta, fetal membrane, embryo, testis, efferent ducts, epididymis, vas deferens, seminal vesicles and prostate
AQP2	Uterus, ovary, testis, efferent ducts, epididymis and vas deferens
AQP3	Uterus, cervix, ovary, placenta, fetal membrane, embryo, epididymis and prostate
AQP4	Uterus, cervix and ovary
AQP5	Ovary, uterus, cervix, oviduct, granulose cells, embryo and epididymis
AQP6	Embryo
AQP7	Ovary, embryo, testis, epididymis, spermatids, testicular and epididymal spermatozoa and ejaculated sperm
AQP8	Uterus, cervix, ovary, oviduct, placenta, fetal membranes, embryo, testis and epididymis
AQP9	Ovary, oviduct, uterus, granulose cells of follicles, placenta, fetal membrane, embryo, testis, efferent ducts, epididymis, vas deferens, prostate and coagulating gland
AQP10	Testis, efferent ducts and epididymis
AQP11	Testis
AQP12	N/A

N/A, data not available

4.2.8 Role of AQPs in reproductive pathophysiology

Few studies have been undertaken to elucidate the involvement of AQPs in reproductive disorders. Alterations in the expression, function and regulation of AQPs have already been demonstrated to be at the basis of some forms of male sub-fertility and infertility (reviewed by Huang *et al*. 2006a). AQPs are related with abnormal sperm motility (AQP7) (Saito *et al*. 2004) and varicocele (AQP1) (Nicòtina *et al.* 2005). In the case of female reproductive disorders, it has been demonstrated that the reduced expression of AQP9 in the human fallopian tube may contribute to tubal ectopic pregnancy (Ji *et al*. 2013). On the other hand, AQPs have also been associated with other different reproductive disorders such as polycystic ovary syndrome (PCOS) (Qu *et al*. 2010) and ovarian cancer, as AQP5 and AQP9 have been found to be up-regulated in malignant ovarian tumours (reviewed by Frede *et al*. 2013). Moreover, another study has shown that AQP3 is involved in the migration and invasion of breast cancer cells (Huang *et al*. 2015).

5. Generalities of sperm cryopreservation

As AQPs are mainly involved in the transport of water and other solutes, a crucial issue that attracts the attention from researchers is the involvement of these proteins during cryopreservation of gametes and embryos. As the current Thesis dissertation is focused upon the localisation and function of AQPs in mammalian spermatozoa, the present section aims at introducing the main features of sperm cryopreservation, the damages that this technique inflicts upon the sperm cell, and its main advantages and disadvantages.

The first knowledge about sperm cryopreservation dates back 1776, when the Italian scientist Lazzaro Spallanzani reported to have achieved the maintenance of human sperm motility after exposure to low temperatures (Spallanzani, 1979; Walters *et al.* 2009). Nevertheless, it was not until the 20th century when this technology experienced some relevant progresses (reviewed by Yeste, 2016). In 1949, Polge and their colleagues in the United Kingdom made a serendipitous discovery with the use of glycerol as a cryoprotectant permeable agent (CPA; Polge *et al.* 1949; Walters *et al.* 2009). This finding allowed identifying crucial elements for the development of Cryobiology and represented a defining momentum for sperm cryopreservation (Baust

et al. 2009; Walters *et al*. 2009). At that time, Polge and his colleagues could not imagine that their work would have far-reaching for reproductive biology and genetic improvement in human and livestock animals.

5.1 Sperm cryoinjury

5.1.1 Principles of cryoinjury during freezing and thawing

It is well known that semen cryopreservation is the best method to store sperm for long periods of time. In addition, this technology enables preserving genetic resources from livestock animals and facilitates international trade. However, spermatozoa experience osmotic, chemical and mechanical stress when are frozen and thawed, and this ultimately results in cryoinjuries (FAO, 2008).

Freezing inflicts a direct damage related to the formation of intracellular ice and cell dehydration. Taking this into account, Mazur *et al.* (1972) proposed the two-factor hypothesis. According to this hypothesis, intracellular ice crystals are produced at high cooling rates due to the fact that intracellular water is not able to leave cells completely. Conversely, most of the water flows out at low cooling rates resulting in an increase in the concentration of intracellular solutes, cell dehydration and contraction of organelles and membranes (Mazur *et al*. 1972; reviewed by Yeste, 2016; **Figure 17**). In addition, it is worth mentioning that these effects are inverted along thawing. Under low thawing rates, recrystallization occurs. Moreover, high thawing rates induce an osmotic stress since CPAs are unable to leave the cell fast enough. This leads to water inlet into the cell that may cause cell membrane disruption (reviewed by Holt, 2000b; Casas & Flores, 2013). For these reasons, many efforts have been focused upon finding the optimal cooling/freezing and thawing rates in order to prevent cryoinjuries as much as possible (Holt *et al.* 2005; Juarez *et al.* 2011).

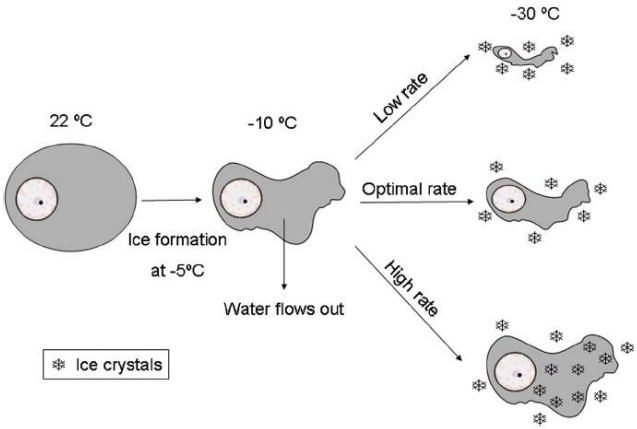

Figure 17. Schematic representation of cell injuries according to the two-factor hypothesis proposed by Mazur *et al*. (1972). Source: Yeste, 2016.

5.1.2 The relevance of cryoprotectants

Apart from cooling rates, the permeability of plasma membrane to water and cryoprotectants (CPAs) is crucial for cell survival during cryopreservation (Tan *et al*. 2013). Cryoprotectants are compounds used to decrease the stress derived from freezing protocols. Nevertheless, they may produce osmotic and toxic damages at certain concentrations and, thus, finding the appropriate concentrations is essential (Okazaki *et al*. 2009).

Classification of CPAs is made according to their diffusion capacity across cell membranes and distinguishes between permeating and non-permeating CPAs. Non-permeating CPAs are compounds that are not able to pass through plasma membrane by simple diffusion and, thus, act extracellularly. The most commonly used non-permeating CPAs are milk and egg yolk proteins, sugars (mainly disaccharides) and other compounds of high molecular weight, such as polyvinylpyrrolidone (PVP), hydroxyethyl starch (HES), polyethylene glycol (PEG) and dextrans (reviewed by Benson *et al*. 2012). Egg yolk is routinely included in cryopreservation extenders to protect boar sperm from cold shock (reviewed by Benson *et al*. 2012). When combined with Orvus ES Paste® (Equex), a surfactant, egg yolk proteins provide better protection to sperm, as this detergent facilitates the interaction of these proteins with the sperm plasma membrane (reviewed by Holt, 2000a and Rodríguez-Martinez & Wallgren, 2010). In bulls, egg yolk- (~20%) and milk-based extenders were used in the past to protect sperm from the detrimental effects of cryopreservation. Nevertheless, other alternatives are currently

used, as the risk of introducing exotic diseases through transporting egg yolk based products has been perceived. In addition, egg yolk has been suggested to be able to interfere with sperm evaluation and the presence of particulate material in the extender may reduce fertility (reviewed by Layek *et al*. 2016). Against this background, IMV Technologies has introduced a new product for bovine sperm cryopreservation called Bioxell™, an egg yolk free extender that contains other CPAs like glycerol. It is worth mentioning that a cryoprotecting solution should contain both permeating and non-permeating CPAs, because while the latters do not provide full protection to the cell, they reduce the levels required for permeating CPAs (reviewed by Yeste, 2016).

As far as permeating CPAs are concerned, the most used are glycerol, dimethylsufoxide (DMSO), ethylene glycol (EG), methanol, propylene glycol (PG) and dimethylacetamide (DMA). These compounds cross cell membranes, are able to alter their properties, induce changes in diffusion rates and modify cytoplasm viscosity (reviewed by Holt, 2000a). Related with this, glycerol is the most used CPA for sperm cryopreservation since no permeating CPA has demonstrated to yield better results. Nevertheless, this CPA can result toxic and can affect plasma membrane fluidity when added at concentrations higher than 4% (Buhr *et al.* 2001).

5.1.3 Effects on plasma membrane

As discussed in Section 3, any plasma membrane contains phospholipids which confer fluidity and cholesterol which provide rigidity and stability (reviewed by Yeste, 2016). The lipid component of the sperm membranes is responsible for the fluidity, sperm maturation, spermatogenesis, capacitation, acrosome reaction and membrane fusion (reviewed by Sanocka & Kurpisz, 2004 and Keber, 2013). Related to this, it has been reported that differences in the fatty acid composition of sperm membrane between species are an important factor on gamete freezability. Higher resilience of mammalian spermatozoa to cold shock, which includes the dramatic effects ensuing from the destabilization of the plasma membrane at temperatures equal or lower than 5ºC, has been perceived for species in which the cholesterol:phospholipid ratio and the degree of saturated fatty acids in the phospholipid fraction are high (reviewed by Mandal *et al*. 2014). Indeed, bull spermatozoa, which is more cryoresistant than boar spermatozoa, presents higher cholesterol:phospholipid (0.45 *vs.* 0.26) and lower plasma membrane

protein:phospholipid molar ratios (0.80 *vs.* 1.26) than boar spermatozoa (Parks & Lynch 1992). Therefore, boar sperm are very sensitive to cold shock because their plasmalemma contains high levels of unsaturated phospholipids and low cholesterol:phospholipid molar ratio (reviewed by Casas & Flores, 2013).

It is well known that at low temperatures, lipids experience alterations in physical phases (i.e. fluid- and gel-phase lipids) resulting in an increase of gel phases. Different studies have reported that the presence of sterols inhibit these phase-changes, that is why the amounts of cholesterol present in the plasma membrane are very relevant (reviewed by Holt, 2000a). Restriction of lateral movements of membrane phospholipids has been reported at temperatures lower than 5ºC resulting in a transition from fluid to gel phases. Since phospholipids present different transition temperatures, phase separations may occur. As a result, lipids are restructured, integral membrane proteins are clustered and some molecules of cholesterol are released. This leads to a disruption of lipid and protein interactions and the loss of function of some proteins such as ion channels (reviewed by Yeste, 2016).

5.1.4 Effects on sperm nucleus

Other cryoinjuries affect sperm nucleus and chromatin integrity leading the destabilization of nucleoproteins and DNA damage (Flores *et al.* 2009; Yeste *et al.* 2013a). Sperm DNA is made up of DNA and nucleoproteins (mostly protamines; P1 and P2). Freeze-thawing protocols disrupt disulphide bridges (S-S) between cysteine radicals of protamines (Flores *et al.* 2011). In addition to the disruption of disulphide bridges, freeze-thawing procedures also increase the levels of sperm DNA fragmentation (Yeste *et al.* 2013a). The extent of this cryoinjury differs between species and is linked to whether sperm chromatin only contains P1 or it also contains P2. Thus, species having P1 and P2 (human, stallion and mouse) present higher levels of DNA fragmentation than those presenting only P1 (boar, ram and bull) (Gosálvez *et al.* 2011).

5.1.5 Effects on mitochondrial function and ROS production

Sperm mitochondrial status is very important because of its relationship with sperm motility and the energy status of cells (Mazur *et al.* 2000). Freeze-thawing procedures induce changes in the mitochondrial membrane potential (reviewed by Yeste, 2016).

Indeed, it has been described that chilling and freezing reduce mitochondrial activity in spermatozoa from boars (Flores *et al*. 2010) and other mammalian species, such as equine (reviewed by Peña *et al*. 2015).

Cryopreservation can increase the intracellular levels of reactive oxygen species (ROS), which are mainly produced in mitochondria. This increase in sperm ROS synthesis after freezing and thawing procedures has been described in bovine (Chatterjee & Gagnon, 2001; Gürler *et al.* 2016) and equine (Baumber *et al*. 2003; Ortega Ferrusola *et al.* 2009; Yeste *et al.* 2015a). In bull, ROS attacks the long chains of PUFAs present in their plasma membrane which initiate a lipid peroxidation cascade that results in deleterious effects on sperm function (Abavisani *et al*. 2013). However, the effects of cryopreservation upon ROS production in boar sperm are less clear (Flores *et al*. 2009; Casas & Flores, 2013).

It is noteworthy that small amounts of ROS are important to drive the tyrosine phosphorylation cascades related with sperm capacitation (reviewed by Aitken *et al*. 2010). Nevertheless, ROS in high quantities cause peroxidative damage to sperm plasma membrane and can cause single or double-strand DNA breaks (reviewed by Aitken & Krausz, 2001 and Spiropoulos *et al*. 2002). Thus, ROS-mediated damage affects the sperm fertilizing potential and the ability to create a normal and healthy embryo (reviewed by Aitken *et al*. 2010).

5.1.6 Effects on sperm motility

A dramatic reduction of sperm motility is very apparent following freeze-thawing (Estrada *et al*. 2014; Yeste *et al.* 2013a; 2013b; 2014). As commented above, mitochondrial function is crucial for sperm motility and its decrease after freeze-thawing procedures has been attributed to damages to the mitochondrial membranes since the ATP generated by oxidative phosphorylation in mitochondria is transferred to the microtubules to drive motility (Mazur *et al.* 2000; O'Connell et *al*. 2002). Nevertheless, it is important to take into account that, prior to capacitation, sperm show very low mitochondrial activity together with a very high glycolytic rate. This is probably due to the fact that after ejaculation sperm encounter a mostly anaerobic environment in the female genital tract and mitochondrial respiration requires aerobic conditions to work. In spite of this, mitochondrial activity is necessary for sperm

capacitation and hyperactivation through ways that do not appear to be directly linked to energy production, but rather to the regulation of intracellular redox balance and calcium stores (reviewed by Rodríguez-Gil, 2013).

5.1.7 Effects on mRNAs and microRNAs

Cryopreservation affects sperm mRNAs. In boars, there are two studies that linked the abundances of certain transcripts encoding for different proteins with cryopreservation protocols (Zeng *et al*. 2014a; 2014b). In other species, such as humans and bovine, different studies have found that certain mRNAs that are involved in post-fertilization events and pregnancy success are reduced following freeze-thawing (Valcarce *et al*. 2013; Card *et al*. 2013). On the other hand, sperm microRNAs, which appear to modulate gene expression in post-fertilization events, have also been found to be affected by cryopreservation procedures in pigs (Zhang *et al*. 2015).

5.1.8 Changes of sperm proteins

Finally, cryopreservation procedures also induce changes in levels, localisation, function and tyrosine-phosphorylation of certain sperm proteins involved in capacitation, adhesion, energy supply and sperm-oocyte binding and fusion. These effects have been observed in boar, humans and in bulls (reviewed by Yeste, 2016).

5.2 Freezability Markers

According to the literature, sperm cryosurvival highly varies between species. In addition, a high individual variability between and within ejaculates and even between fractions of the same ejaculate exists in the sperm ability to sustain cryopreservation (cryotolerance or freezability; Holt *et al*. 2005; Peña *et* al. 2006; Waterhouse *et al.* 2006; Dorado *et al*. 2010; Yeste *et al*. 2015). As a consequence, ejaculates are usually classified as good (GFE) or poor freezability ejaculates (PFE) according to their post-thaw sperm survival and motility (Casas *et al*. 2009; Yeste *et al*. 2013a; Vilagran *et al.* 2014). In this context, a main inconvenient is the low association between conventional sperm quality parameters evaluated before cryopreservation and ejaculate freezability (Roca *et al.* 2006; Casas *et al*. 2009; Yeste *et al*. 2013a; Vilagran *et al*. 2014). The reason why conventional parameters fail to detect GFE and PFE is because they do not

contemplate the cell mechanisms facing to stressful conditions, but rather reflect the sperm physiology at 37ºC (reviewed by Yeste, 2016).

The mechanisms underlying the differences between GFE and PFE remain largely unknown. Nevertheless, different studies focused on boar spermatozoa have been aimed at elucidating the molecular basis of sperm freezability. The study conducted by Thurston *et al*. (2002) explored the hypothesis that inter-individual variation was genetically determined. These authors found 16 molecular markers linked to genes related with freezability variations using the Amplified Fragment Length Polymorphism (AFLP) technique. Moreover, other works have identified separate sperm and seminal proteins as freezability markers. Specifically, heat-shock protein 90 (HSP90AA1), acrosin-binding protein (ACRBP), triosephosphate isomerase (TPI), voltage-dependent anion channel 2 (VDAC2), fibronectin (FN1), N-acetyl-β-hexosaminidase (β-HEX), superoxide dismutase 1 (SOD1), outer dense fibre protein 2 (ODF2), and α-kinase anchor protein 3 (AKAP3) have been reported as markers for predicting boar ejaculate freezability (Casas *et al.* 2009; Casas *et al.* 2010; Vilagran *et al.* 2013; Chen *et al*. 2014; Vilagran *et al.* 2014; Vilagran *et al.* 2015; Wysocki *et* al. 2015; see **Table 4**). However, it is yet to be addressed why the abundance of these proteins differs between samples since, thus far, no work has determined whether differences in gene expression occur during spermatogenesis (reviewed by Yeste, 2015).

With regard to bull spermatozoa, different works have reported the association of certain proteins with sperm freezability **(Table 4)**. Concretely, Jobim *et al*. (2004) studied the seminal plasma of high and low bull freezability ejaculates through two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and identified three proteins that were more abundant in seminal plasma samples collected from bulls with high semen freezability. These proteins were bovine seminal plasma (BSPA1/A2), acidic seminal fluid protein (aSFP) and bovine clusterin precursor. These authors also identified another protein, lipocaline-like prostaglandin D synthase (PGDS), which was found to be more abundant in seminal plasma samples from bulls with low sperm freezability. Moreover, other studies have demonstrated that bulls with lower freezability and fertility present higher levels of glutamic oxaloacetic transaminase (GOT) and glutamate pyruvate transaminase (GPT) in seminal plasma (Belorkar, 1987; Herman & Madden, 1953; Vander Horst *et al*. 1979).

In any case, there is great interest in identifying freezability markers in fresh sperm, either of protein nature or not, as this may predict the ejaculate cryotolerance and avoid the cryopreservation of those sperm samples with poor freezability.

Table 4. The main freezability markers for boar sperm reported thus far.

Protein	Species	Marker	Relation with Cryotolerance	Reference (cryotolerance)	Function	Reference (function)
Acrosin binding protein (ACRBP)	Boar	Sperm	Higher levels in GFE than in PFE	Vilagran *et al.* 2013	Regulates acrosome reaction avoiding premature capacitation and degenerative acrosome exocytosis	Vilagran *et al.* 2013
Fibronectin 1 (FN1)	Boar	Seminal plasma	Higher levels in GFE than in PFE	Vilagran *et al.* 2015	It is related with total sperm motility and sperm-fertilizing ability	Wennemuth *et al.* 2001
Heat Shock Protein 90 (HSP90AA1)	Boar	Sperm	Higher levels in GFE than in PFE	Casas *et al.* 2010	Provides resistance and protection against cell oxidative and thermal stress	Fukuda *et al.* 1996; Huang *et al.* 1999; Wang *et al.* 2005; Powers *et al.* 2008
N-acetyl-β-hexosaminidase (β-HEX)	Boar	Seminal plasma	Enzyme activity negatively correlated with sperm cryotolerance	Wysocki *et al.* 2015	Enables sperm cells to penetrate the zona pellucida by removing N-acetylglucosamine residues in ZP glycoproteins	Miller *et al.* 1993
Triosephosphate isomerase (TPI)	Boar	Sperm	Lower levels in GFE than in PFE	Vilagran *et al.* 2013	Involved in energy production in the glycolytic pathway. Concretely, it leads the conversion of dyhydroxyacethone phosphate to glyceraldehyde phosphate	Vilagran *et al.* 2013

Voltage-dependent anion channel 2 (VDAC2)	Boar	Sperm	Higher levels in GFE than in PFE	Vilagran *et al.* 2014	Involved in osmotic regulation and suggested to play vital roles during spermatogenesis, sperm maturation, motility, acrosome reaction and fertilization	Liu *et al.* 2010; Shoshan-Barmatz *et al.* 2010
Superoxide dismutase 1 (SOD1)	Boar	Sperm	Higher levels in frozen-thawed than in extended semen	Chen *et al.* 2014	Eliminates superoxide radicals	Tsunoda *et al.* 2012
Outer dense fibre protein 2 (ODF2)	Boar	Sperm	Higher levels in frozen-thawed than in extended semen	Chen *et al.* 2014	Involved in sperm progressive motility, sperm tail elasticity and protection against shearing epididymal forces	Donkor *et al.* 2004
α-kinase anchor protein 3 (AKAP3)	Boar	Sperm	Higher levels in frozen-thawed than in extended semen	Chen *et al.* 2014	Regulates sperm motility, capacitation, and acrosome reaction	Hillman *et al.* 2013
Bovine seminal plasma A1/A2 BSPA1/A2	Bull	Seminal Plasma	Higher levels in GFE than in PFE	Jobim *et al.* 2004	Potentiates sperm capacitation by binding to capacitation factors such as heparin and by stimulating sperm membrane cholesterol efflux	Moreau *et al.* 1998
aSFP (spermadhesin)	Bull	Seminal Plasma	Higher levels in GFE than in PFE	Jobim *et al.* 2004	Plays a role as decapacitation factor and/or ZP-binding molecule	Dostàlovà *et al.* 1994

Bovine seminal plasma A1/A2 BSPA1/A2	Bull	Seminal Plasma	Higher levels in GFE than in PFE	Jobim *et al.* 2004	Potentiates sperm capacitation by binding to capacitation factors such as heparin and by stimulating sperm membrane cholesterol efflux	Moreau *et al.* 1998
Bovine clusterin precursor	Bull	Seminal Plasma	Higher levels in GFE than in PFE	Jobim *et al.* 2004	Given its localisation in sperm membrane and its role in lipid transport and re-distribution, this protein may have biological functions similar to those of BSP	Jobim *et al.* 2004
Lipocaline-like prostaglandin D synthase (PGDS)	Bull	Seminal Plasma	Higher levels in PFE than in GFE	Jobim *et al.* 2004	Although the role of PGDS is still not clear, it has been suggested to act on sperm PUFAs, regulating membrane fluidity.	Reviewed by Caballero 2011
Glutamic oxaloacetic transaminase (GOT)	Bull	Seminal Plasma	Higher levels in PFE than in GFE	(Belorkar *et al.*, 1987; Herman & Madden, 1953; Vander Horst *et al.* 1979)	Amino acid metabolism	Reviewed by Huang *et al.* 2006b
Glutamate pyruvate transaminase (GPT)	Bull	Seminal Plasma	Higher levels in PFE than in GFE	(Belorkar et al., 1987; Herman & Madden, 1953; Vander Horst et al. 1979)	Plays a key role in the intermediary metabolism of glucose and amino acids	Reviewed by Huang et al. 2006b

5.3 Importance of AQPs for the cryopreservation of male and female gametes

Nowadays, fertility preservation is an essential part of reproductive science that not only refers to gametes (sperm and oocytes) and embryos, but also to reproductive organs such as ovarian and testicular tissues. It is well know that cryopreserved cells may be stored for long-periods of time without changes in their functionality or genetic information, this method becoming highly attractive for reproductive purposes (reviewed by Bagchi *et al*. 2008; reviewed by Chian *et al.* 2013).

Currently, slow freezing and vitrification are the most utilized methods for gamete cryopreservation. The first is characterized by the use of low concentrations of CPAs, which is associated with little toxicity to cells and low cooling rates. Vitrification method uses high cooling rates in combination with high concentrations of cryoprotectants and an abrupt reduction in temperature (reviewed by Sales *et al.* 2013). Although vitrification is the best method to store oocytes and embryos (reviewed by Mukaida & Oka, 2012) the most efficient method for long-term sperm preservation is slow freezing (reviewed by Mocé *et al.* 2016).

As previously mentioned in other sections, the permeability of plasma membrane to water and CPAs is crucial during cryopreservation (Tan *et al*. 2013). The transport of water and cryoprotectants across the plasma membrane takes place via simple diffusion through the lipid bilayer and facilitated diffusion through channel proteins. Transport rates of water and CPAs are low in simple diffusion, but they are much higher in facilitated diffusion (Jin *et al*. 2011). Related to facilitated diffusion, and as aforementioned, it is widely known that AQPs are critical in regulating the transport of water and CPAs across cell membranes and in preventing osmotic damage (Kumar *et al*. 2015). Moreover, because glycerol is the most used permeable CPA, the presence of GLPs might be relevant for the survival of cryopreserved cells. For all these reasons, many efforts have been made to elucidate the role of these proteins during cryopreservation procedures. Specifically, Edashige *et al*. (2003) demonstrated that the injection of cRNA encoding for *Aqp3* into mouse oocytes increased glycerol permeability and augmented cell survival after vitrification. Morató *et al*. (2014) reported that the exogenous expression of AQP3 in mature porcine oocytes also increased the permeability to water and ethylene glycol. Moreover, another study has

demonstrated that AQP7 plays an important role in the tolerance to hyperosmotic stress and in the survival of the human oocytes during cryopreservation (Tan *et al*. 2013).

With regard to embryos, Edashige *et al.* (2006) and Jin *et al.* (2011) described that cell permeability to water and CPAs in mouse and bovine morulae was associated with the expression of AQP3, both at mRNA and protein levels. Related with this, Jin *et al.* (2013) found that the expression of *AQP3* was significantly higher in pig blastocysts than in oocytes and morulas, which indicates that the higher permeability of water and CPAs of pig blastocysts and their higher cryotolerance is, at least in part, related to the presence of a high number of AQP3-channels.

6. State of art of AQP3, AQP7 and AQP11 in sperm

The three AQPs studied in this work were chosen because, as described below, their presence had previously been demonstrated in other mammalian species. Furthermore, these three proteins belong to the groups of GLPs (AQP3 and AQP7) and superAQPs (AQP11), both involved in the transport of water and CPAs.

Spermatozoa have higher water permeability than other cells like red blood cells and renal tubular epithelial cells (reviewed by Huang *et al.* 2006a). There are only two studies conducted in human and murine sperm about the presence of AQP3 and its role in sperm osmoregulation (Chen *et al*. 2010; Chen & Duan, 2011). These functional studies with *Aqp3*-knockout mice have reported normal sperm motility but higher vulnerability to hypotonic stress, which produces cell swelling and increases tail bending after entering the uterus. These abnormalities difficult the sperm migration throughout the oviduct and decrease fertilization rates.

Regarding AQP7, Saito *et al.* (2004) conducted a study comparing fertile and infertile men and reported that while ejaculated sperm from all fertile men presented AQP7, spermatozoa were devoid of AQP7 in 23% of infertile men. In addition, sperm motility of infertile patients lacking AQP7 in ejaculated spermatozoa was significantly lower than that of sperm exhibiting positive AQP7-staining. This suggests that AQP7 is involved in the maintenance of sperm motility and the absence of AQP7 in ejaculated spermatozoa may be an underlying cause for male infertility. In agreement with these data, Yeung *et al.* (2010) found that spermatozoa from 10% of infertile human patients showed no clear AQP7-staining. In contrast to these findings, other studies have

demonstrated that knockout mice for *Aqp7* are fertile and produce normal functional spermatozoa, possibly due to the fact that their function is compensated by other AQPs (Sohara *et al.* 2007). Furthermore, Kondo *et al.* (2002) have shown that a homozygous mice for a non-functional mutation in AQP7 remains fertile implying that this protein could not be indispensable in the regulation of fertility.

As far as AQP11 is concerned, Yeung & Copper (2010) have described that this protein is present in the cytoplasm of elongated spermatids and is essential for sperm production during spermatogenesis and spermiation in mice. Unfortunately, *Aqp11*-null mice develop polycystic kidneys causing their death due to a severe renal failure (reviewed by Matsuzaky *et al*. 2016). Therefore, addressing how the efflux of water and non-metabolizable substances is performed via AQP11 is much difficult as, thus far, there is no viable *Aqp11* knock-out mouse model.

Despite all of the aforementioned, the localisation, presence and role of AQPs in mammalian spermatozoa is still poorly studied. Taking this into account and given the importance of these proteins in several processes, the current dissertation has focused on the study of two GLPs (AQP3 and AQP7) and one superaquaporin (AQP11) in the sperm of the two major species (bovine and porcine) in livestock production.

Objectives

Objectives

Against the background introduced in the previous section, the present Thesis dissertation has three main aims that look for addressing the presence and function of AQPs in the sperm of two major livestock species such as boar and bull.

In response to the following objectives, five papers, as referred at the end of each aim.

1. The identification of three separate AQPs, AQP3, AQP7 and AQP11 in boar and bull sperm by immunoblotting. (**PAPER I, PAPER II, PAPER IV** and **PAPER V**).
2. The determination of the precise localisation by immunocytochemistry procedures. (**PAPER I**, **PAPER II**, **PAPER IV** and **PAPER V**).
3. Elucidate their putative role on sperm function through the evaluation of the relationship of these three proteins with different sperm functional parameters, sperm cryotolerance and fertilizing ability. (**PAPER I**, **PAPER II**, **PAPER III, PAPER IV** and **PAPER V**).

Paper Compendium

Paper 1

Aquaporins 7 and 11 in boar spermatozoa: detection, localisation and relationship with sperm quality.

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ABSTRACT

Aquaporins (AQPs) are integral membrane water channels that allow transport of water and small solutes across cell membranes. Although water permeability is known to play a critical role in mammalian cells, including spermatozoa, little is known about their localisation in boar spermatozoa. Two aquaporins, AQP7 and AQP11, in boar spermatozoa were identified by western blotting and localised through immunocytochemistry analyses. Western blot results showed that boar spermatozoa expressed AQP7 (25 kDa) and AQP11 (50 kDa). Immunocytochemistry analyses demonstrated that AQP7 was localised in the connecting piece of boar spermatozoa, while AQP11 was found in the head and mid-piece and diffuse labelling was also seen along the tail. Despite differences in AQP7 and AQP11 content between boar ejaculates, these differences were not found to be correlated with sperm quality in the case of AQP7. Conversely, AQP11 content showed a significant correlation ($P < 0.05$) with sperm membrane integrity and fluidity and sperm motility. In conclusion, boar spermatozoa express AQP7 and AQP11, and the amounts of AQP11 but not those of AQP7 are correlated with sperm motility and membrane integrity.

Additional keywords: immunostaining, pig, sperm membrane integrity, sperm motility, water channels, western blot.

Paper II 

Aquaporins in boar spermatozoa. Part II: detection and localization of Aquaglyceroporin 3

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ABSTRACT

The proteins belonging to the aquaporin family play a fundamental role in water and solute transport across biological membranes. While the presence of these proteins has been extensively studied in somatic cells, their function in mammalian spermatozoa has been studied less. The present study was designed to identify and localise aquaglyceroporin 3 (AQP3) in boar spermatozoa. With this purpose, 29 fresh ejaculates from post-pubertal Piétrain boars were classified into two groups based upon their sperm quality and subsequently evaluated through western blot and immunofluorescence assessments. Western blotting showed the specific signal band of AQP3 at 25 kDa, whereas immunofluorescence assessments allowed us to identify two different AQP3 localisation patterns: (1) spermatozoa presenting a clear labelling located only in the mid-piece and (2) spermatozoa exhibiting a distribution pattern in the head and along the entire tail. The first staining pattern was predominant in all studied ejaculates. Despite individual differences in AQP3 content and localisation between boar ejaculates, these differences were not correlated with sperm quality. In conclusion, although AQP3 is present in boar spermatozoa in two different localisation patterns, neither the AQP3 content nor its localisation have been found to be associated with conventional sperm parameters.

Additional keywords: aquaglyceroporin 3, boar sperm, mid-piece, sperm tail, western blot.

Paper III

Relationship of aquaporins 3 (AQP3), 7 (AQP7) and 11 (AQP11) with boar sperm resilience to withstand freeze-thawing procedures

Noelia Prieto-Martínez, Ingrid Vilagran, Roser Morató, Joan E. Rodríguez-Gil, Sergi Bonet & Marc Yeste

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Title

Relationship of aquaporins 3 (AQP3), 7 (AQP7) and 11 (AQP11) with boar sperm resilience to withstand freeze-thawing procedures

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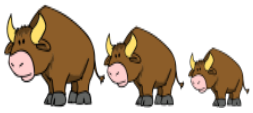
Abstract

Cryopreservation is the most suitable method to preserve boar sperm over long-term storage. However, freeze-thawing protocols inflict extensive damage to sperm cells, reducing their viability and compromising their fertilizing ability. In addition, high individual variability is known to exist between boar ejaculates, which may be classified as of good (GFE) or poor (PFE) freezability. While conventional spermiogram parameters fail to predict sperm cryotolerance in fresh sperm, high levels of certain proteins, also known as freezability markers, have been found to be related to the sperm resilience to withstand freeze-thawing procedures. In this context, the hypothesis of the present study was that aquaporins AQP3, AQP7 and AQP11 could be linked to boar sperm cryotolerance. Twenty-nine ejaculates were evaluated and subsequently classified as GFE or PFE based upon their sperm viability and motility at post-thawing. Fourteen ejaculates resulted to be GFE, whereas the other fifteen were found to be PFE. Relative abundances of AQP3, AQP7 and AQP11 and their localization patterns were evaluated in all fresh and frozen-thawed ejaculates through immunoblotting and immunocytochemistry. Prior to cryopreservation, relative amounts of AQP3 and AQP7 were found to be significantly ($P<0.05$) higher in GFE than in PFE. In contrast, no significant differences ($P>0.05$) between freezability groups were found for AQP11, despite GFE tending to present higher levels of this protein. The localization of AQP7, but not that of AQP3 or AQP11, was observed to be affected by cryopreservation procedures. In conclusion, these results suggest that AQP3 and AQP7 are related to boar sperm cryotolerance and may be used as freezability markers.

Keywords: Boar sperm; Cryotolerance; AQP3; AQP7; AQP11; Freezability markers.

Paper IV 

Aquaglyceroporins 3 and 7 in bull spermatozoa: identification, localisation and their relationship with sperm cryotolerance

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ABSTRACT

Aquaporins (AQPs) are integral membrane water channels that allow transport of water and small solutes across cell membranes. Although water permeability is known to play a critical role in mammalian cells, including spermatozoa, little is known about their localisation in boar spermatozoa. Two aquaporins, AQP7 and AQP11, in boar spermatozoa were identified by western blotting and localised through immunocytochemistry analyses. Western blot results showed that boar spermatozoa expressed AQP7 (25 kDa) and AQP11 (50 kDa). Immunocytochemistry analyses demonstrated that AQP7 was localised in the connecting piece of boar spermatozoa, while AQP11 was found in the head and mid-piece and diffuse labelling was also seen along the tail. Despite differences in AQP7 and AQP11 content between boar ejaculates, these differences were not found to be correlated with sperm quality in the case of AQP7. Conversely, AQP11 content showed a significant correlation ($P < 0.05$) with sperm membrane integrity and fluidity and sperm motility. In conclusion, boar spermatozoa express AQP7 and AQP11, and the amounts of AQP11 but not those of AQP7 are correlated with sperm motility and membrane integrity.

Additional keywords: immunostaining, pig, sperm membrane integrity, sperm motility, water channels, western blot.

Paper V

Aquaporin 11 is related to cryotolerance and fertilising ability of frozen-thawed bull sperm

Roser Morató, Noelia Prieto-Martínez, Rodrigo Muiño, Carlos O. Hidalgo, Joan E. Rodríguez-Gil, Sergi Bonet & Marc Yeste

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Aquaporin 11 is related to cryotolerance and fertilising ability of frozen-thawed bull sperm

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Abstract

The present study sought to identify and localise aquaporin 11 (AQP11) in bull sperm and to investigate the relationship between relative AQP11-content, sperm cryotolerance and fertilising ability of frozen-thawed semen. With this purpose, twenty bull ejaculates were classified into two groups of good and poor freezability according to their post-thaw sperm quality and assessed through immunocytochemistry and immunoblotting analyses before and after cryopreservation. Evaluations by immunocytochemistry allowed us to localise AQP11 throughout the entire tail and along the sperm head. These results were confirmed through immunoblotting, which showed a specific band of about 50 kDa corresponding to AQP11. Furthermore, immunoblotting revealed that bull ejaculates with good freezability characteristics presented significantly ($P<0.05$) higher relative amounts of AQP11 both in fresh and frozen-thawed sperm than those with poorer freezability. In addition, *in vitro* oocyte penetration rates were correlated with the relative AQP11-content in fresh sperm. In conclusion, AQP11 is present in the head and tail of bull sperm and their relative amounts in fresh and frozen-thawed sperm are related to the resilience to withstand cryopreservation and the fertilising ability of frozen-thawed sperm. Future research is needed to elucidate the actual role of sperm AQP11 on bovine fertility.

Keywords: AQP11; aquaporins; bull; sperm; cryopreservation; in vitro fertilisation

Discussion

Discussion

Water is the main component of all living cells and, for this reason, is involved in the regulation of most biological processes, such as the response to osmotic gradients (Gravelle *et al.* 2013). Nevertheless, and due to the hydrophobic nature of lipid membranes, water penetrates slowly this structure by simple diffusion (reviewed by Huang *et al.* 2006a and Matsuzaki *et al.* 2002), which does not explain the high water exchange rates observed in erythrocytes, renal tubular epithelial cells and spermatozoa (Noiles *et* al. 1993; Huang *et al.* 2006a). These observations supported the hypothesis raised by Sidel and Salomon in 1957, who suggested the existence of selective water pores in biological membranes (Sidel & Salomon, 1957). However, it was not until the early nineties when Peter Agre and their colleagues discovered AQPs, a family of water-specific transmembrane channel proteins. This research provided the molecular basis for understanding water transport through facilitated diffusion (reviewed by Agre, 2004 and Agre, 2006). Since then, the study of AQPs has received particular consideration providing new insights into the structure, function and regulation of these proteins in living organisms (reviewed by Gomes *et al*. 2009).

It is well established that AQPs are ubiquitously distributed across mammalian tissues. In particular, their presence in the reproductive tract of both males and females (reviewed by Huang *et al*. 2006a) indicates that AQPs play an important role in reproductive physiology (Thoroddsen *et al.* 2011). Nevertheless, and in contrast to the deep knowledge and numerous studies on AQPs in somatic cells, the understanding of sperm AQPs is limited. Concretely, AQPs have been studied in the mammalian spermatozoa of rodents (Yeung & Cooper, 2010; Chen & Duan, 2011) dogs (Ito *et al.* 2008) and humans (Yeung *et al.* 2010; Moretti *et al*. 2012). However, the identification, localisation and function of these proteins in boar and bull spermatozoa are yet to be reported.

Against this background, one of the purposes of the present Thesis dissertation was to identify three separate members of the AQP family, AQP3, AQP7 and AQP11 in ejaculated boar and bull sperm by immunoblotting (**PAPER I**, **PAPER II**, **PAPER IV** and **PAPER V**). The results obtained prove the presence of these three proteins in the sperm of both livestock species. Regarding boar sperm, the results of Western blot showed the presence of one specific band for AQP3 (25 KDa), AQP7 (25 KDa) and

AQP11 (50 KDa). In the case of bull sperm, immunoblotting showed the presence of two specific bands for AQP3 (30 and 60 KDa) and only one for AQP7 (25 KDa) and AQP11 (50 KDa). It is noteworthy that the weight of reactive bands for AQP3 differed between the two species. In the same way, when comparing the data obtained in the present work with studies conducted in other species, the results suggest that molecular weights of AQP7 and AQP11 are also species-specific. For example, the study conducted by Yeung *et al*. (2010) in human sperm found the existence of four separate AQP7 isoforms (27 KDa, 29 KDa, 30 KDa and 40 KDa), which corresponded to different glycosylation patterns. On the other hand, and despite using the same extraction method, molecular weights of AQP11 were seen to differ within rodent species as whilst a single isoform of 33 KDa was observed in rat spermatozoa, three different isoforms of 27, 34 and 43 KDa were identified in mouse spermatozoa (Yeung & Cooper, 2010). The fact that bull sperm present two specific bands could be related to the existence of two AQP3-isoforms of different molecular weights in a similar fashion to the described for humans and mice. In contrast, boar sperm would only have a single AQP3-isoform. Another possible explanation would be related to the presence of monomeric (30 KDa) and dimeric (60 KDa) forms of AQP3 in bull sperm. However, the use of denaturants and reducing agents in lysis and Laemmli buffers should have, in principle, denaturized all proteins, making less likely the observation of dimeric forms. That being said, one should also note that while samples were boiled at 90ºC for 5 min, some protocols suggest that it is better to heat proteins at 70ºC when dealing with multi-pass membrane proteins, as these proteins tend to aggregate when boiled and the aggregates may not enter the gel efficiently. Therefore, it should not be excluded that the presence of the 60 KDa-band in bull sperm is artefactual. For this reason, more research including different lysis buffers and conditions for gel electrophoresis is required to address this issue.

In the context of the bands and molecular weights identified by immunoblotting, it should be noted that even studies performed in the same species have reported different results, as in the case of the study conducted by Vicente-Carrillo *et al*. (2016). All these discrepancies can be, at least in part, due to the different techniques utilized for preparing the sperm samples before immunoblotting analysis. Nonetheless, positive controls and peptide competitive assays led us to conclude the specificity of the antibodies and the reliability and accuracy of our data. This indicates that all the bands obtained correspond to the proteins of interest. Thus, taking into account the combined results of the three proteins studied and the previous data obtained by Yeung *et al.* (2010) and Yeung &

Cooper (2010), it seems that molecular weights of AQP3, AQP7 and AQP11 are species-specific. In any case, more research including mass spectrometry analysis through Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry peptide mapping (MALDI-TOF MS) is required as this technique provides much higher accuracy in the identification of immunoblotted-proteins (Egelhofer *et al.* 2002).

After confirming the presence of AQP3, AQP7 and AQP11 in the sperm of both species, the present Thesis also sought to evaluate the distribution and localisation of these proteins through immunocytochemistry and confocal laser microscopy (**PAPER I**, **PAPER II**, **PAPER IV** and **PAPER V**). The obtained results demonstrate the presence of these three proteins in all fresh studied ejaculates with a homogeneous distribution and localisation in both species. Specifically, AQP3 is present in two different localisation patterns in boar sperm. The first one corresponds to clear labeling at the mid-piece and the acrosomal region and was predominant in all studied ejaculates (~70%; unpublished data). In the second pattern, spermatozoa exhibit an AQP3-distribution along the entire tail and the acrosomal region. In contrast to these findings, AQP3 was only found in the mid-piece of bull sperm. On the other hand, whereas AQP7 was mainly found at the connecting-piece and partially at the mid-piece of boar sperm and it was detected in both post-acrosomal region and mid-piece of bull sperm. Finally, AQP11 was found to be localized throughout the sperm head and tail in both species.

Immunolocalisation of AQP3, AQP7 and AQP11 was not only performed in fresh/liquid-stored but also in frozen-thawed boar and bull sperm (**PAPER III**, **PAPER IV** and **PAPER V**). This goal could be considered as a double-edged sword because not only does give us information on whether AQPs-distribution may be affected by cryopreservation procedures but does also investigate the participation of these proteins in this process, as discussed later. In a similar way to the observed in fresh ejaculates, AQP3, AQP7 and AQP11 were detected in all frozen-thawed samples. However, it is worth noting that while distributions of AQP3, AQP7 and AQP11 in bull sperm and those of AQP3 and AQP11 in boar sperm were homogeneous, without changes before and after cryopreservation, a different localisation pattern for AQP7 was observed between fresh and frozen-thawed boar sperm. Indeed, whereas AQP7 was mainly found at the connecting piece of boar spermatozoa before cryopreservation, this protein was also found to be present at the sperm mid-piece and in the acrosomal region following freeze-thawing. Therefore, AQP7-distribution in boar sperm appears to be affected by cryopreservation procedures. Similar to these results, the study conducted by Tan *et al.*

(2013) described that the hyperosmosis induced by the addition of CPAs during cryopreservation procedures led to redistribution of AQP7 in the plasma membranes of mouse oocytes. These authors also observed that AQP7 interacted with F-actin, which could facilitate that translocation. In boar sperm, Vicente-Carrillo *et al.* (2016) have also observed that freeze-thawing relocates the labeling of AQP7 to the sperm head. Therefore, and despite more research still being required to elucidate this issue, the redistribution of AQP7 observed during freeze-thawing procedures in boar spermatozoa is consistent with other previous studies.

It is noticeable that the most predominant pattern for AQP3 in boar sperm, i.e. localized in the sperm mid-piece, coincided with the findings obtained in bulls. However, this is not in accordance with the localisation reported in other mammalian species, as AQP3 has been found to be mainly confined to the principal piece of the sperm tail in human (results obtained by immunofluorescence and immunogold) and mouse sperm (data acquired using immunofluorescence; Chen *et al.* 2011; Chen & Duan, 2011). On the other hand, the staining results for AQP11 coincided between boars and bulls. This localisation contrasted with the reported for rat sperm, obtained by immunocytochemistry using a biotinylated secondary antibody with a streptavidin-HRP conjugate, where AQP11 was found at the end-piece of sperm flagellum (Yeung & Cooper, 2010). Regarding AQP7, frozen-thawed boar sperm exhibited a similar pattern to that found by Vicente-Carrillo *et al*. (2016) in frozen-thawed boar sperm when the same antibody was used. The same pattern was also observed in fresh and frozen-thawed bull semen. In spite of this, it is worth mentioning that localisation patterns of AQP7 in fresh sperm differed from those obtained by Vicente-Carrillo *et al*. (2016), the observations being performed under a confocal microscope in both cases. In effect, while Vicente-Carrillo *et al*. (2016) found AQP7 in the mid-piece of fresh boar spermatozoa, localisation of AQP7 in the current Thesis was mainly restricted to the connecting piece, the AQP7-labeling in the mid-piece being marginal. In order to clarify these inconsistencies, we repeated all experiments and we obtained exactly the same results following our protocol and the set by Vicente-Carrillo *et al*. (2016). In addition, these results are in contrast with those obtained in human spermatozoa, where AQP7 has been found in other sperm compartments, including the pericentriolar area, the equatorial segment, and the mid- and main pieces (Saito *et al.* 2004; Yeung *et al.* 2010; Moretti *et al.* 2012).

In this conflicting area, one should note that the discrepancies in the localisation of AQPs could be due to the use of different antibodies, cell preparation and/or

immunostaining methods. Although control experiments performed without the primary antibody and with specific blocking peptides asserted both the specificity and reliability of the staining in the context of the current Thesis, further research using immunogold and transmission electron microscopy are required to determine the precise localisation of these proteins. These analyses may provide a possible explanation on whether AQP7-localisation pattern changes in response to cryopreservation or rather this observation is an artifact of freeze-thawing protocols. Related to this, and to the best of our knowledge, only one study has used immunogold to localize AQP7 in boar sperm (Vicente-Carrillo *et al*. 2016). However, the authors that conducted this work used an antibody of dubious specificity that was not properly confirmed using a blocking peptide. Yet the immunoblots performed in the study of Vicente-Carrillo *et al*. (2016) differed when different AQP7-antibodies were used. Again, this clearly supports the suggestion that these authors should have confirmed the specificity of all their antibodies, especially in the case of immunogold.

Once demonstrated both the presence and localisation of AQP3, AQP7 and AQP11 in boar and bull sperm, the subsequent aim of the present dissertation was to analyze whether any relationship between sperm quality and these three proteins existed. In the first instance, this relationship was studied in boar spermatozoa (**PAPER I** and **PAPER II**) by correlating the relative levels of AQP3, AQP7 and AQP11 and different sperm quality parameters evaluated in extended boar semen. These parameters were plasma membrane integrity (evaluated through SYBR14/PI), membrane lipid disorder (M540/YO-PRO-1) and acrosome integrity (PNA-FITC/PI). While relative levels of AQP3 and AQP7 were not found to be correlated with any of the sperm parameters evaluated, relative AQP11-content was significantly and positively correlated with sperm motility and integrity evaluated through % $SYBR14^{+}/PI^{-}$, % $PNA\text{-}FITC^{-}/PI^{-}$, % $M540^{-}/YO\text{-}PRO\text{-}1^{-}$ and % of total and progressive motile spermatozoa. In contrast to these results, Chen *et al*. (2011) demonstrated that an *Aqp3*-knockout mouse model presented normal motility but higher susceptibility to face a hypotonic ambient (i.e. the female reproductive tract; ~310 $mOsm \cdot Kg^{-1}$). The sperm produced by this *Aqp3*-null mouse model showed an increment in tail bending after entering the uterus, which hindered the sperm migration within the oviduct and resulted in decreased fertilization rates. With regard to AQP7, whilst some studies conducted in human related the distribution of this protein with sperm motility and morphology (Yeung *et al*. 2010; Moretti *et al*. 2012), other works conducted in mice found that AQP7 was not indispensable for the regulation

of sperm function and fertility (Kondo *et al.* 2002; Yang *et al.* 2005; Sohara *et al.* 2007; Yeung *et al.* 2009). Indeed, a genetic deletion of AQP7 did not produce apparent defects in sperm. All that being said, and despite the fact that AQP3 and AQP7 have not been found to be related with the sperm quality of fresh/extended boar semen in this study, another crucial issue is the role of these two GLPs as glycerol transporters. As glycerol is the most used permeable CPA, it could be that these two GLPs were related with the sperm resilience to withstand freeze-thawing procedures. The present Thesis also purported to address such a relationship in both boar and bull sperm, and this issue will be taken it up again later in this Discussion. As far as AQP11 is concerned, Shannonhouse *et al.* (2014) conducted a study in which they identified different changes in the testicular transcriptome of Syrian hamster related to photoperiodic modulation of fertility. In this work, they found that transcript levels of *Aqp11* were positively correlated with those of three genes (*Catsper1*, *Pgk2*, and *Tnp2*) involved in the regulation of sperm motility. These findings are in agreement with the present Thesis and could contribute to explain why relative AQP11-content in boar spermatozoa is positively correlated with total and progressive sperm motility, at least in fresh semen. Thus, the differences in the relationship between AQP3, AQP7 and AQP11 amounts and sperm-quality parameters appear to be species-specific. Finally and still dealing with fresh/liquid-stored semen, the present dissertation has also investigated fresh bull semen. Apart from the fact that no correlation was observed between sperm quality parameters and the relative levels of AQP3, AQP7 and AQP11, bull semen is mainly stored in frozen state, so that emphasis is here made on the putative role of these three AQPs in the cryotolerance of bovine semen.

Semen preservation technologies seek to keep the sperm fertilizing capacity for a period whose duration depends on the method and material used. In the case of long-term conservation, frozen storage in liquid nitrogen (LN_2) is the most used system (reviewed by Rodríguez-Gil, 2006). Nevertheless, in spite of the potential advantages of long-term semen storage, porcine sperm are notoriously sensitive to cold temperatures, because of the low cholesterol/phospholipid ratio of their membranes (reviewed by Johnson et al. 2000 and Bailey *et al.* 2008). For this reason, the method for routine sperm preservation in pigs (up to 12-15 days) is liquid-storage at 15-17°C (reviewed by Rodríguez-Gil, 2006). Moreover, the timing of ovulation in the pig is another important factor to consider. Indeed, as this process can occur over a long-estrus window, boar spermatozoa may be required to survive up to 40 h in the oviduct. This represents an inconvenient for frozen-

thawed sperm, as their lifespan is dramatically decreased and thus reproductive performance is significantly lower than that of fresh/extended semen (Alm-Packalén, 2009). In contrast, bull sperm cryopreservation is widely and commonly used in the bovine breeding industry (Pons-Rejraji *et al*. 2009). Indeed, oppositely to other species, such as the pig, bull sperm are characterized by superior cryoresistance which enables them to efficiently survive cryopreservation (Slowińka *et al*. 2008). All these factors explain the use of fresh/extended semen in pigs and of cryopreserved semen in cattle. For that reason, and as aforementioned, we were mainly focused upon the relationship of AQP3, AQP7 and AQP11 with cryopreserved rather than fresh bull semen.

As explained in the Introduction of the present dissertation, the role of some AQPs is not restricted to the transport of water but these proteins are also involved in the traffic of certain small, uncharged solutes such as glycerol, urea, ammonia and arsenite (Borgnia *et al*. 1999; Liu *et al*. 2002; Saparov *et al*. 2007), enabling a 10-100 fold increase in transport rates across plasma membranes (reviewed by Agre *et al*. 2002). It is well established that the permeability of the plasma membrane to water and cryoprotectants (CPAs) is crucial for cell survival during cryopreservation (Tan *et al*. 2013). Hence, given the role of AQPs in water transport (Barcroft *et al*. 2003; Knepper *et al*. 2004) and taking into account that glycerol has been the most used CPA for freezing sperm from most animal species (reviewed by Holt, 2000a) including boar (at concentrations of 2-3%; Bianchi *et al*. 2008) and bovine semen (Forero-Gonzalez *et al*. 2012), we hypothesized that AQPs could be involved in sperm cryotolerance (**PAPER III**, **PAPER IV** and **PAPER V**).

In order to address whether variations in the relative content of AQP3, AQP7 and AQP11 in fresh sperm could be related to the sperm resilience to withstand freeze-thawing procedures, the relative amounts of these three proteins were evaluated by immunoblotting in fresh and in frozen-thawed boar and bull sperm. Furthermore and according to the literature, sperm cryotolerance highly varies between species, individuals from the same species, ejaculates from the same individual and even between fractions of the same ejaculate (Holt *et al*. 2005; Peña *et* al. 2006; Waterhouse *et al*. 2006; Dorado *et al*. 2010; Yeste *et al*. 2015). Taking this into account, we also found interesting to study whether boar and bull ejaculates with higher cryotolerance were those that presented higher relative levels of AQP3, AQP7 and AQP11 in fresh sperm (**PAPER III**, **PAPER IV** and **PAPER V**). This kind of research also has a practical angle, as identifying the ejaculates of poor freezability before freeze-thawing procedures take place may avoid the

extra costs incurred when PFE are cryopreserved. In this case, and following an approach previously deployed in other studies of our research group (Casas et al. 2009; Yeste et al. 2013a; 2014a; Vilagran et al. 2013; 2014), boar and bull ejaculates were classified into GFE and PFE groups through a hierarchical cluster analysis and on the basis of their viability and motility at post-thawing.

For AQP3, our data demonstrated that the relative amounts of this protein were significantly higher in GFE than in PFE in both fresh and frozen-thawed boar sperm. Furthermore, the relative abundances of AQP3 in fresh/extended boar semen were significantly correlated with sperm viability at 30 and 240 min post-thawing and with progressive and total motility assessed at 30 min post-thawing **(PAPER III)**. In contrast, relative AQP3-content in bull sperm did not differ between GFE and PFE either before or after freeze-thawing and relative AQP3-amounts were not correlated with any bull sperm parameter at post-thawing. Therefore, although the present dissertation had hypothesized that AQP3 could be involved in the cryotolerance of boar and bull spermatozoa, this hypothesis was only confirmed in the case of boars **(PAPER III)** but not in that of bulls **(PAPER IV)**.

The relationship of AQP3 with boar sperm cryotolerance could be explained by their properties as a member of the aquaglyceroporin subfamily. AQP3 has been identified in reproductive organs such as uterus, cervix, ovary, placenta, fetal membrane, epididymis and prostate (reviewed by Zhang *et al*. 2012). In addition, their presence in mouse and pig oocytes (Edashige *et al.* 2003; Morató *et al.* 2014), pig expanded blastocysts (Jin *et al*. 2013), and mice morulae (Edashige *et al.* 2006) has also been reported to play an important role during cryopreservation, as their exogenous expression increases the cell permeability to water and CPA resulting in higher tolerance to cryopreservation. Furthermore, two studies conducted by Chen & Duan (2011) and Chen *et al*. (2011) described this protein as a part of an osmosensing system responsible of detecting early events in cell swelling. These researchers described that the lack of this protein is related with a higher vulnerability to physiological hypotonic changes occurring upon sperm entrance into the female reproductive tract and with sperm tail deformation, which hampers sperm motility and ultimately reduces fertilizing ability. Thus, all these studies demonstrated the importance of this protein in mammalian reproduction. Taking into account the participation of AQP3 in all these processes, it is not surprising that this protein does also seem to be involved in the cryopreservation success of boar sperm.

On the other hand, it is not possible, at this moment, to explain why AQP3 has not been found to be related to bull sperm cryotolerance. Unfortunately, no data regarding the role of AQP3 on mammalian sperm cryopreservation is available. When trying to formulate a hypothesis to explain the results obtained in **PAPER IV**, which denote that there is no relationship between the relative content of AQP3 in fresh bull semen and the sperm resilience to withstand cryopreservation, we should consider that the expression of *AQP3* was not altered when bovine embryos were exposed to a hypertonic medium during in vitro culture (Camargo *et al.* 2011). Therefore, the fact that AQP3 is not related to the response of bovine embryos to high hypertonic media could be an explanation on why bull sperm with higher relative AQP3-abundance do not exhibit better cryotolerance when exposed to a cryopreservation medium of high osmolality.

The relative levels of AQP7 in fresh boar sperm were significantly higher in GFE than in PFE and were also found to be correlated with the sperm viability and progressive motility evaluated at post-thawing. Similar results were obtained in bull spermatozoa as, again, fresh ejaculates with higher cryotolerance (GFE) and thus higher sperm survival following freeze-thawing presented higher relative amounts of AQP7 than PFE. In addition, another statistical approach which consisted of a combination of PCA and linear regression analyses showed that the relative abundances of AQP7 in fresh bull sperm could predict the sperm survival at post-thawing. In a similar fashion to the case of AQP3, the relationship of AQP7 with sperm cryotolerance of both boar and bull ejaculates could also be explained by its properties as a glycerol transporter. In fact, cryoprotectants have been reported to up-regulate the expression of AQP7 in mouse oocytes but not that of AQP3 and AQP9 which are also GLPs (Tan *et al.* 2013). The increased expression of this protein facilitates water diffusion and is essential for the cell to face the osmotic shock linked to cryopreservation via Aurora A/CPEB phosphorylation mediated by PI3K and PKC pathways (Tan *et al.* 2013). The present Thesis has found that ejaculates with higher relative amounts of AQP7 exhibit higher cryotolerance and thus, their sperm survival at post-thawing is higher. Therefore and bearing in mind that spermatozoa are transcriptionally silent cells and that the osmolality of freezing medium LEYGO (1,650-1,750 $mOsm \cdot Kg^{-1}$) is very high, the fact that a given spermatozoon has more relative levels of AQP7 makes it more resilient to withstand the osmotic shock and thus freeze-thawing procedures.

Regarding to AQP11, their levels were higher in GFE than in PFE but not significantly different either in fresh or frozen-thawed sperm. Besides, relative AQP11-

abundances in fresh or frozen-thawed sperm were found not to be significantly correlated with sperm quality at post-thawing. This indicates that, for the time being, AQP11 is not able to predict boar sperm cryotolerance. Related to this, it is noticeable that, as aforementioned, the present Thesis also demonstrated that relative AQP11-content was positively correlated with sperm quality in boar fresh semen (Prieto-Martínez *et al.* 2016a; **PAPER I)**. As conventional sperm analyses are not useful to predict boar sperm freezability (Hernández *et al*. 2006; Casas *et al*. 2009), one should not be surprised by the fact that a protein marker that is related to conventional quality parameters in fresh semen has no relationship with sperm cryotolerance. While conventional sperm quality parameters fail to detect GFE and PFE because they do reflect the sperm physiology at 37ºC rather than contemplating the cell mechanisms facing adverse/stressful conditions (reviewed by Yeste, 2016), further research should investigate which the exact role of AQP11 in the physiology of epididymal, ejaculated/liquid-stored and capacitated sperm is. On the other hand, and as far as the results obtained in bulls are concerned, a significant and positive correlation was observed between sperm quality evaluated at post-thawing and relative AQP11-levels in fresh and frozen-thawed sperm. In addition, both before and after freeze-thawing, GFE presented significantly higher relative AQP11-levels than PFE. Therefore, bull spermatozoa with low relative AQP11-amounts appear to be less able to withstand freeze-thawing procedures.

Different studies have been reported the expression of AQP11 in several rat tissues, such as kidney, liver, testes and brain (Gorelick *et al.* 2006) and in human adipocytes, where despite AQP11 not belonging to the aquaglyceroporin subfamily, it has been suggested to be a glycerol channel (Madeira *et al.* 2014). A main inconvenient when trying to find an explanation for the role of AQP11 during sperm cryopreservation is that fact that this protein is one of the latest discovered AQP family members, whose function has been difficult to set due to its primarily intracellular location. Taking this into account, AQP11 channels could be important to keep organelle viability after cryopreservation, when intracellular compartments are subjected to dehydration and to rehydration. Nevertheless, considering our data, the implication of AQP11 in boar sperm cryotolerance has not been clearly established in the present Thesis **(PAPER III)**. In contrast, this protein plays an important role in bull sperm cryotolerance. Therefore, more research is warranted to elucidate whether a high number of boar samples should be evaluated to confirm the small differences between GFE and PFE or the ability of AQP11 to predict ejaculate freezability is species-specific. This latter hypothesis would also be

in agreement with the fact that AQP3 is related with boar but not with bull sperm freezability. In fact, it is quite likely that the sperm resilience to withstand freeze-thawing protocols does not rely only upon a specific AQP but rather more than one, and that the combination of these AQPs is species-specific (AQP3-AQP7 in boars and AQP7-AQP11 in bulls).

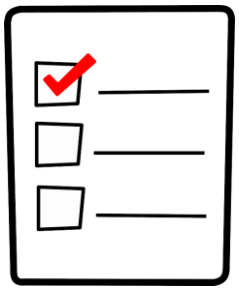
Cryopreservation is an efficient way to store spermatozoa and plays a major role in genetic improvement, economization of breeding programs in the livestock industry and preservation of endangered species (Yoon *et al*. 2015). Nevertheless, although this technique is extensively used in cattle industry for AI purposes since it produces the same calving rates than those of fresh semen (Hiemstra, 2005), the use of frozen-thawed sperm for AI is limited in swine because the fertility of cryopreserved semen is significantly lower than that of fresh semen (Roca *et* al. 2006). In order to improve the success of this technique, several studies have been focused on the research of protein markers linked to the sperm resilience to withstand freeze-thawing procedures (Thurston *et al.* 2002; Casas *et al.* 2009; 2010; Vilagran *et al.* 2013; 2014; 2015). It is in this context that the results obtained in the **PAPER III** are of great interest. In effect, these results support the reliability of AQP3 and AQP7 as cryotolerance markers in boar sperm as both are involved in sperm cryotolerance and are able to predict the ejaculate freezability. Regarding bull sperm, the findings reported herein could also have practical implications since, as aforementioned, frozen-thawed sperm is routinely used for AI (Curry, 2000). Therefore, we suggest that the relative abundances of AQP7 and AQP11 in bull sperm could be used as cryotolerance markers and that quantitative analysis of these proteins in fresh sperm could contribute to select the semen samples with higher quality prior to cryopreservation and AI (Daigneault *et al.* 2015; Lecewicz *et al.* 2015) (**PAPER IV** and **PAPER V)**. Finally and still related with the reliability of AQP11 as a cryotolerance marker, one of the most interesting results obtained in the present work is the capability of this protein to predict the fertilizing ability of frozen-thawed bull sperm **(PAPER V)**. In this sense, we investigated whether the relative AQP11-abundance in fresh bull semen was associated with the ability of frozen-thawed bull sperm to penetrate *in vitro* matured bovine oocytes. The results showed that relative AQP11-abundance in fresh semen is correlated with penetration rates of frozen-thawed sperm and that penetration rates were significantly higher in GFE than in PFE. Hence, under a practical point of view the analysis AQP11 could also contribute to improve the selection of higher quality semen samples prior to AI. Nevertheless, this topic warrants further research in order to ascertain

whether the relationship of relative AQP11-levels with fertilizing ability of frozen-thawed bull sperm was due to the role exerted by this protein during freeze-thawing or it was due to the previous selection of intact sperm through discontinuous gradient washing, with higher recovery in GFE than in PFE. This could be addressed by determining the relationship between relative AQP11 levels and penetration rates of fresh bull semen. In addition, further research is also necessary to determine whether a correlation between relative AQP11-content in fresh semen and reproductive outcomes following AI with frozen-thawed bull sperm also exists. On the other hand, taking into consideration that around 99% of semen doses used for pig AI are refrigerated (reviewed by Johnson *et al*. 2000), it could also be interesting to investigate the relationship between the relative AQP11-abundance in fresh/extended boar semen of boars and IVF and AI outcomes.

Future studies should also scrutiny the suitability of other methods such as ELISA. This technique can be used for quantitative determination of proteins and represents a more accurate estimation than semi-quantitative approaches such as Western blotting. This would be followed by the development and optimization of kits determining the quantity of AQP3, AQP7 and AQP11 in fresh boar and bull semen, which could allow ultimately selecting the samples with higher cryotolerance and/or the fertilizing ability, thereby reducing cryopreservation costs and optimizing the use of seminal doses both in porcine and bovine.

Taking all above into consideration, we can conclude that the present dissertation has reported, for the first time, the existence and localisation of AQP3, AQP7 and AQP11 in both boar and bull sperm. Moreover, localisation patterns for these three AQPs have been reported to present species-specific peculiarities, since their distribution differs between mammalian species. On the other hand, although cryopreservation does not induce major changes in the localisation patterns of AQP3 and AQP11 in either boar or bull sperm, it appears to alter that of AQP7 at least in boars. As far as the relationship between sperm quality and these three proteins is concerned, only relative AQP11-levels are significantly correlated with sperm motility and membrane integrity in fresh/extended boar sperm. In addition, regarding the relationship between these three AQPs and sperm cryotolerance, the present dissertation has demonstrated that relative AQP7-content in fresh sperm is related to their resilience to withstand freeze-thawing procedures and is able to predict ejaculate freezability in boars and bulls. On the other hand, while AQP3 is involved in the cryotolerance of boar spermatozoa, AQP11 is only related to that of bull

spermatozoa. Finally, bull fresh ejaculates with higher amounts of AQP11 seem to exhibit also higher *in vitro* fertilizing ability at post-thawing.

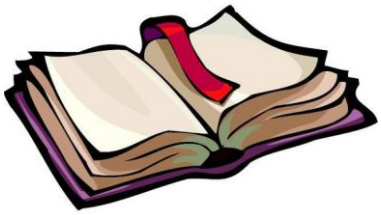


Conclusions

Conclusions

1. AQP3, AQP7 and AQP11 are present in boar and bull spermatozoa and their localisation are species-specific **(PAPER I**, **PAPER II**, **PAPER IV** and **PAPER V**).
2. AQP3, AQP7 and AQP11 have a homogeneous distribution in the ejaculated sperm of both species and cryopreservation procedures do not affect the localization of these proteins, except for AQP7 in boars (**PAPER I**, **PAPER II**, **PAPER III**, **PAPER IV** and **PAPER V**).
3. While relative amounts of AQP11 are significantly correlated with the quality of boar extended semen, AQP3 and AQP7 are correlated with no sperm quality parameter (**PAPER I** and **PAPER II**).
4. Relative levels of AQP3 in fresh boar semen are significantly higher in GFE than in PFE. Thus, the semen from boars with high AQP3 content should be used for cryopreservation procedures (**PAPER III**).
5. In contrast, relative AQP3-content in fresh bull sperm does not differ between GFE and PFE. Therefore, AQP3 is discarded as freezability marker for bull sperm (**PAPER IV**).
6. Relative abundance of AQP7 in fresh of both boar and bull spermatozoa is significantly higher in GFE than in PFE. Therefore, AQP7 is a marker for boar and bull sperm freezability, as predicts the sperm ability to withstand freeze-thawing protocols before these procedures take place (**PAPER III** and **PAPER IV**).
7. The amounts of AQP11 in fresh boar semen do not differ significantly between GFE and PFE. Therefore, AQP11 content is not able to predict boar sperm cryotolerance (**PAPER III**).
8. In contrast, in fresh bull sperm, GFE presented significantly higher relative AQP11-levels than PFE. Thus, this protein predicts the ejaculate freezability in bull fresh sperm (**PAPER V**).
9. The relative AQP11-abundance in fresh bull semen is associated with the ability of frozen-thawed bull sperm to penetrate *in vitro* matured bovine oocytes and penetration rates are significantly higher in GFE than in PFE (**PAPER V**).

10. While the results obtained in the current dissertation support the relevance of AQPs for boar and bull sperm cryopreservation, further research is warranted to address through which precise mechanism each AQP exerts its function and to explain why, whereas the involvement of AQP7 in sperm cryotolerance is concurrent in both species, that of AQP3 and AQP11 differs.



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