A MOLECULAR APPROACH ON SPERM CHANGES DURING EPIDIDYMAL MATURATION, EJACULATION AND IN VITRO CAPACITATION OF BOAR SPERMATOZOA.
APROXIMACIÓ MOLECULAR ALS CANVIS QUE ES PRODUEIXEN DURANT LA MADURACIÓ EPIDIDIMÀRIA, EJACULACIÓ I CAPACITACIÓ *IN VITRO* EN ESPERMATOZOIDES PORCINS

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Dipòsit legal: Gl. 1387-2012
http://hdl.handle.net/10803/84064

Universitat de Girona

DOCTORAL THESIS / TESI DOCTORAL

A molecular approach on sperm changes during epididymal maturation, ejaculation and *in vitro* capacitation of boar spermatozoa

Aproximació molecular als canvis que es produeixen durant la maduració epididimària, ejaculació i capacitació *in vitro* en espermatozoides porcins.

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2012

Programa de Doctorat en Tecnologia

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Memòria presentada per optar al títol de Doctora per la Universitat de Girona

La realització d'aquest treball ha estat possible gràcies a la beca de recerca de la UdG (BR07/12)

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Que aquesta tesi titulada " **A molecular approach on sperm changes during epididymal maturation, ejaculation and in vitro capacitation of boar spermatozoa"**, presentada per Anna Fàbrega Coll per optar al grau de Doctora per la Universitat de Girona, s'ha realitzat sota la nostra direcció i, considerant aquesta acabada, autoritzem la seva presentació perquè sigui jutjada per la Comissió corresponent.

I per tal que així consti als efectes que correspongui, firmen la present,

Dr. Sergi Bonet Marull Dra Elisabeth Pinart Nadal

La doctorand : Anna Fàbrega Coll

Girona, gener 2012

A vosaltres família

A vosaltres Quimeta i Núria

Agraïments

Arribat a aquest apartat, que és l'últim en escriure's però el primer en aparèixer, només em queda agrair-vos a tots els que heu fet possible que hagi arribat aquí. De ben segur que la meva memòria despistada oblidarà a algú però espero haver estat més desperta el dia a dia i haver-vos sabut agrair la vostra ajuda en cada moment, perquè sigui "gràcies, gracias, merci, thanks..." us ho ben mereixeu!

En primer lloc, vull agrair-te, Sergi, l'oportunitat i la confiança que vas tenir amb mi des del primer dia que vaig aparèixer pel vostre despatx. En aquell moment no ho sabia, però ara, puc afirmar que no només em vas donar la possibilitat d'aprendre i formar-me al laboratori sinó l'oportunitat de conèixer i compartir uns anys "estupendos" amb un gran equip humà. Gràcies de veritat!

A tu, Beth, et dec un agraïment molt especial per la teva ajuda i la teva paciència en cadascuna de les etapes i correccions però sobretot per entendre'm i valorar-me en cada pas d'aquest camí, gràcies per creure en mi!

A vosaltres, Mailo i Sílvia, gràcies perquè des del primer moment m'heu acollit i fet sentir part d'aquesta "família".

A vosaltres TS, perquè sou les/els "millors"!!, perquè seguiu ajudant-me a evitar que allò que "sovint la distància fa l'oblit" no es compleixi... us trobo i us trobaré sempre a faltar! Evita, la fada multilingüe de Sant Martí Sapresa! "Danke" pels tants moment des de Bremen fins al Lab 3; Marc, perquè has estat molt més que un bon promotor per a mi, merci!!! Marta P, gràcies per tot el que m'has ensenyat, perquè ets molt bona en la feina i encara millor com a persona, no ho oblidis ni ara ni mai! Estela, des de Fricorsa fins aquí, qui ens ho havia de dir, somriu i no deixis de creure en tu i endavant! Eva T, no puc imaginar-me una millor companyia en tants trajectes dins i fora del cotxe (sense desmerèixer el pobre Misto), per a mi "ni ets normal, ni ets molar" ets una amiga de veritat, gràcies, gràcies i gràcies per escoltar-me, i sobretot per fer-me reflexionar! Míriam, ets la més "petita" de la meva "mainada" i amb qui no sols comparteixo algunes manies de "sagitari".... és genial adonar-te amb els anys com algú pot escoltar i valorar el que dius, gràcies per obrir-me els ulls! Lili, ningú com tu fa anar millor la impressora, les PCRs i els "bitxos" pel laboratori, ni ningú com tu sap treure el millor somriure quan més el necessites, ànims i molta sort perquè et mereixes el millor! Eva Flores, qui m'havia de dir en aquelles visites a l'UAB que al final acabaríem compartint poiata i tants moments, força, molta força per seguir endavant i Marrecs! Íngrid, hem compartit pocs moments però potser dels més intensos, molta sort per endavant i gaudeix d'aquest grup, suposo que a hores d'ara ja has pogut comprovar que són genials! Isa, ja et pensaves que m'oblidava de tu Dra. Houses Little Rock, ... tu ets sense dubte un dels millors miralls en el que una es voldria veure reflectida, que et puc dir que no sàpigues ja si ets la meva "jefa petita".

Anna P, tot i que varem compartir poc temps en aquest llarg camí, no vull deixar d'agrair-te aquells primers mesos pels teus consells i la teva ajuda que m'han acompanyat al llarg dels anys. Cesca, Gabi, Ferran i Sara, Yolanda i Anna C, personal del PCiT, de BioGirona i de la Facultat de Ciències que en petits o llargs períodes de temps heu estat una gran ajuda i sovint us he demanat impossibles i tot i així ho heu

aconseguit, moltes gràcies! A la resta de becaris de Ciències que tot i estar lluny sempre us recordeu de les del Parc, als companys de Biologia amb qui continuem compartint l'esperit biòleg cada 31 d'octubre, als del màster, amb els quals vaig iniciar aquest camí, als companys de l'UAB que sovint heu patit les nostres visites i els nostres dubtes, a tots i a cadascun dels que heu passat per TS en pràctiques, en estades, ... com estudiant o com a companys, i als nous companys de feina, amb qui des de fa quasi un any comparteixo una bona part del dia, a tots, moltes gràcies! I com no a vosaltres, "petites", també us dec un especial agraïment perquè sempre i sou Mireia i Núria!

Je ne peux pas vous oublier. Je remercie toutes les personnes d'INRA à Nouzille, Tours, (Benoît, Guillaume, JL Gatti, Lucie, Valérie, Xavier...) ainsi que mes camarades de l'Auberge de Jeunesse (Agnieszka, Eveline, Hayat, Hannah, Huber...). C'est avec vous que j'ai beaucoup appris au labo, que j'ai fait une de mes meilleures expériences de ma vie. Je n'oublierais jamais votre chaleureux accueil et vous avez fait le nécessaire pour que je me sente pendant ce séjour comme chez moi. Je remercie spécialement Jean-Louis et Françoise qui m'ont montré à aimer la France et avec qui j'ai découvert les petits trésors de la Vallée de la Loire.

A Santa Pau, a vosaltres, als de sempre i per sempre! Gràcies per ser la "colla" (Anna, Esther, Gemma, Jordi, Maion, Marc, Nuri, Núria, Oriol, Sandra, Tt, Vero,... i tota la resta que us aneu incorporant), per ser-hi aquí i en la distància! I a les garrotxines que des de l'insti sempre busquem aquell moment per passar juntes!

I finalment vosaltres família, a qui dedico l'agraïment més profund perquè sou el que més estimo, sobretot vosaltres pares (Maria i Pere) i germà (Albert), però també als avis, tiets i cosins que sovint heu estat molt més que això passant a ser uns pares i uns germans més per mi. Sou el meu pilar més sòlid i la pota que mai em falla.

A tots, **moltíssimes gràcies**!

"Sovint em sento atrapat
pel meu propi malson
i tinc ganes de cridar
cada dia en algun lloc
surt el tren de mitjanit [...]

[...] Si ets llunàtic i estàs espantat
si vius als núvols i estàs deprimit
si la boira ja t'ha acompanyat
cada dia en algun lloc
pots pujar el tren de mitjanit.

N'he conegut molts com tu i jo
és bo saber que no estem sols,
això és bo.
No sempre s'està de sort
no sempre trobaràs el mar
darrera el port [...]"

Sau- El tren de mitjanit (1991)

List of Papers

This thesis is presented as a compendium of four papers:

Paper I

Fàbrega A, Puigmulé M, Bonet S & Pinart E
"Epididymal maturation and ejaculation are key events for further in vitro capacitation of boar spermatozoa."
Accepted for publication in Theriogenology 28/03/12. (Impact factor 2,045; Q1 Veterinary Sciences)

Paper II

Fàbrega A, Puigmulé M, Yeste M, Casas I, Bonet S & Pinart E
"Impact of epididymal maturation, ejaculation and in vitro capacitation on tyrosine phosphorylation patterns exhibited of boar (*Sus domesticus*) spermatozoa"
Theriogenology 76 (2011) 1356–1366. (Impact factor 2,045; Q1 Veterinary Sciences)

Paper III

Fàbrega A, Puigmule M, Dacheux J-L, Bonet S & Pinart E
"Glycocalyx Characterization and Glycoprotein expression of *Sus domesticus* Epididymal Sperm Surface Samples"
Reproduction, Fertility and Development (Impact factor 2,553; Q1 Zoology)
http://dx.doi.org/10.1071/RD11064.

Paper IV

Fàbrega A, Guyonnet B, Dacheux J-L, Gatti J-L, Puigmule M, Bonet S & Pinart E
"Expression, immunolocalization and processing of fertilins ADAM-1 and ADAM-2 in the boar (sus domesticus) spermatozoa during epididymal maturation"
Reproductive Biology and Endocrinology, 2011, 9 96. (Impact factor 1,695; Q3 Reproductive Biology)

Abbreviations

ACE	Angiotensin converting enzyme I
ADAM-1 and ADAM-2	Fertilin alpha and beta (or PH-30 alpha and beta)
ADAM-3	Cyritestin 1
α-Fuc	α-fucose
α- and β-Gal	α- and β-galactose
α- and β-GalNAc	α and β-N-acetyl-D- galactosamine
α- and β Glc	α-D- and β-glucose
α- and β-GlcNAc	α and β-N-acetyl-D-glucosamine
α-Man	α-D-manose
ALH	Amplitude of lateral head displacement
AP	Alkaline phosphatase
APS	Ammonium persulfate
AR	Acrosome reaction
BCF	Beat cross frequency
BME, 2BME or β-met	2-Mercaptoethanol, β-mercaptoethanol or 2-Hydroxy-1-ethanethiol
BSA	Bovine serum albumine
BTS	*Beltsville* thawing solution
cc	Cubic centimetre or cubic centimeter
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
CETP	Cholesteryl ester transfer protein
CRISP	Cysteine-rich secretory proteins
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DTT	Dithiotreitol
E-3	Epididymis-specific secretory protein 3
EDTA	Ethylenediaminetetraacetic acid
E-RAPB	Epididymal retinoic acid-binding protein
FI	Fluorescence intensity
FITC	Fluorescein isothiocyanate-labelled
FL1, FL2, FL3	Flow cytometer photodetectors 1, 2 and 3
Fluo-3 AM	Fluo-3-acetomethoxy ester
FSC	Forward-scatter
Fuc	Fucose
gACE	Gametic form of angiotensin converting enzyme I
Gal	Galactose
GalNAc	N-acetylgalactosamine

Glc	Glucose
GlcNAc	N-acetylglucosamine
Glc/Man	glucose/mannose
GST-P	Glutathione S-transferase P
GPx	Glutathione peroxidase
HEP64	Hamster epididymal protein 64
HPA	*Helix pomatia* agglutinin
HRP	Horseradish peroxidase
IEF	Isofocalization
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
KCl	Potassium chloride
$KHCO_3$	Potassium bicarbonate
KH_2PO_4	Potassium dihydrogen phosphate
LCA	*Lens culimaris* (lentil) agglutinin-A
LF	Lactoferrin
LIN	Linearity index percentatge
M540	Merocyanine 540
Man	Mannose
MEPs	Mouse epididymal proteins
MOT	Total motility percentage
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Disodium hydrogen orthophosphate
$Na_3C_6H_5O_7{\cdot}2H_2O$	Sodium citrate tribasic dihydrate
Na_3VO_4	Sodium orthovanadate (sodium tetraoxovanadate(V)),
Neu5Ac	N-acetylneuraminic acid
P26h	Hamster sperm protein of 26 kDa
P47	Lactadherin P47
p95	95-kDa protein
PBS	Phosphate buffer saline
pCD	Procathepsin D
PGDS	Prostaglandin D2 syntase
PH-20	Hyaluronidase PH-20(or SPAM1)
PH-30 alpha and beta	Fertilin alpha and beta (or ADAM-1 and ADAM-2)
PHA-L	*Phaseolus vulgaris* (red kidney bean) leucoagglutinin
PI	Propidium iodure
PMSF	Phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
PNA	*Arachis hypogae* (peanut) agglutinin
PRG	Progressive motility percentage
PSA	*Pisum sativum* (pea) lectin

PVDF	Polyvinylidene fluoride
sACE	Somatic form of angiotensin converting enzyme I
SBA	*Glycine max* (soybean) agglutinin
SDS	Sodium dodecyl sulphate
SMA-4	Sperm maturation antigen 4
SOD	Superoxide dismutase
sp17	Sperm surface protein of 17 kDa
sp38	Sperm surface protein of 38 kDa
sp56	Sperm surface protein of 56 kDa
SPAM1	Sperm adhesion molecule 1 (or PH-20)
Spz	Spermatozoon/ Spermatozoa
SSC	Side-scatter
STR	Straightness index percentage
TBS	Tris buffer saline
TEMED	Tetramethylethylenediamine
TPx	Thioredoxin peroxidase
Tween 20	Polysorbate 20
UEA-I	*Ulex europaeus* agglutinin I
VAP	Average velocity
VCL	Curvilinear velocity
VSL	Straight-linear velocity
WGA	*Triticum vulgari* (wheat) germ agglutinin
WOB	Oscillation index percentage
YP1	Yo-Pro®-1
ZP	Zona pellucida

Contents

Versió en català "Aproximació molecular als canvis que es produeixen durant la maduració epididimària, ejaculació i capacitació *in vitro* en espermatozoides porcins."

Abstract

Mammalian spermatozoa acquire functionality during epididymal maturation and ability to penetrate and fertilize the oocyte during capacitation. In the present work, the sperm quality, the phosphotyrosine and surface carbohydrate patterns of spermatozoa and, the expression and processing of a specific sperm plasma membrane protein throughout the boar epididymal duct have been assessed, in order to better understand their specific roles in the sperm maturation and/or in further stages of reproductive function, as well as to define new potential sperm maturation markers.

The accurate **quality assessment** of epididymal, ejaculated and *in vitro* capacitated spermatozoa highlighted that boar spermatozoa acquired their ability to move in the epididymal corpus; however their motility was not linear until the ejaculation. Although epididymal spermatozoa showed low membrane fluidity and intracellular calcium content, ejaculation lead to and increased calcium content, whereas membrane fluidity is maintained low. Acrosome integrity remained constant throughout the epididymal duct and after ejaculation and *in vitro* capacitation. The frequency of viable spermatozoa with intact mitochondrial sheath was higher in caput and ejaculated samples than in corpus and cauda samples; in contrast, the frequency of spermatozoa with high membrane potential was significantly higher in cauda samples. *In vitro* capacitation resulted in a decreased frequency of viable spermatozoa with intact mitochondrial sheath and an increased frequency of spermatozoa with high membrane potential in ejaculated samples. Results indicated that both **epididymal maturation** and **ejaculation** are **key events** for further **capacitation**, because **only ejaculated spermatozoa** are **capable to undergo** the set of changes leading to **capacitation**.

Assessing the phosphotyrosine patterns we demonstrated that **epididymal maturation** *in vivo* is associated with a **progressive loss of phosphotyrosine residues** of the sperm head followed by a subtle **increase** after *in vitro* **capacitation**. As cells pass from caput to cauda **epididymis**, tyrosine phosphorylation becomes confined to a triangular region over the **posterior part of midacrosome** (equatorial) region, whereas *in vitro* **capacitation** causes a spread labeling over the **whole head**. Different bands with phosphotyrosine residues were detected during epididymal maturation and after *in vitro* capacitation: 1) **93, 66 and 45 kDa** bands with specific phosphotyrosine expression in **immature spermatozoa**; 2) **76, 23 and 12 kDa** bands with specific phosphotyrosine expression in **mature spermatozoa**, they being significantly higher after *in vitro* capacitation; 3) **49, 40, 37, 30, 26 and 25 kDa constitutive** bands that increased their phosphotyrosine expression after maturation and/or *in vitro* capacitation; and 4) **28 and 20 kDa** bands with a specific phosphotyrosine expression in *in vitro* **capacitated spermatozoa**. Expression, location and proteomic results demonstrated the importance of phosphotyrosine residues in acquisition of the competent status for oocyte fertilization.

The characterization of the major sperm glycocalix carbohydrates residues, showed a significant **increase** of sperm **galactose, glucose/mannose and N-acetyl-D-glucosamine** residues **distally in the**

epididymis. Moreover, the sperm head, cytoplasmic droplet and mid-piece were recognized by most of the lectins tested, whereas only HPA and WGA bound to the principal pieces and terminal pieces of the sperm flagellum. Fourteen sperm surface proteins were observed, with different patterns of lectin expression among epididymal regions. The pattern of sperm glycocalyx modifications provides new insights into the molecular modifications associated with epididymal maturation; therefore the evaluation of **glycosiltion pattern** of ejaculated spermatozoa could be used as a **marker of epididymal maturation**.

As an example of the changes occurring during the epididymal maturation in some plasma membrane proteins, fertilin expression, location and proteomic pattern was assessed. Fertilin alpha (ADAM-1) and beta (ADAM-2) are integral membrane proteins of the ADAM family that form a fertilin complex involved in key steps of the sperm-oocyte membrane interaction. Results highlight that ADAM-1 and ADAM-2 mRNAs were **highly produced in the testis**, but also in the **vas efferens** and the **epididymis**. **ADAM-1** subunit appeared as a main reactive band of **~50-55 kDa** corresponding to the occurrence of different isoforms throughout the epididymal duct, especially in corpus region where isoforms ranged from acidic to basic pI. In contrast, **ADAM-2** was detected as several bands of ~90 kDa, ~75 kDa, ~50-55 kDa and ~40 kDa. The intensity of high molecular mass bands decreased progressively in the distal corpus where lower bands were also transiently observed, and only the **~40 kDa** was observed in the **cauda**. The presence of bands with different molecular weights likely results from a **proteolytic processing occurring mainly in the testis for ADAM-1**, and **throughout the caput epididymis for ADAM-2**. Immunolocalization showed that **fertilin migrates from the acrosomal region to the acrosomal ridge during the sperm transit from the distal corpus to the proximal cauda**. This migration is accompanied by an important change in the extractability of a part of ADAM-1 from the sperm membrane. This suggests that the fertilin surface migration may be triggered by biochemical changes induced by the epididymal posttranslational processing of both ADAM-1 and ADAM-2. Characterization of such fertilin complex maturation patterns is an important step to develop fertility markers based on epididymal maturation of surface membrane proteins in domestic mammals.

All together these results establish a basis for the **sperm assessment** (quality parameters, phosphotyrosine and carbohydrate patterns) in order to obtain reliable and robust information about **the maturative status** of the ejaculated sperm and, the also open new perspectives in the characterization of **potential proteins markers of the sperm maturation and/or fertility**, such as fertilin, glycosilation and phosphotyrosilation patterns.

Introduction

1. The boar spermatozoon

In **boars,** the mature **spermatozoon** is a gamete of 45 µm length, with three different morphological regions: the **head**, the **connecting piece (neck)** and the **tail** (Bonet *et al.* 2000) (Figure 1), and three different functional regions: 1) the sperm head, involved in sperm-oocyte interaction, 2) the tail midpiece, formed by mitochondria involved in energy production, and 3) the flagelar structure of the tail, involved in motility (Flesch & Gadella 2000).

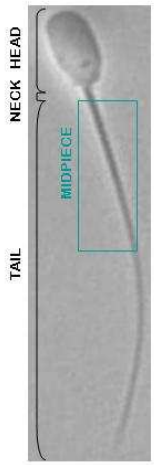


Figure 1. Mature porcine spermatozoa. Phase contrast microscope view of a mature spermatozoon obtained at x20 magnification. Three morphological regions can be distingued: the head, the connecting piece (neck) and the tail; as well as another functional region, the midpiece which contains the mitochondrial sheath.

The **head** is flat and oval and, with around 7 µm in length, 3.7 µm in wide and 0.4 µm in thick. The two faces of the head are different: one is flat and the other presents an apical protuberance like a half moon, of 0.4 µm wide and 1.2 µm long. Two major domains of the plasma membrane can be distinguished, the acrosomal region (anterior) and the postacrosomic region (posterior). The head contains the nucleus, the perinuclear fibrous material, the postacrosomal dense lamina, and the acrosome (Eddy & O'Brien 1993, Bonet *et al.* 2000).

The **nucleus** has a highly condensed and electron dense chromatin and its volume is significantly lower than that of somatic cells. Being of 6.6 µm in length, its thick increases from 220 nm in the acrosomal region to 320 nm in the postacrosomal region. The two meiotic divisions that occur during spermatogenesis result in the sperm containing only one copy of each chromosome (Eddy & O'Brien 1993, Bonet *et al.* 2000).

The **acrosome** is a vesicle localized in the acrosomal region forming a cap-like covering over the 80% of the nucleus length. The acrosomal matrix consists of an amorphous cytoskeletic material, homogeneously distributed and rich in hydrolases which participate in the acrosomic reaction and the fusion of spermatozoon and oocyte membranes (Fawcett 1975, Bonet *et al.* 2000). Despite the acrosomic membrane is continuous, two domains can be differentiated: the internal (inner) membrane and the external (outer) membrane (Fawcett 1975, Holt 1984, Bonet *et al.* 2000). The inner membrane overlies the anterior part of the nuclear envelope, while the outer membrane lies close to the inner surface of the plasma membrane in the anterior region of sperm head (Eddy & O'Brien 1993). The acrosome is divided into three morphological segments corresponding to the three plasma membrane domains of the acrosomal region: apical, principal and equatorial (Figure 2). The apical segment, in the anterior part of the acrosome, is large expanded and the acrosomic membrane overlays under the plasma membrane. The principal segment represents the major proportion of the acrosome; the equatorial segment, in the posterior part of the acrosome, is more electrondense than the rest of acrosomal regions. The apical and

principal segments together are frequently referred to as the anterior acrosome or acrosomal cap (Eddy & O'Brien 1993, Bonet *et al.* 2000).

The **perinuclear fibrous material** is a specialized cytoskeletal structure that forms a rigid net between the nuclear membrane and the acrosome. In the postacrosomic region the perinuclear fibrous material contains the **postacrosomal dense lamina** (Olson 2002), an homogeneous, fibrous and electrondense material that lies parallel beneath to the plasma membrane an overtakes 20% of the nuclear length, coinciding with the nuclear region non covered by the acrosomal vesicle (Bonet *et al.* 2000). The rigid structure of the perinuclear fibrous membrane contributes to the maintenance of the nucleus form and, it has a mechanical function during the penetration process of the spermatozoon into the oocyte; moreover, this structure also contains factors implicated in the oocyte activation (Kimura *et al.* 1998, Perry *et al.* 1999). During the epididymal transit the perinuclear fibrous sheath is highly modified: material condensation (Bedford & Nicander 1971, Jones 1971), formation of disulfur bridges that stabilize the structure (Calvin & J. 1971, Bedford & Calvin 1974), and adoption of a serrated form in the border of the postacrosomal dense lamina (Juárez-Mosqueda & Mújica 1999).

The space between the nucleus and the internal acrosomal membrane or the internal face of the postacrosomal dense lamina is designed as subacrosomic or perinuclear space. This space is occupied by a low-electrondense matrix of fibrous perinuclear material, which becomes more electrondense and more developed in the supranuclear region (Bonet *et al.* 2000).

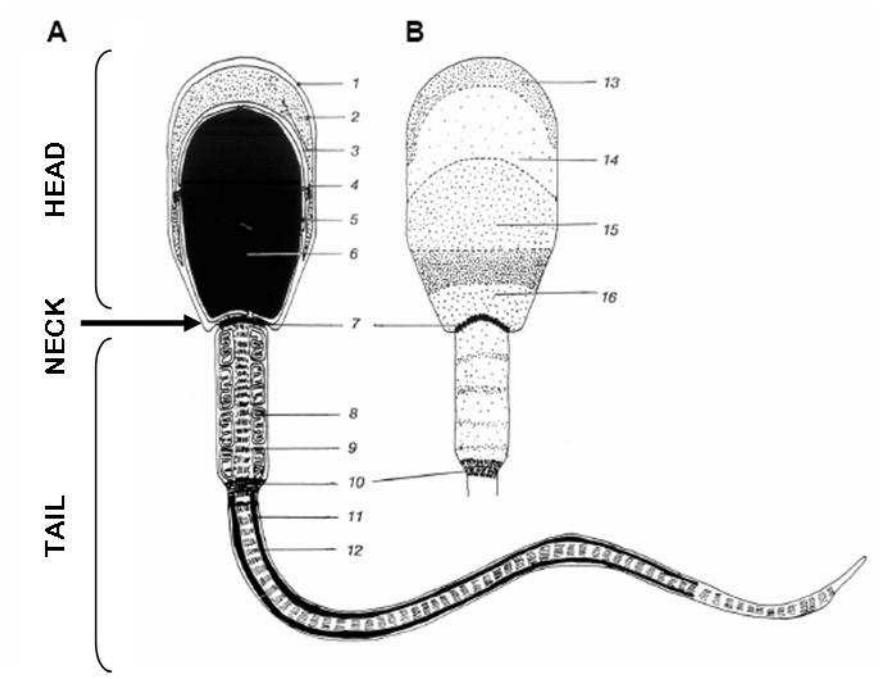


Figure 2. Schematic representation of the boar sperm cell. (A). Sectional view of the sperm cell. All solid lines represent membrane bilayers 1) plasma membrane, 2) outer acrosomal membrane, 3) acrosomal matrix, 4) inner acrosomal membrane, 5) nuclear membrane, 6) nucleous, 7) connecting piece (neck), 8) midpiece, 9) mitochondria and axoneme, 10) *annulus* (Jensen's ring), 11) fibrous sheath and 12) axoneme and outer dense fibres. (B). Surface view of the sperm head acrosomic region subdomains: 13) apical ridge, 14) principal segment and 15) equatorial segment; and surface view of the sperm head 16) postacrosomic region. Modified from Gadella, 1994 **(Gadella *et al.* 2008)**.

The **connecting** piece or **neck** of the spermatozoon is a short linking segment located between the base of the nucleus and the first mitochondrion of the midpiece. The connecting piece has the following components: basal plate, laminar bodies, *capitulum*, segmented columns, basal body, and axoneme. The basal plate consists of an electrondense material (similar to the postacrosomal dense lamina) adhered to the outer membrane of the nuclear envelope. The laminar bodies are pronounced folds of redundant nuclear envelope enclosing chromatin-free nuclear space, which begin in the perimeter of the nuclear base and terminus on the first mitochondria of the midpiece. The *capitulum* is a dome-shaped structure lying beneath the basal plate. The segmented columns arise from the extrems of the *capitulum*; initially they are attached together and form an extension of the *capitulum*, and then become individual structures forming 9 columns attached to the outer dense fibres of both midpiece and main piece. The basal body is located in the base of the convexity of the *capitulum*. From the basal body, the axoneme is organized across the space limited by the segmented columns. The axoneme presents the tipical 9+2 microtubular pattern and each peripheral microtubule doublet is associated with an outer dense fibre (Bonet *et al.* 2000).

The **tail** is filamentous and cylindrical and contains three regions or pieces (Fawcett 1975): the midpiece, the main piece and the terminal piece.

The **midpiece** stretches form the distal end of the connecting piece to the *annulus* or Jensen's ring, an electrondense ring marking the junction of the midpiece and main piece. In the midpiece and main pieces the structure formed by the central axoneme and the outer dense fibres is maintained. A mitochondrial sheath lies just beneath to the plasmalemma. The mitochondrial structure is stabilized by disulfur bridges, and peripheral granules are localized between dense fibres in the proximal region.

In the **main** piece the mitochondrial sheath is replaced by two continuous longitudinal axes or columns which are coplanar with respect the central microtubule pair, and join together by a series of circumferential ribs, forming a fibrous sheath. In the distal parts the fibrous axes begin to join (Bonet *et al.* 2000).

The **terminal** piece is the last and shortest segment of the spermatozoon's tail. This piece has no cytoskeletal structures; it is constituted by a disorganized axoneme enclosed by the plasmalemma. The axoneme disorganizes progressively as it stretches down from the principal piece (Bonet *et al.* 2000).

2. Testis and efferent ducts

2.1. Anatomy

The **testes** of all mammals are paired encapsulated ovoid organs consisting of **seminiferous tubules** separated by interstitial tissue. Their size varies depending on the species from 20-22 cm^3 in man to 0.062-0.077 cm^3 in mouse (Kerr *et al.* 2006).

Spermatozoa are formed in the seminiferous tubules and leave to the rete testis. In mammalian species, the Sertoli cells comprise the main cell type component of the seminiferous epithelium. Sertoli cells offer physical support to germ cells, providing them nutrients and growth factors, as well as protection and allowing their specific maturation and differentiation into mature spermatozoa (Amann 1989, Pinart *et al.* 2000, Pinart *et al.* 2001a). Adjacent Sertoli cells are joined by specialized occluding junctions creating a barrier (blood-testis barrier), that divides the epithelium into a basal compartment and an adluminal compartment. The basal compartment contains the early sperm-forming cells, spermatogonia and early spermatocytes. The adluminal compartment contains meiotic and postmeiotic stages of sperm development. So, the blood-testis barrier isolates the differentiating germ cells from the general extracellular fluid compartment, but it allows that some substances cross the barrier and reach the germ cells from the bloodstream.

The **rete testis** forms a complicated network of convergent tubules that connects the seminiferous tubules with the **efferent ducts** (Figure 3). During their transit, spermatozoa are involved in a fluid media, and they are carried from the dorsal pole of the testis to the epididymis' enter (Setchell *et al.* 1994).

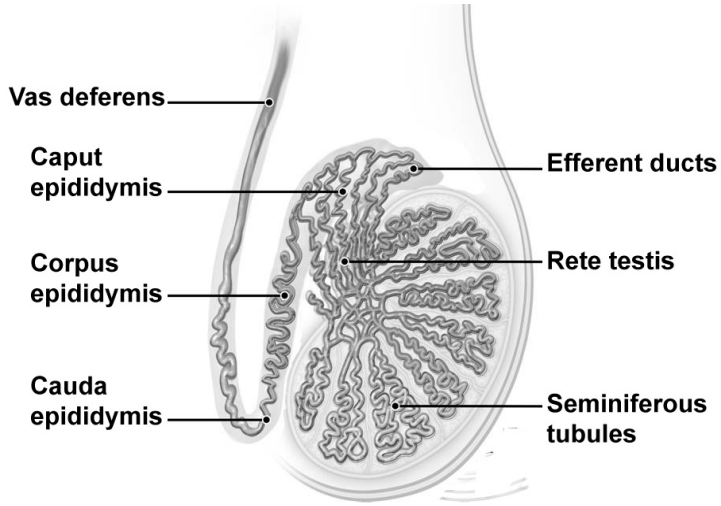


Figure 3. Schematic representation of testicular structure. View of the seminiferous tubules connected with the efferent ducts by means of the rete testis. The epididymis is composed by a single duct (caput, corpus and cauda region) that finished into the vas deferens.

In domestic animals, the number of efferent ducts ranges between 13 and 20 (Saitoh *et al.* 1990). In pigs, the efferent ducts can be divided into the proximal or intratesticular segment and the distal or epididymal segment, the structure being thicker and then longer and more flexuous. The efferent duct

epithelium is mostly composed by ciliated and non-ciliated cells, and also intraepithelial lymphocytes (Arrighi *et al.* 1993, Setchell *et al.* 1994). Efferent duct cells have a low capacity for protein synthesis and secretion, but non-ciliated cells appear to be actively involved in fluid re-absorption and in fluid-phase (not modulated by extracelular ligands or by other known natural inducers) and adsorptive (modulated by a ligand binding to plasma membrane receptors) endocytosis (reviewed in (Setchell *et al.* 1994)).

2.2. Spermatogenesis

The main function of testis is the **production** of **male gametes** and steroid hormones. The term **spermatogenesis** concerns all the set of processes by which spermatozoa are formed from spermatogonial cell population in the seminiferous tubules of adult males (Figure 4). In domestic pig (*Sus domesticus*), differing to his ancestor, wild pig (*Sus scrofa*), this process takes place along all the year, decreasing its reproductive efficiency in summer as a result of the increased of temperature and photoperiod (Sancho *et al.* 2004, Sancho *et al.* 2006).

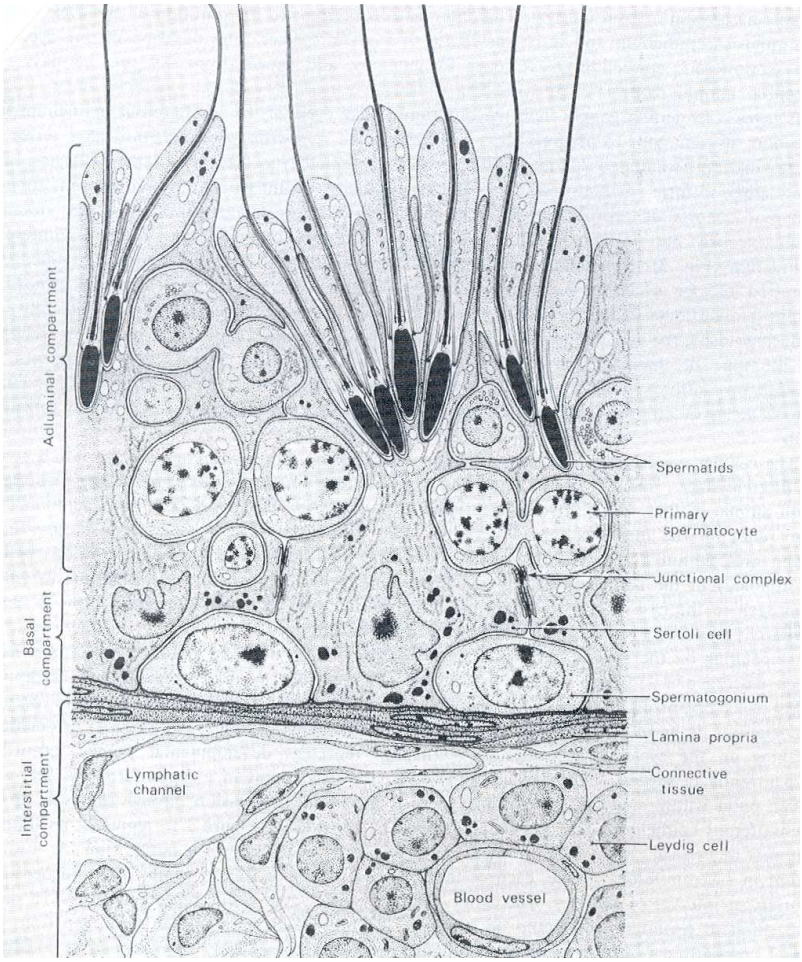


Figure 4. Schematic representation of spermatogenesis in the seminiferous tubules. Representation of the diferents sperm cells presents in the basal compartment and adluminal compartment of the seminiferous epithelium. Figure from (Amann 1989).

The seminiferous epithelium has a dual role: it produces immature spermatozoa continuously, and it also replaces the population of cells that give rise to the process, the spermatogonia. Thus, the entire process of spermatogenesis occurs in three sequential phases of cell proliferation and differentiation: 1) mitotic phase (spermatocytogenesis), 2) meiotic phase, and 3) post-meiotic phase (spermiogenesis), which involves stepwise progression of morphologically undifferentiated spermatids to highly differentiated spermatozoa. The duration of spermatogenesis is about 73 days in humans, 40 days in mice, and 34 days in boars (Amann 1989).

During the **mitotic phase** primitive spermatogonia proliferate by mitosis to give rise to several successive generations of spermatogonia, each generation being more differentiated than the preceding one. Two major types of spermatogonia are distinguished: A spermatogonia, which are characterized by the absence of heterochromatin and, B spermatogonia that possess abundant heterochromatin and further divide to give primary spermatocytes after the last mitotic division of spermatogenesis (Pinart *et al.* 2001b). A and B types undergo a series of divisions that result into many intermediate generations of cells. Moreover, an specific area of a seminiferous tubule become committed to produce the same differentiated spermatogonia stage synchronously (De Rooij & Grootegoed 1998).

The **meiotic phase** starts when primary spermatocytes enter into prophase I of meiotic division and chromosomes condense. During meiosis the chromosomes are linearly paired and interchanges of genetic segments between homologous chromatids occur (*crossing-over*). This is followed by two rapid successive meiotic divisions without DNA replication to produce spermatids, which are remodeled into spermatozoa.

The final phase of differentiation of male germ cells is the **post-meiotic phase (spermiogenesis)** prior to their release (spermiation) from the seminiferous epithelium. During this phase the undifferentiated haploid round spermatids undergo complex morphological, biochemical and physiological changes that result in the formation of asymmetric flagellated spermatozoa. Formation of both acrosome and flagellum are the two major transforming events occurring during spermiogenesis. Spermiogenesis is a relatively long process in mammals (~22 days in humans, ~14 days in mice, and ~9 days in boar). Despite spermiogenesis is a continuous process, it has been divided into a number of morphological events according to the changes occurring in the gamete cell: Golgi phase, cap phase, acrosome phase and maturation phase (Figure 5). During the Golgi phase, several granules from Golgi complex fuse among them to form the acrosomal vesicle. This acrosomal vesicle is attached to the nuclear envelope. Also, the centrioles translocate to the opposite pole of the developing acrosome, and a longitudinal polarity is established in the cell. One of the centrioles is implicated in the flagellum formation, and the other one in the connecting piece formation. Another morphological event in Golgi phase is the clustering and redistribution of mitocondria which are around the nucleus. During the cap phase, the acrosomal sac flatterns and form a cap attached to the nuclear envelope through the acroplaxome (an F-actin-containing cytoskeletal plate), and initiates its descent along the nucleus. The spermatide rotates with the acrosome pointing to the basal lamina and the developing axonema extending into the lumen of the seminiferous tubule. In the acrosome phase a set of cytoplasmic tubules arise and form a cylindrical structure called manchette, which is directly implicated in the elongation of the spermatid. The manchette is a transient organelle, not present in the spermatozoa. During the maturation phase, acrosome completes its differentiation covering half of the nuclear surface, the mitochondria sheath is

formed, the nucleus is condensed, and the manchette's structure disappears. Finally, during the maturation phase the tail structure is assembled the excess cytoplasm is released, and the chromatin remodeling continues and it characterizes by the condensation and stabilization of the chromatin with the replacement of histones by protamines (Pinart *et al.* 2000, Pinart *et al.* 2001b, Gupta 2005b).

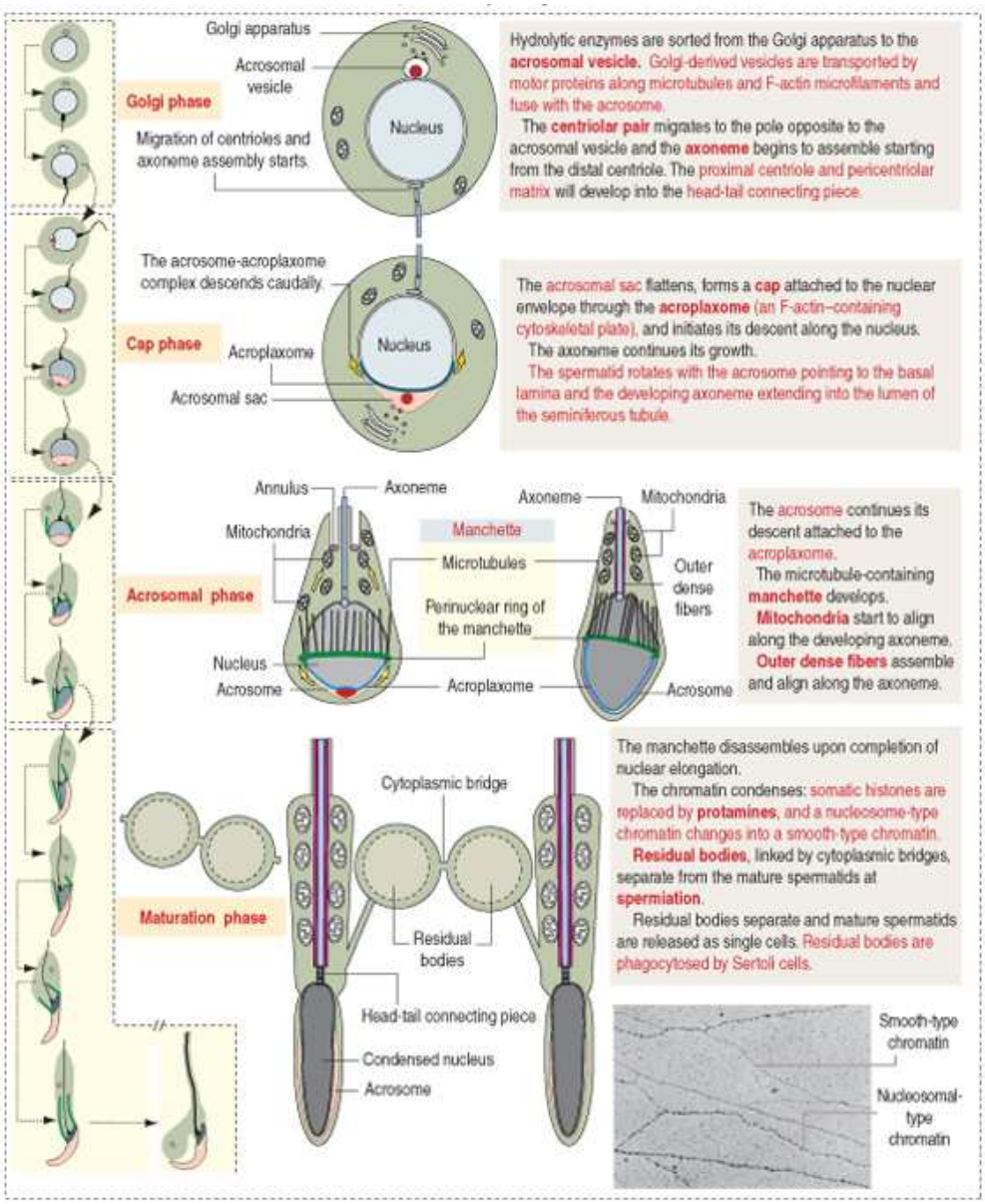


Figure 5. Schematic representation of the different spermiogenesis phases. The spermiogenesis is a continuous process divided into: Golgi phase, cap phase, acrosomal phase and maturation phase. Figure from (Elsevier. Kierszenbaum: Histology and Cell Biology: An Introduction to Pathology 2e)

3. Posttesticular maduration

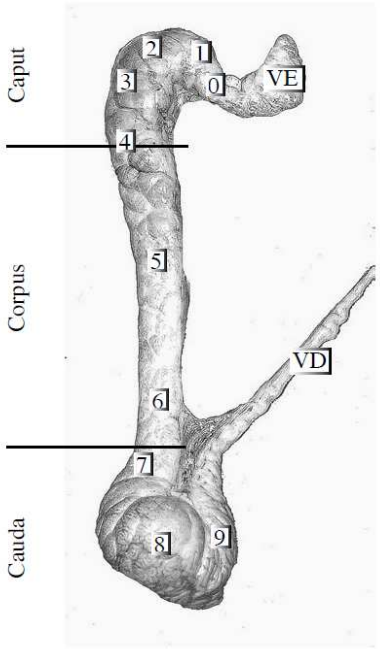
3.1. Epididymis

The epididymis is a single highly convoluted duct, originated from the convergence of 5 or 6 efferent ducts (Saitoh *et al.* 1990). It is closely applied to the surface of the testis extending from the anterior to the posterior pole, and held to the tunica albuginea by the connective tissue. The duct is coiled into segments demarcated by connective tissue septa and envolved by a capsule of fibrous tissue. Both, epididymis and testis are hanged in the scrotum. This localization results in the maintenance of a temperature some degrees below the body temperature (Setchell *et al.* 1994, Pruneda 2006). In boar, the epididymal duct is 85 g weight and 27 cm long in the coiled up form and, 54 m long in unwind form (Pruneda *et al.* 2006, Pruneda *et al.* 2007). The duct is wider in the caput and in the cauda and, thinner in the corpus. The epididymis consists of three distinct compartments: 1) the ductal lumen, containing spermatozoa and epididymal plasma, 2) the wall lining the lumen formed by and epithelium, a connective tissue and a muscle layer (smooth-muscle fibers), and 3) the extraductal tissue.

The **epididymal epithelium** is a complex dynamic structure dependent on androgens for the maintenance of a differentiated state of its epithelial cells (Bedford 1972). It contains various cell types, each varying as a proportion of the total population at different points along the duct. In the adult male these cells may vary in their cytological and histochemical nature according to the region of the duct in which they are situated (Bedford 1972). The predominant cells in mammals are the principal cells, tall, narrow and columnar cells which bear apical stereocilia that decrease and become shorter and less abundant through the distal end of the epididymis; Stereocilis participate in both absorption and secretion processes. Other cell types are apical cells in the caput region, basal cells in the corpus and cauda, clear cells that have not been described in boar epididymis, and halo cells that are present throughout the epididymis but decreasing in number from caput to cauda (Goyal & Williams 1991, Briz *et al.* 1993a, Setchell *et al.* 1994). Basal cells are small spherical cells, and apical cells have cytoplasmic processes to the basal part and can be implicated in the acidification and alcalinization of the luminal fluid.

The epididymal duct is surrounded by a **connective tissue** that contains fibroblasts, collagen and elastic fibers, blood and lymphatic vessels, nerve fibers, macrophages, wandering leucocytes, and 7 to 10 concentric layers of **smooth-muscle fibers** subjacent to the epithelium (Briz *et al.* 1993a, Setchell *et al.* 1994, Stoffel & Friess 1994).

In several mammals the epididymis is divided into 5 functional and structural regions: initial segment, intermedium zone, caput, corpus and cauda (Reid & Cleland 1957, Hamilton 1972, Hamilton 1975, Robaire & Hermo 1988, Hermo 1995). Even so in mammals the epididymis is divided in three **morphological segments**: **caput**, **corpus** and **cauda**. Divergences among species in the structure and extent of the different segments suggest variations in post-testicular sperm maturation and storage (Jones 2002). In boar, the three morphological segments of the epididymis are subdivided in eleven **regions**: initial segment (region **0**), caput (regions **1-4**), corpus (regions **5-7**) and cauda (regions **8-9**), which finish in the deferent duct (**10**) (Dacheux *et al.* 1984) (Figure 6).



Furthermore, sperm concentration increases form the rete testis fluid to the deferent duct from 10^8 to 10^9 spermatozoa/ml with a maximum of 10^{10} in the first part of the epididymis (Dacheux *et al.* 2003b), as a result of the reabsorption of testicular fluid in the proximal caput (Pruneda 2006). Caput region also concentrates most of the protein secretion activity throughout the epididymal duct (Dacheux *et al.* 2009). In the corpus this process is reversed and the sperm concentration decreases again. The high cell concentration of the luminal fluid is accompanied by a high protein concentration, low salt levels corresponding to less than 50% of osmotic pressure, low oxygen tension and absence of energy substrates, such as glucose. The high cell concentration facilitates interactions among germ cells, luminal components and the epididymal cell surface (Dacheux *et al.* 2005).

Figure 6. Adult boar epididymis. View of the boar epididymis indicating the different regions: VE, vas efferens or efferent ducts; 0 to 8/9, epididymis; VD, vas deferens, and the three main classical regions: caput, corpus, cauda. Figure from (Dacheux *et al.* 1984).

Basic **functions** of the epididymis are: 1) **sperm maturation**, which not only provides the spermatozoon of motility, but also the ability to recognize and bind to the zona pellucida (ZP), and to fuse with the oocyte; this process takes from one to two weeks depending on mammal species, 2) sperm **transport** through peristaltic movements of the smooth-muscle fiber below the epithelium and, 3) sperm **storage** in a quiescent state until the ejaculation.

3.2. Duct deferens

The duct or vas deferens begins at the point where the epididymal duct straightens and reverses direction toward the inguinal canal. It has a complex epithelium with a thick concentric muscle layers involved in the sperm ejaculation (Setchell *et al.* 1994).

3.3. Epididymal maturation

During their transit throughout the epididymis, spermatozoa acquire the capacity to move and to fertilize oocytes, and then they are stored in a high cell concentration in the cauda until the ejaculation. There is some acceptance that the epididymal cauda of mammals plays a role in sperm competition because it is adapted to store sperm. Epididymal cauda provides a unique milieu that keeps the sperm in a metabolic quiescent state prior to ejaculation (reviewed in (Jones *et al.* 2007b)). After ejaculation the spermatozoa undergo capacitation and acrosome reaction in the female reproductive tract. These processes enable them to fertilize the oocyte and are accompanied by several re-localitzation of many proteins. The ability of sperm to fertilize develops as a result of the **sperm maturation** along the

epididymis, which includes interactions with certain epididymal secretions, besides a complex cascade of changes involving the remodeling of sperm surface, the induction of chromatin condensation, the acquisition of movement, and development of the potential for capacitation (reviewed in (Olson 2002, Gupta 2005a)).

In vivo, epididymal sperm maturation is described as an androgen dependent process, despite the biochemical basis of the process is largely unknown (Gupta 2005a). Microscopic studies demonstrated that during epididymal transport, spermatozoa undergo remodeling that includes changes in the dimension and appearance of the acrosome and nucleus, structural changes in intracellular organelles, and in some species migration of the cytoplasmic droplet along the tail (Olson 2002). These structural changes in morphology are accompanied by biochemical modifications in the molecular form and the antigenicity of several intra-acrosomal and cell surface proteins (Cooper 1986, Jones 1998). Although secretions of the epididymal epithelium are clearly important for sperm maturation and survival, their role in this process has yet to be fully determined. Modifications in epididymal sperm membranes may result from the incorporation of protein, sugar and lipid determinants. Being a dynamic structure, the sperm surface is modified even after spermatozoa leave the male tract. The sperm surface modifications of spermatozoa include changes in membrane fluidity during the passage of spermatozoa through the epididymis, remodeling of lipid profile with a significant decline in total content of lipids, phospholipids and glycolipids of the sperm membrane. Therefore, the plasma membrane of mature spermatozoa from cauda shows greater neutral lipid content than that of the immature spermatozoa from caput. The sperm maturity is associated with a marked increase of sterol and steryl ester, and a decrease of other membrane bound neutral lipids. Cholesterol/phospholipid and saturated/unsaturated fatty acid ratio increases greatly in maturing sperm membrane (reviewed in (Gupta 2005a)).

Epididymal maturation also involves DNA stability and increases of cyclic adenosine monophosphate (cAMP) levels in spermatozoa of ram, bovine, boar and goat during epididymal transit (Gupta 2005a). Intracellular cAMP levels depen on the relative rates of cAMP synthesis and degradation (Gupta 2005a). In bovine the activation of sperm motility involves at least two events, an elevation in the intrasperm content of cAMP during epididymal transit and the production of specific forward motility proteins by epididymal cells (Brandt *et al.* 1978). Furthermore, throughout the epididymis spermatozoa change from ineffective swimming motion into a vigorous unidirectional and progressive pattern of motility. Thus, spermatozoa taken from the epididymal caput of various species show a variety of circular twitching or swimming motions. Spermatozoa isolated from the caput of rat, rabbit and guinea pig show circular swimming patterns, while those taken from the caput of bull, man and non-human primates display a pattern of motion that ranges from near immotility to a weak whiplash-like motion of the tail (reviewed in (Schirren 1980)). It has been suggested that the intra-sperm pH may have a role in the regulation of sperm motility initiation in the epididymis (Gupta 2005a).

3.4. Epididymal fluid

In boar, the proteins present in the epididymal fluid are the resultant of: 1) rete testis compounds that enter through efferent ducts in the proximal part of the epididymis, 2) epididymal secretion and

absorption, 3) proteolysis of pre-existing proteins within the fluid and, 4) to some extent, metabolic activity of spermatozoa (Dacheux *et al.* 2005). The epididymal fluid provides a milieu for gametes analogous to blood plasma providing a milieu for cell tissues. Due to the presence of the blood-testis and blood-epididymis barriers, most of the blood proteins are not found in the epididymal plasma (albumin, transferrin and certain other being exceptions) (Dacheux *et al.* 2009). In contrast to the constant concentrations of blood plasma proteins, the protein concentration in epididymal fluid varies greatly along the duct: from 2 to 4 mg/ml in the initial segment of the epididymis, a maximum of 50 to 60 mg/ml in the distal caput and, 20 to 30 mg/ml in corpus and cauda (Syntin *et al.* 1996, Fouchecourt *et al.* 2000). Several hundreds of epididymal proteins were described electrophoretically, but only a 10% of them have been identified. About 15-20 proteins make up more than 60-80% of the total protein concentration (review in (Dacheux *et al.* 2009)). Most common proteins found are lactoferrin (LF), procathepsin D (pCD), cholestherol transfer protein (CETP), glutathione peroxidase (GPx), beta-N-acetyl-hexosaminidase, mannosidase, galactosidase, prostaglandin D2 syntase (PGDS), cysteine-rich secretory proteins (CRISP) and epididymal retinoic acid-binding protein (E-RAPB). This protein composition changes continuously throughout the duct; lactoferrin, mannoside, PGDS and albumin are present in high concentrations in stallion, boar, ram and human, respectively, but GPx and PGDS are virtually absent in human and boar, respectively. Most of the epididymal proteins are characterized by their numerous isoforms which result from their high degree of glycosylation. It is probable that major epididymal proteins which surround gametes are more involved in sperm preservation than in inducing specific and localized modifications on the sperm surface. The high concentrations of proteins involved in gamete protection from oxidative stress, such as GPx5, thioredoxin peroxidase (TPx), glutathione S-transferase P (GST-P) and superoxide dismutase (SOD) probably contribute to sperm survival during epididymal storage (review in (Dacheux *et al.* 2009)).

3.5. Contribution of the accessory organs

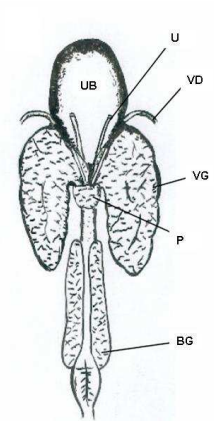


Figure 7. Schematic representation of boar accessory glands. Bulbouretrhral gland (BG); Prostate (P); Urethra (U); Urinary bladder (UB); Vas deferens (VD); Vesicular gland (VG).

During ejaculation, semen is formed from the secretions released by the seminal vesicles, the prostate gland, and Cowper's (bulbourethral) glands (Figure 7). The **seminal vesicles** are paired structures located dorsal to the trigonal area of the urinary bladder. They discharge a fructose-rich product to the urethra via the ejaculatory ducts in human and directly to the urethra in boars. The **prostate** surrounds the basis of the urethra and releases its contents into the urethra during ejaculation via a number of small ducts. The prostate secretes a clear fluid that has a slightly acid pH, and it is rich in acid phosphates, citric acid, zinc and a number of proteolytic enzymes. The **bulbourethral** or **Cowper's glands** empty into the bulbous urethra. They provide a lubricating fluid that protects the urethral mucose and neutralize urine remnants. The principal role of seminal vesicular and prostatic secretions is the sperm transport at the time of ejaculation, but they may also contribute briefly to the metabolism of spermatozoa (Mastroianni & Coutifaris 1990).

4. Mammalian fertilization

4.1. Transport of spermatozoa in the female genital tract

In boars, the ejaculate is released in three different and consecutive fractions (Sancho 2002, Yeste 2008, Casas 2010): 1) **Pre-spermatic fraction**, of 25 cc, has a clear or transparent appearance and a foul smelling. Formed by secretions from the prostate, it serves to flush urine and bacteria out of the boar's urethra and it does not contain spermatozoa. 2) **Spermatic** or **rich fraction** has a white appearance and a high sperm concentration around $0.5\text{-}1\cdot10^9$ spermatozoa per ml (80-90% of the total sperm). This fraction is usually of 40-100 cc, and it contains secretions produced by both the prostate and seminal vesicles. 3) **Post-spermatic** or **poor fraction**, with a volume ranging to 10-400 cc, contains secretions from prostate and seminal vesicles. It has a white pale appearance and low sperm concentration, under 10^6 spermatozoa per ml. Post-spermatic fraction components have an essential role in the activation of quiescent spermatozoa, despite they are often discarded in order to prevent over dilution of the sperm rich fraction. Finally, an ultimate fraction can be distingued in natural service, the **gelatinous phase**, 20-40 cc, which has a gelatinous consistency and contains the mucous secretions of bulbourethral glands, implicated in the formation of the "mucous plug", which functions to effectively seal the ejaculate volume inside the female uterus.

In a number of species, including the horse, dog, pig and mouse, the efficiency of transport of spermatozoa into the female reproductive tract is assured, because the semen is ejaculated directly into the uterus. In other species as human, rabbit, cow, sheep and goat, semen is ejaculated into the posterior vaginal fornix near the external cervix, so spermatozoa must find their way from the vagina into the upper reproductive tract (review in (Suarez & Pacey 2006) (Suarez 2006)).

4.2. Capacitation

Epididymal and ejaculated spermatozoa are not able to fertilize oocytes. As a requisite for binding the ZP of the oocyte and achieving successful fertilization, ejaculated spermatozoa need to undergo a sequence of events that result in changes in the plasma membrane composition and structure (reviewed in (Yanagimachi 1994) and (Eddy & O'Brien 1994)). This process takes place in the female tract, and it was firstly described simultaneously and independently by both Austin and Chang in 1951 (Austin 1951, Chang 1951). Shortly thereafter, the process was termed as "**capacitation**" by Austin (Austin 1952).

Principal events of capacitation include: 1) the destabilization of the sperm plasma membrane with the elimination or alteration of substances absorbed in the plasma membrane; 2) the increase of the influx of calcium inside of the spermatozoa as a consequence of the membrane destabilization (Yanagimachi 1994); 3) the activation of the hyperactive movement (Yanagimachi 1970) that allows the spermatozoa to pass throughout the oviductal fluid and, 4) the activation of the second messenger pathways (de Lamirande *et al.* 1997). All these events prepare the spermatozoa for the **acrosome reaction** and the penetration to the ZP of the oocyte.

In vivo, capacitation is initiated after mating in the female tract. However, the exact site where this process begins varies according to the site of semen deposition. In species such as the bovine in which the semen is deposited in the anterior vagina, capacitation begins during sperm migration through the cervix. In other species with uterine ejaculation, the oviduct seems to be the major site of sperm capacitation (Review in Bosch and Wright, 2005). In boar, *in vivo* capacitation and sperm storage in the reservoir takes place in the oviductal istmus (Hunter 1981). Spermatozoa are capacitated into the female tract and they take 5-6 hours to acquire the ability to penetrate the oocyte (Polge 1978). Ejaculated spermatozoa exposed to lipid-binding components of the female genital tract undergo changes in lipidic composition of the plasma membrane due to the loss of cholesterol composition. Decreased cholesterol content causes an increased disorder among the membrane phospholipids, which initiates a chain of events, such a rise in intracellular pH, calcium and bicarbonate, activation of adenylate cycalse, a rise in cyclic adenosine monophosphate (cAMP) levels, and phosphorylation of specific proteins (Harrison 2004) (Figure 8). All these events are synchronized with the occurrence of ovulation (Hunter & Rodriguez-Martinez 2004). In pig, only few miles of spermatozoa achieve the isthmus and just few of them arrive to the fecundation point in order to minimize the polyspermic effect (Hunter 1991).

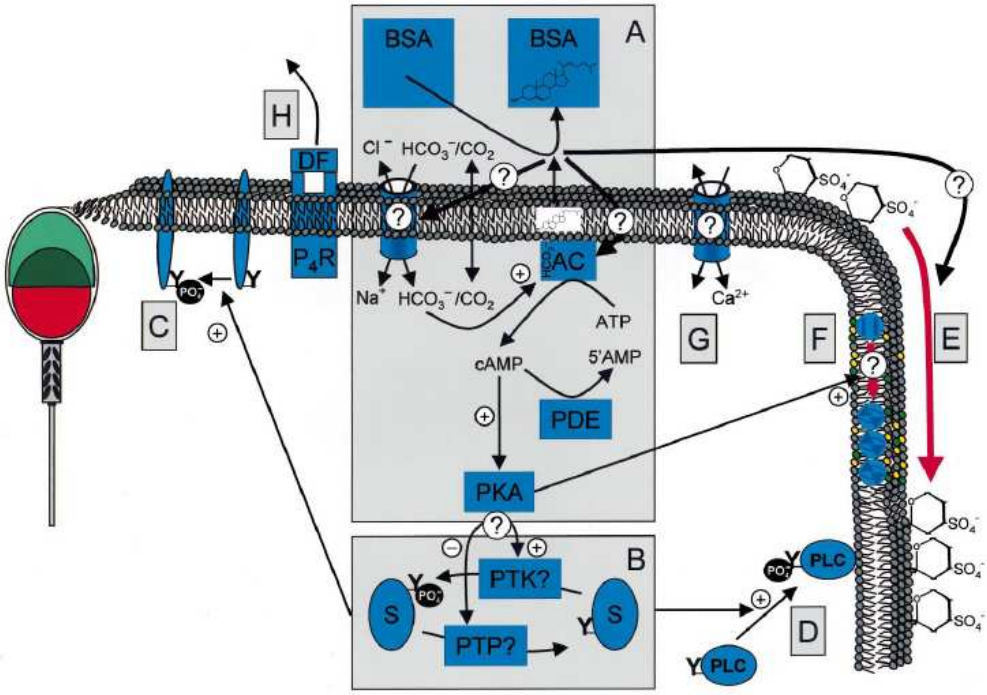


Figure 8. Proposed sequences of mammalian sperm capacitation. (A) Bicarbonate may enter to sperm cells via ion channels or via diffusion as carbon dioxide. Intracellular bicarbonate switches on Adenylate Cyclase (AC) and concomitant production of cyclic adenosine monophosphate (cAMP) activates Protein kinase A (PKA). The role of cholesterol efflux in the activation of PKA is unclear. Cholesterol efflux may induce increased bicarbonate entry or may affect AC. (B) PKA induces tyrosine phosphorylation (Y) of several substrates (S) most likely via activation of protein tyrosine kinase (PTK) or inhibition of protein tyrosine phophatases (PTP). (C) Sperm-ZP binding proteins and other plasma membrane proteins become tyrosine phosphorylated via the bicarbonate induced activation of PKA. (D) Cytosolic Phospholipase C (PLC) is tyrosine phosphorylated via the bicarbonate-PKA pathway. Tyrosine phosphorylated PLC is subsequently translocated to the plasma membrane. (E) PKA activation induces plasma membrane changes like lateral redistribution of seminolipid and translocation of aminophospholipids. (F) Aminophospholipids are translocated by the PKA dependent activation of a postulated scramblase. Most likely the efflux of cholesterol is involved in these plasma membrane transitions. (G) The entry of small amounts of calcium into sperm cells plays possibly an important role in capacitation. (H) Decapacitation factors (DF) are removed from the sperm cell surface, uncovering receptors like the postulated progesterone receptors (P_4R). Figure from (Flesch & Gadella 2000).

4.3. *In vitro* capacitation

At present, it is extremely difficult to evaluate the influence of oviduct cells on spermatozoa *in situ* and at the proper period in the reproductive cycle (Smith 1998, Flesch & Gadella 2000). However, capacitation can be **induced *in vitro*** in epididymal and ejaculated spermatozoa under defined media and physical conditions (Saravia *et al.* 2007, Ded *et al.* 2010, Puigmulé *et al.* 2011), that mimesis the electrolytic composition of the oviductal fluid. Such capacitation media typically include **energetic sources** (pyruvate, lactate and glucose), a protein component (usually **bovine serum albumin**, BSA), **calcium** and sodium **bicarbonate**. BSA contribute to *in vitro* capacitation as a membrane cholesterol acceptor, while bicarbonate regulates different sperm functions acting directly over the cAMP production (Harrison & Miller 2000) and the tyrosine phosphorylation of several proteins (Harrison 2004) (Visconti *et al.* 1995a). In fact, bicarbonate seems to be the key player in triggering tyrosine phosphorylation of proteins in capacitating mammalian sperm (Flesch & Gadella 2000). Calcium was reported to act in synergy with bicarbonate in the cAMP production (Litvin *et al.* 2003), despite its role in boar tyrosine phosphorylation, plasma membrane changes and motility increase is still known. The immediate effects of bicarbonate on pig spermatozoa are the alteration of the plasma membrane architecture (Harrison *et al.* 1996), and the increase of the progressive motility (Holt & Harrison 2002). However, while the ability to induce capacitation *in vitro* has enabled scientists to study the changes that take place within the sperm cell during this process, it is quite likely that some of these changes differ to those occuring *in vivo*.

4.4. Acrosome reaction

The **acrosome reaction** (AR) is an irreversible exocytic event initiated immediately after primary binding of capacitated spermatozoa to the oocyte. The plasma membrane of the sperm head fuses with the underlying outer acrosomal membrane at multiple sites, and this results in the formation of fenestrations in the plasma membrane by which the acrosome content, including numerous hydrolyzing and proteolytic enzymes, is released (Flesch & Gadella 2000). Hydrolytic enzymes dissolve the ZP-matrix around the penetrating spermatozoon and this allows it to enter to the perivitelline space (Gadella *et al.* 2001). Acrosomal contents are released by calcium-mediated (a massive increase in intracellular calcium levels is required) exocytosis in response to specific signals (Yanagimachi 1994). Importantly, the plasma membrane of the equatorial domain of the sperm head does not fuse with the acrosomal outer membrane, and it seems to be the specific site involved in oolema interaction (Flesch & Gadella 2000). However, if the acrosome reaction is initiated earlier (i.e. prior to ZP-binding) the enzymes will be lost and the spermatozoon will be no longer capable of zona penetration and, therefore, of fertilization (Gadella *et al.* 2001) (Figure 9).

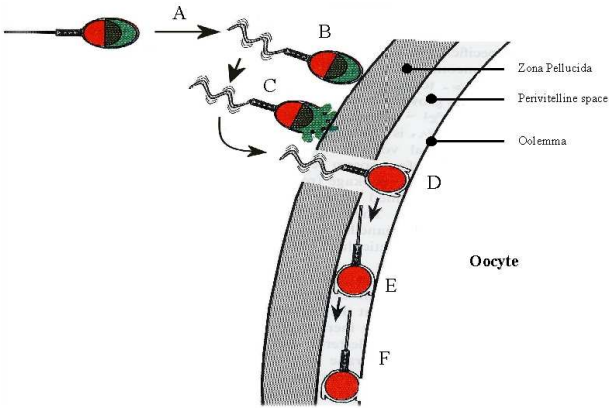


Figure 9. Mammalian fertilization events. Inside the female genital tract spermatozoa undergo capacitation (A) and get hyperactivated (B). The hyperactive motility enables spermatozoa to move away from the oviductal epithelium and provides the motile thrust needed for penetration of the ZP. Spermatozoon binds to the ZP (B) and their acrosome react (C) releasing hydrolytic enzymes which lyse the ZP. Subsequently the sperm can cross the perivitelline space and binds to the oolemma initially with the apical tip (D) and later sideways (E). The sperm cell then fuses with (F) and gets incorporated in the oocyte. Figure from (Flesch & Gadella 2000).

In vitro acrosome reaction can be induced by the combination of calcium with calcium inophore, inducing the fusion of plasma membrane with the underlying acrosome membranes; non-capacitated sperm cells with rigid plasma membranes are not capable to fuse these membranes under the same conditions (Cheng *et al.* 1996). Moreover, dramatic differences exist between spontaneous acrosome reaction, calcium ionophore induced and physiologically induced acrosome reaction.

4.5. Fertilization

Fertilization is defined as the process of union of two germ cells, oocyte and spermatozoon, whereby the somatic chromosome number is restored and the development of a new individual is initiated. Final steps of mammalian oogenesis and spermatogenesis prepare oocytes and spermatozoa, respectively, for fertilization.

The plasma membrane of sperm head is the primary locus of interaction with the oocyte and thus it is likely to contain many functionally important proteins. Each of the sperm head surface regions have an specific role that allow the spermatozoon to fertilize the oocyte (Gadella *et al.* 2008) (Figure 10). Mammalian spermatozoa interact with the oocyte on four different levels during fertilization: 1) the cumulus layer; 2) the ZP which induces exocytosis of acrosome contents; 3) the oocyte plasma membrane, which begins with the adhesion to the oocyte plasma membrane and concludes with the fusion of the sperm and oocyte membranes; and 4) the cytoplasm where the decondensation of the sperm nucleus takes place (reviewed in (Evans 2002)). In mammals, sperm-oocyte interaction appears to be relatively species-specific, and it includes several membrane receptors implicated in reorganization of glycoproteins from the ZP. The ZP is composed of three highly specific glycoproteins with a large amount of glycans (Reviewed in (Detlef *et al.* 2005)). According to the coding gens three types of glycoproteins are described, which are processed and modified posttranslationally: ZP1 (~ 200 kDa; dimer), ZP2 (~120 kDa) and ZP3 (~ 83 kDa) (Wassarman 1999, Wassarman 2008).

Figure 10. Schematic representation of the sequence of interactions between the male and female gamete leading to fertilzation. (1) Sperm binding to the ZP (restricted to the apical ridge subdomain area); (2) the acrosome reaction (fusion restricted to the apical ridge and pre-equatorial subdomain areas); (3) the penetration of the ZP (equatorial membranes remain intact and mixed vesicles resulting from the acrosome reaction are shed from the penetrating spermatozoon); (4) binding and fusion with the oocyte plasma membrane (restricted to the equatorial subdomain area); (5) activation of the fertilized oocyte by soluble sperm factors; (6) polyspemy blockage by the cortical reaction. Modified from Flesch et al., 2000) (Gadella *et al.* 2008)

Gamete recognition, binding and fusion are highly regulated processes that involve a number of biochemical changes. Primary binding between capacitated spermatozoa with intact acrosome and ZP involves many specialized molecules of the apical plasma membrane of the sperm head: lactadherin P47 (P47), zona adhesion proteins, galactosyltransferase, sperm surface protein of 56 kDa (sp56) and, 95-kDa protein (p95) (Reviewed in (Wassarman 1999, Mori *et al.* 2000, Detlef *et al.* 2005)); being present in the acrosome-intact spermatozoa, all these molecules recognize and bind with **primary receptors** associated with O-linked carbohydrate moieties of the ZP (ZP3 glycoproteins). This initial binding between spermatozoa and the ZP induces the acrosome reaction, as a result of which **secondary ligands** and receptors present on the acrosomic matrix and/or the inner acrosomal membrane become exposed on the sperm surface (Wassarman 1999, Howes & Jones 2001, Detlef *et al.* 2005). This secondary binding also triggers the hyperactivation of the sperm flagellum and the lytic-like action in acrosomic matrix, meanwhile the sperm head penetrates towards the ZP and migrates through the perivitalline space of ZP (Bedford 1998). Proacrosin, sperm protein of 38 kDa (sp38) and 17 kDa (sp17), and sperm adhesion molecule 1 (SPAM1 or PH-20) are known to mediate the secondary tight binding with ZP2 glycoproteins (Wassarman 1999, Mori *et al.* 2000, Detlef *et al.* 2005). Acrosin and PH-20 have, besides the mentioned ZP affinity, proteinase and hyaluronidase activities (Urch & Patel 1991, Gmachl *et al.* 1993).

Finally, the third binding and fusion step includes the participation of the **third** kind of sperm **receptors**, such as the receptors for the integrin alpha-6-beta-1 of oocyte and fertilin (PH-30 alpha/beta or ADAM-1/2), or integrins and C9 receptors of the oolemma (Howes & Jones 2001). Other inner acrosomal membrane proteins, such as cyristestin acrosomal matrix proteins, such as cyristestin 1 (ADAM-3), have also been descrived to participate in this process (Forsbach & Heinlein 1998).

The ZP of porcine oocyte is also composed by a specific group of three glycoproteins which are referred as ZPA (~ 90-70 kDa), ZPB (~55-59 kDa) and ZPC (~55-46kDa) (Harris *et al.* 1994, Mori *et al.* 2000, Sinowatz *et al.* 2001). Based on the sequence data, ZPA, ZPB and ZPC are homologues to the ZP2, ZP1 and ZP3 glycoproteins described in mouse, respectively (Harris *et al.* 1994). Moreover, in porcine oocytes an associative ZPB-ZPC heterocomplexes were reported (Yurewicz *et al.* 1998). ZPC and ZPB have an independent binding activity, but ZPB-ZPC heteromers become functional and structural units of the gamete-specific extracellular matrix with high affinity to molecules present in boar sperm membrane (Yurewicz *et al.* 1998, Sinowatz *et al.* 2001). Protein binding assays of the ZP showed that binding proteins are concentrated in the acrosomal ridge of the spermatozoa. These studies also observed that corpus spermatozoa are already able to fertilize oocytes (Burkin & Miller 2000).

5. Sperm cell composition

5.1. Proteomic composition

The spermatozoon is a highly polarized and specialized cell with low content of cytosol and organelles (Eddy & O'Brien 1994, Yanagimachi 1994), but abundant proteins. Spermatozoa do not contain endoplasmatic reticulum, Golgi apparatus, lysosomes, peroxisomes or ribosomes, therefore they have lost the potential gene expression (both transcription and translation processes are completely silenced) (Boerke *et al.* 2007).

The proteomic content of sperm cells is constituted by miles of proteins, as demonstrated the proteomic identification of human sperm proteins reported by Martínez-Hereida (Martínez-Heredia *et al.* 2006), including a large amount of tail proteins (reviewed in (Oliva *et al.* 2008, Oliva *et al.* 2009)) such as tubulin (Mohri 1968), acrosomal proteins (Tanii *et al.* 2001, Yoshinaga *et al.* 2001) such as acrosin (Brown & Harrison 1978, Puigmulé *et al.* 2011), mitochondrial proteins (NagDas *et al.* 2005, Aitken *et al.* 2007), nuclear matrix and cytoskeletal proteins (Shaman *et al.* 2007), the most abundant sperm nuclear proteins, protamines (Felix 1960, Oliva 2006) and, membrane proteins (Peterson & Russell 1985, Hunnicutt *et al.* 1996).

5.2. Composition of the plasma membrane

Sperm **plasma membrane** is a very dynamic structure (for review see (Flesch & Gadella 2000)). During the transit of spermatozoa through the epididymis, the composition of plasma membrane changes by the releasing redistribution, modification, and adsorption of proteins and lipids. A lateral polarized distribution has been described in the sperm plasma membrane; moreover freeze-fracture studies indicated that each domain contains different concentration and distribution of intramembranous particles which represent transmembrane proteins (Flesch & Gadella 2000). This reorganization also takes place along the female genital tract in order to achieve the ability to fertilize the oocyte.

Lipid composition and implication in surface sperm

Surface membrane of mature spermatozoa is not in contact with intracellular membranes because vesicle mediated membrane transport is blocked. The only exception to this occurs when the apical plasma membrane fuses with the underlying outer acrosomal membrane, during the highly regulated acrosome reaction. The unusual composition and organization of lipids on the plasma membrane are probably reflections of these specific sperm properties (for review see (Flesch & Gadella 2000)). Although there is considerable variation among mammalian species, in general the plasma membrane contains approximately 70% phospholipids, 25% neutral lipids and 5% glycolipids (on molar base); major variable factor is the amount of cholesterol. Human sperm cells for instance contain rather high

amounts of cholesterol (40 mol percent of total lipids) whereas boar sperm cells contain much less cholesterol (22 mol percent) (Mann & Lutwak-Mann 1982). Low cholesterol to phospholipids molar ratio, as well as the high content of unsaturated phospholipids makes sperm plasma membrane of boars particularly prone to form jellified phases (Parks & Lynch 1992). This particular composition of the plasma membrane is probably derived from the sperm maturation in the epididymis, which is known to decrease the cholesterol content of membranes (Nikolopoulou *et al.* 1985). At this respect, it is demonstrated the boar spermatozoa become more sensitive to cold-shock as they mature in the epididymis and after ejaculation (Simpson *et al.* 1987).

Proteomic composition and implication in sperm surface

Plasma membrane **proteins** have essential functions for cells, interacting with both cellular and extracellular components, structures and signaling molecules (Josic & Clifton 2007).

During maturation, proteins on the sperm surface are highly processed according to their specific function: 1) proteins located to specific domains, such ADAM2 or PH-20 are processed and relocated (Primakoff *et al.* 1985, Overstreet *et al.* 1995, Blobel 2000), in order to participate in oocyte-sperm interaction and fecundation (Jury *et al.* 1997, McLaughlin *et al.* 1997, Waters & White 1997); 2) some proteins secreted by epithelial epididimal cells are apparently added to the sperm surface in transit through the epididymis (review in (Gupta 2005a)), such as mouse epididymal proteins (MEPs) or sperm maturation antigen 4 (SMA-4) in mouse, epididymis-specific secretory protein 3 (E-3) in rat, hamster epididymal protein 64 (HEP64) or hamster sperm protein of 26 kDa (P26h) in other rodents, and a 135 kDa protein in large domestic animals (Okamura *et al.* 1992); 3) finally, other proteins are released of the cleaved protein into the epididymal medium, as for angiotensin-convering enzyme (ACE) (Gatti *et al.* 1999).

Futhermore, during sperm capacitation the organization of plasma membrane proteins and lipids also changes dramatically resulting in the capacitated state, which characteristically allows the sperm to bind to the ZP and thereafter to acrosome react (Gadella *et al.* 2008).

Glycocalyx

The surface of mammalian spermatozoa is covered by a dense coating of carbohydrate-rich molecules forming a 20-60 nm thick **glycocalyx**. The glycocalyx comprises carbohydrate residues linked to lipids and other molecular structures. These structures can be intercalated or anchored within the lipid bilayer, or superficially associated with the membrane via polar groups or by hydrophobic interactions (Schroter *et al.* 1999). Moreover, the majority of sugar residues are attached to proteins which are either integrated within the sperm membrane, or are more or less loosely associated with it. It is estimated that the glycocalyx may be comprised by several hundreds of different glycoproteins, some of which are synthesized within the testis. Others, however, are produced by the epithelia of the efferent ducts, epididymis and possibly accessory glands, and become associated with post-testicular spermatozoa during the transit through and storage in the male tract.

The acquisition of the mature glycocalyx is associated with the attainment of full sperm fertilizing ability. The glycocalyx could play a major role in intercellular gamete communication because it forms the extracellular coating of the spem's surface (Flesch & Gadella 2000). Until its complete molecular structure is elucidated, the complex function of the glycocalyx remains obscure, though it may be related with membrane maturation and immunoprotection in the female tract, as well as with sperm-ZP binding and fertilization (Schroter *et al.* 1999).

The majority (~92 %) of extracellular membrane proteins of all cells, also in spermatozoa, are glycosylated. The oocyte is also encompassed by a heavily glycosylated matrix, the ZP, which differs from sperm glycocalyx in both thick (16 μm in pig) and complexity. The oocyte glycosylated investment is comprised just for three main glycoproteins (ZP1, ZP2, and ZP3), whereas sperm glycocalyx is constituted by 50 to 150 different glycoconjugates (Schroter *et al.* 1999).

Lectins are proteins, usually of vegetal origin, able to recognize specific oligosaccharide structures (Table 1). The conjugation of lectins with fluorochromes or biotin systems leads to investigate cell-surface sugars and the changes that these sugars undergo during cell growth, differentiation or malignancy (Lis & Sharon 1998) and, they are also useful aids for the structural characterization of carbohydrate moieties of glycoproteins, as they can discriminate different glycoconjugates in the spermatozoon surface.

Lectin binding studies provide evidence that glycan moieties of plasma membrane glycoproteins undergo extensive modifications during the epididymal transit of spermatozoa, by proteolytic processing of fluid fucosyltransferase and beta-D-galactosidase (Gupta 2005a).

Table 1. Lectin carbohydrate affinity and inhibitory sugar.

Lectin	Specificity	Inhibitory sugar	Carbohydrate Group
Arachis hypogaea (Peanut) agglutinin (PNA)	β-Gal	Lactose (200 mM)	Gal
Lens Culinaris Agglutinin (LCA)	α-Man; α-Glc	Methyl α-D-mannopyranoside (200 mM)	Glc/man
Pisum Sativum Agglutinin (PSA)	α-Man; α-Glc	Methyl α-D-mannopyranoside (200 mM)	
Wheat germ agglutinin (WGA)	β-GlcNAc ; Neu5Ac	N-Acetyl-D-glucosamine (500 mM)	GlcNAc
Helix pomatia Agglutinin (HPA)	α-GalNAc	N-Acetyl-D-galactosamine (200 mM)	GalNAc
Phytohemagglutinin-L (PHA-L)	Gal ; GalNAc	N-Acetyl-D-glucosamine (500 mM)	
Glycine max (Soybean) agglutinin (SBA)	α- or β-GalNAc	N-Acetyl-D-galactosamine (200 mM)	
Ulex europaeus Agglutinin I (UEA-I)	α-Fuc	L-fucose (500 mM)	Fuc

Carbohydrate affinities abbreviations: Galactose (Gal); Glucose (Glc); Mannose (Man); N-acetylglucose (GlcNAc); N-acetylgalactose (GalNAc); Fucose (Fuc); B-galactose (β-Gal) ; α-D-manose (α-Man); α-D-glucose (α-Glc); β-N-acetyl-D-glucose (β-GlcNAc); N-acetylneuraminic acid (Neu5Ac) ;α or β N-acetyl-galactosamine (α- or β-GalNAc); α-fucose (α-Fuc).

5.3. Angiotensin converting enzyme I (ACE)

Angiotensin converting enzyme I (ACE; EC 3.4.15.1) is a plasma membrane anchored zinc-metallopeptidase that typically cleaves C-terminal dipeptides from several oligopeptide substrates, including circulating angiotensin I, bradykinin and the haemoregulatory peptide N-acetyl-SDKP. This enzyme is usually related with the regulation of blood pressure, although some studies demonstrated that it has an important role in reproductive function (review in (Sabeur *et al.* 2001)). A gametic form of ACE (gACE) has been reported in spermatozoa. An important role of gACE in fertility was demonstrated

in subfertile gACE knockout male rats in which gACE absence could interfere both building and remodeling of sperm membrane (Esther *et al.* 1997, Metayer *et al.* 2002, Thimon *et al.* 2005), despite the precise stages at which the absence of ACE interferes in male fertility has not been elucidated yet (Gatti *et al.* 1999, Metayer *et al.* 2002).

In male mammals two isoforms of angiotensin-converting enzyme are produced, a somatic isoform and a germinal isoform. Both isoforms are coded by the same gene (Metayer *et al.* 2001). The **somatic form (sACE)** has a molecular weight of approximately 140-180 kDa. It is expressed in many tissues and plays an important role in the control of blood pressure by acting on the bradykinin and angiotensin peptides. Its sequence is composed of two redundant domains (N- and C-terminal domains) each having a zinc-binding site and a functional catalytic site (review in (Metayer *et al.* 2001)). The **germinal ACE (gACE)** isoform is expressed in male haploid germ cells and, generated by the activity of a testis-specific promoter situated within the 12th intron of the somatic gene (Gatti *et al.* 1999). This germinal isoform of 90-110 kDa is restricted to the C-terminal domain of the sACE, including the hydrophobic membrane anchor (that allows cellular membrane insertion) and the short cytoplasmic tail, but it has a specific N-terminal sequence of 60 amino acids and only one catalytic and one zinc-binding site (Gatti *et al.* 1999, Metayer *et al.* 2001, Thimon *et al.* 2005)(Figure 11).

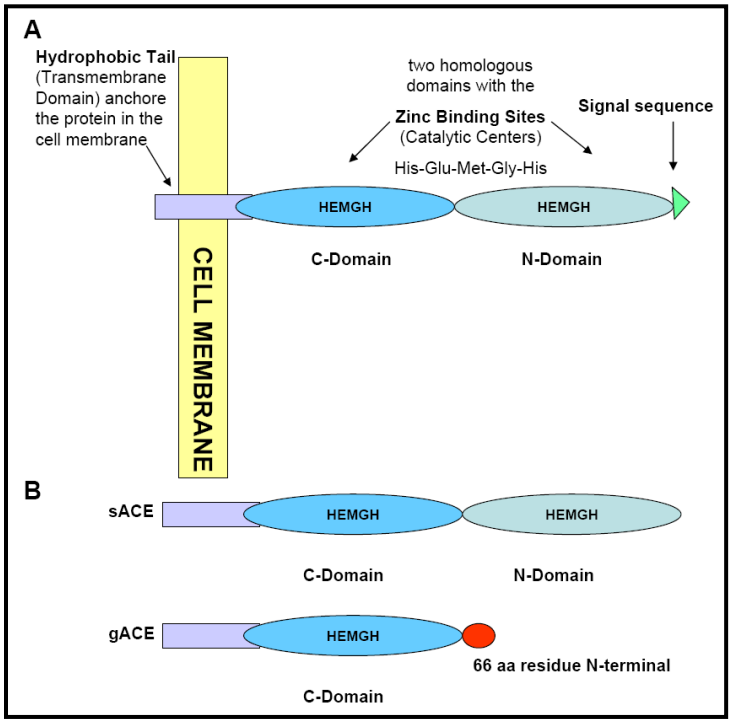


Figure 11. Protein structure of angiotensin-converting enzyme (ACE). A. Somatic ACE consists of two homologous domains flanked by a signal sequence and hydrophobic transmembrane domain, which anchor the protein to the cell membrane. Each homologous domain contains a consensus zinc-binding site His-Glu-Met-Gly-His (HEMGH), which binds one zinc molecule and serves as a catalytic center. The signal sequence is cleaved during processing and not present in ACE mature form. B. Structure of somatic (sACE) and testis (gACE) ACE. gACE form contains only the C domain of sACE plus an additional 66 amino acid residues of testis-specific sequence. Figure adapted from (Esther *et al.* 1997).

Both, sACE and gACE have soluble forms. The sACE soluble form is produced by a specific membrane-associated cleavage secretion process that releases the extracellular domain of this enzyme (Metayer *et al.* 2001, Metayer *et al.* 2002). The soluble epididymal and seminal plasma gACE isoform is derived from the plasma membrane by a proteolytic process of the extracellular domain (Gatti *et al.* 1999, Metayer *et al.* 2002, Thimon *et al.* 2005). It is a 94-105 kDa protein released from sperm in the proximal caput of the epididymis in domestic mammals (Gatti *et al.* 1999) and in rodents (Metayer *et al.* 2002); this released form is the responsible of ACE activity detected in the epididymal fluid (Gatti *et al.* 1999). The localized area of liberation of epididymal sperm ACE suggests the need of a specific environment (Gatti *et al.* 1999). The changes in the molecular weight from 105 KDa to 94 kDa could be due to variations in postsecretory modifications, as changes in the glycosylation state or to

the loss of the plasma membrane anchor (Gatti *et al.* 1999, Metayer *et al.* 2002). Another fluid form of 10 kDa, corresponding to the trans-membrane and intracellular domains of gACE, is generated via active proteolytic cleavage at the membrane level during the gACE releasing in the fluid during the epididymal transit of sperm (Gatti *et al.* 1999, Thimon *et al.* 2005). This active proteolytic process involve serine protease activity (Thimon *et al.* 2005).

Immunolocalization of ACE shows an intense labeling on the midpiece and diffuses on the acrosome of testicular and initial caput spermatozoa. In spermatozoa from further zones of caput, corpus and cauda, as well as ejaculate, ACE is localized in the acrosomal ridge (Gatti *et al.* 1999).

Not all gACE forms of testicular sperm are released during the epididymal transit. Remnant activity and immunolocalization in the acrosomal region of the enzyme was reported in all epididymal regions and in ejaculated sperm (Kohn *et al.* 1998), thus indicating that this intraacrosomal location could protect ACE from proteolytic release in the epididymis (Hagaman *et al.* 1998, Gatti *et al.* 1999)

5.4. Fertilin (PH-30 or ADAMs)

Fertilin is a heterodimeric transmembrane glycoprotein (~75 kDa) and member of **ADAMs** protein family localized in the posterior head of mature guinea pig sperm (Blobel *et al.* 1990, Blobel 2000) (Figure 12).

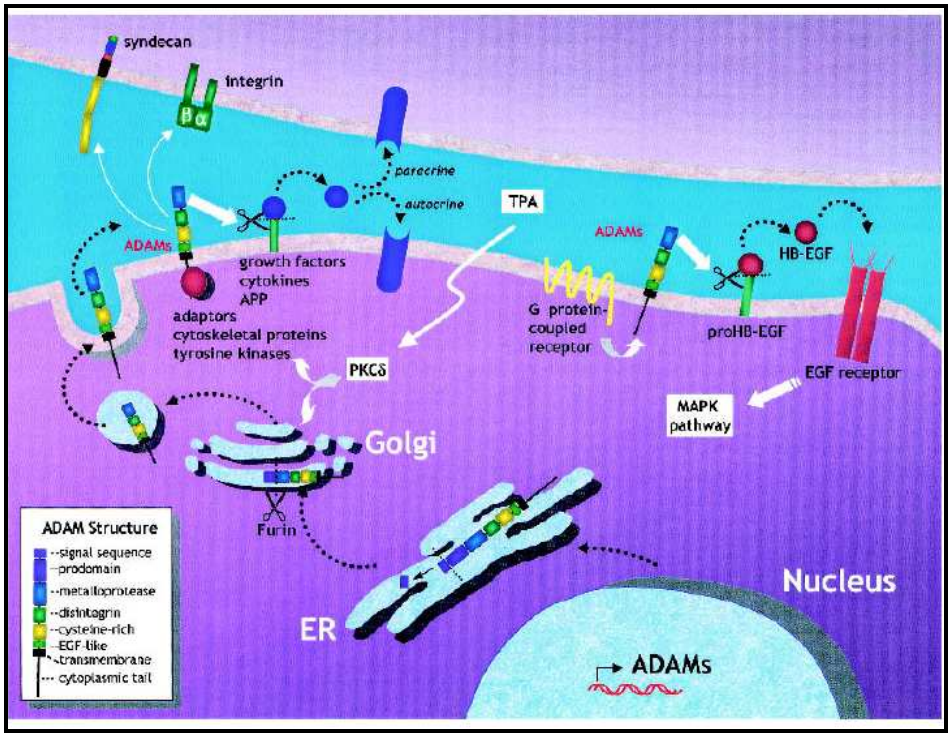


Figure 12. An overview of ADAMs synthesis, processing and function. Several representative activities of ADAMs are schematized. Not all the possible functions of ADAMs are shown. Figure from (Seals & Courtneidge 2003).

ADAMs protein family belongs to the zinc protease superfamily which is essential for fertilization and cellular development, but it also participate in several biological processes such as cancer, inflammation and, neurogenesis (Primakoff & Myles 2000, Seals & Courtneidge 2003). ADAM family proteins have

multi-functional domains consisting of pro-, metalloprotease-, disintegrin-, cystein rich-, epidermal growth factor (EGF) like-, and cytoplasmic-domains which are believed to support distinct functions (Seals & Courtneidge 2003) (Figure 13).

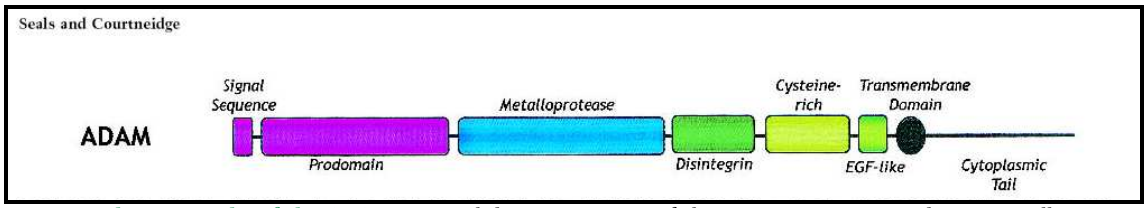


Figure 13. The topography of the ADAMs. General domain structures of the ADAMs proteins: pro-domain, metalloprotease domain, disintegrin domain, cysteine-rich domain, EGF-like domain, transmembrane domain and cytoplasmic tail domain. Figure from (Seals & Courtneidge 2003).

Fertilin is an essential protein required for the migration of spermatozoa through the oviduct, for binding to the ZP, and for the initiation of sperm-oocyte interaction during the sperm fertilization (Primakoff & Myles 2000, Seals & Courtneidge 2003). It is composed of two tightly associated and immunologically differentiated subunits, alpha-subunit (or **ADAM-1**) of ~45 kDa and beta-subunit (or **ADAM-2**) of ~ 27 kDa (Blobel *et al.* 1990, Janice 2001) (Figure 14)

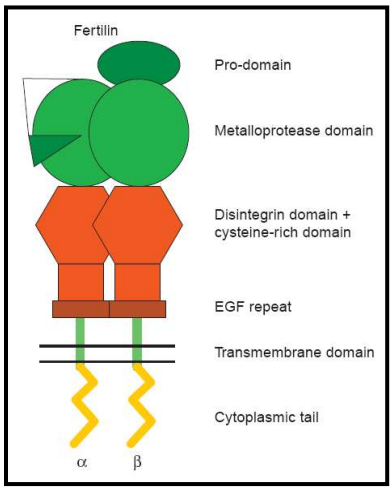


Figure 14. Model of fertilin heterodimer proposed by Bobel, 1990. Figure from (Blobel 2000).

Fertilin subunits are made as large precursors that are processed in different developmental stages. Thus, α-subunit is processed firstly in testis (Blobel *et al.* 1990), whereas β-subunit is processed during the sperm passage through the epididymis, between distal corpus and proximal cauda, when the spermatozoa acquire motility and fertilization competence (Van Gestel *et al.* 2007).

In guinea pig, the apparent molecular mass is ~45 kDa for α-subunit and ~ 27 kDa for β-subunit (Blobel *et al.* 1990). In *Macaca fascicularis* sperm, β-subunit is processed to a 47 kDa mature state from a 100 KDa precursor form during the passage through the epididymis, by forming an midpiece 50 kDa form (Kim *et al.* 2009) (Figure 15). The β-subunit of fertilin has an essential role in fertilization, as it was associated with the gamete fusion by means its cysteine-rich regions, which resemble those present in viral fusion peptides (Janice 2001). In *knockout* mice for β-fertilin, it was subsequently shown that the adhesion step (with the disintegrin domain), not fusion step, is defective; nevertheless these males are still fertile but with low capacity of ZP binding (Seals & Courtneidge 2003, Van Gestel *et al.* 2007). β-fertilin is predicted to be proteolytically inactive, and both prodomain and metalloprotease domain are cleaved prior to the acquisition of fertilization competence, a role for protease activity in fertilization can be ruled out (review in (Seals & Courtneidge 2003)).

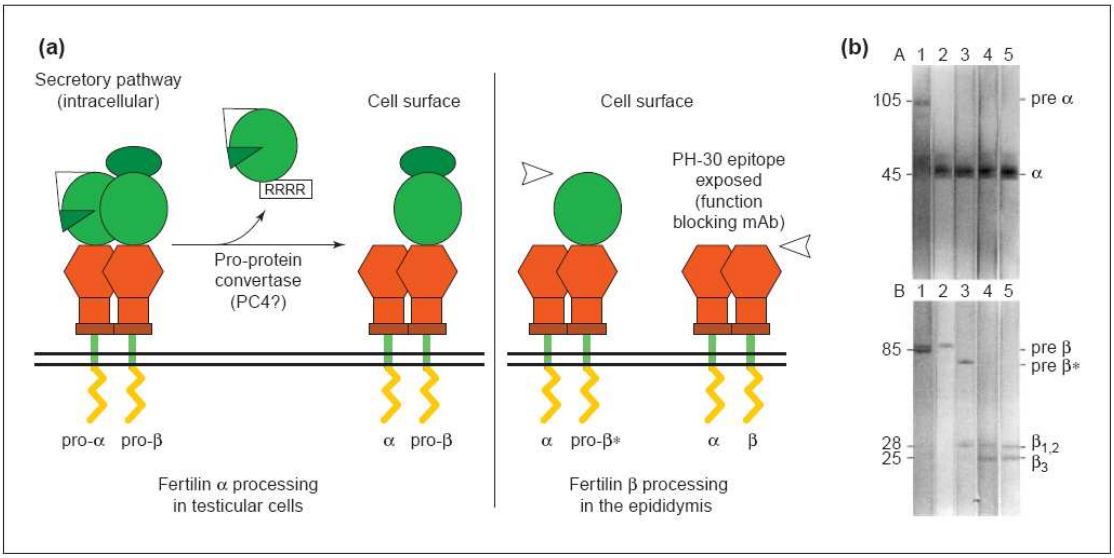


Figure 15. Proteolytic processing of fertlin during sperm maturation in the testis and epididymis. Figure from (Blobel 2000)

The two fertilin subunits are directly associated with an unknown binding domain. Whereas α-subunit is non-functional in some primates, the existence of the fertilin complex remains controversial in primates. Because the targeted deletion of α-subunit prevents the presentation of β-subunit on the sperm surface, it is thought that β-subunit is important in the formation of the fertilin complex on the sperm surface (Kim *et al.* 2009).

In guinea pig, fertilin immunolocalization also differs along the epididymis. The proteolytic processing of fertilin is associated with its redistribution on the sperm surface; in testicular spermatozoa, fertilin distributes over the whole surface spermatozoa, but during the sperm transit through the epididymis it migrates to the posterior sperm head (Blobel 2000).

5.5. Tyrosine phosphorylation of proteins

Tyrosine phosphorylation is a post-translational modification of proteins that allows the cell to control various processes. The phosphorylation state of proteins is controlled by the activity of protein kinases and phosphatases (Urner & Sakkas 2003). In mammalian spermatozoa, the phosphorylation status of proteins is regulated by the activation of compartmentalized intracellular signallig pathways.

The tyrosine phosphorylation process is associated with sperm capacitation in various species, such as mouse (Visconti *et al.* 1995c), human (Luconi *et al.* 1996), bovine (Galantino-Homer *et al.* 1997), goat (Chatterjee *et al.* 2010), and pig (Flesch *et al.* 1999). Protein tyrosine phosphorylation is also known to regulate sperm functions in mammals, such as hyperactivated motility (Vijayaraghavan *et al.* 1997) (Nassar *et al.* 1999), ZP binding (Pukazhenthi *et al.* 1998), acrosome reaction (Pukazhenthi *et al.* 1998), and sperm-oocyte fusion (Urner & Sakkas 2003, Naz & Rajesh 2004).

Capacitated spermatozoa from human, monkey, hamster, rat and mouse (review in (Naz & Rajesh) present an intense tail labeling. In these species, tail phospotyrosine proteins locate in the fibrous sheath,

suggesting they may be involved in hyperactived motility, which is associated with capacitation and/or acrosome reaction (Tardif *et al.* 2001). Meanwhile in porcine sperm capacitation also appears to be associated with tyrosine phosphorylation proteins, it is unrelated with sperm motility changes (Bailey *et al.* 2005).

Epididymal maturation and priming events to acquire functional spermatozoa are also related with tyrosine phosphorylation patterns (Tardif *et al.* 2001). In rat the epididymal maturation involves dephosphorylation patterns (Lewis & Aitken 2001), while the rat and mouse sperm ability to tyrosine phosphorylate the tail increase in order to prepare the spermatozoa for the hyperactivated movement (Aitken *et al.* 2007). Furthermore, epididymal maturation results in a change in tyrosine phosphorylation from the acrosomal domain present in caput spermatozoa to a narrow band at the posterior margin of the acrosomal vesicle in cauda spermatozoa (Lewis & Aitken 2001).

Several proteins are reported to be tyrosine phosphorylated during sperm maturation and/or capacitation. Earlier studies of *in vitro* capacitation of boar sperm (Kalab *et al.* 1998) reported several constitutive phosphotyrosine sperm proteins (p44, p40, p38 and p34), whereas other demonstrated an early increase of a 32 kDa phoshotyrosine protein band after *in vitro* capacitation, p32 (Tardif *et al.* 2001). This protein probably correspond to the 34 kDa phosphotyrosine protein subsequently identified by Kalab (Kalab *et al.* 1998) and Flesh (Flesch *et al.* 1999). The p32 may possess tyrosine kinase-like activity; it is present in the cytosolic fraction of boar spermatozoa, and only appears in the membrane fraction after capacitation (Tardif *et al.* 2001, Bailey *et al.* 2005).

Another phosphotyrosine protein, p21, previously described by Dubé et al., (Dubé *et al.* 2004) as a Chain 1-phospholipid hydroperoxide glutathione peroxidase (PHGPx) was identified in porcine (Esworthy *et al.* 1994), and in hamster capacitated spermatozoa as a 19 kDa protein (NagDas *et al.* 2005). PHGPx is a selenoprotein and a key enzyme in the protection of biomembranes exposed to oxidative stress (Imai & Nakagawa 2003); in capacitated spermatozoa it is present as a disulfide cross-linked, membrane-anchored component of the mitochondrial sheath (NagDas *et al.* 2005). Physiological and molecular implications of PHGPx are poorly known, nevertheless previous data suggested that tyrosine phosphorylation of PHGPx may represent an important event in the signaling cascade associated with capacitation, that may regulate the hyperactivation of sperm motility and/or mitochondrial function (NagDas *et al.* 2005).

6. Flow cytometry

Fundamentals of flow cytometry

Flow cytometry is used primarily to measure the physical and biochemical characteristics of cells or biological particles in suspension. Particle size or complexities are some of the physical characteristics that can be estimated by the dispersion of the laser light after intercepting a particle. To analyze biochemical characteristics by flow cytometry, labeling techniques with fluorochromes are usually applyied. This technology is used to perform measurements on whole cells as well as cellular constituents, such as nucleous and organelles. Flow cytometers are investigative tools for a broad range of scientific disciplines because they make measurements on thousands of individual cells/particles in a matter of seconds. This is a unique advantage relative to other detection instruments that provide bulk particle measurements. Flow cytomety is a complex and highly technical field; therefore, a basic understanding of the technology is essential for all users (Radcliff & Jaroszeski 1997).

Flow cytometres are composed basically by a **light source** (laser and arc lamp), **fluidic system**, **optical flow chamber**, **flow cellular system**, several **optic components** to differentiate and select the light modifications, and some **electronic elements** which amplify and process the resultant signal in the computer (Figure 16).

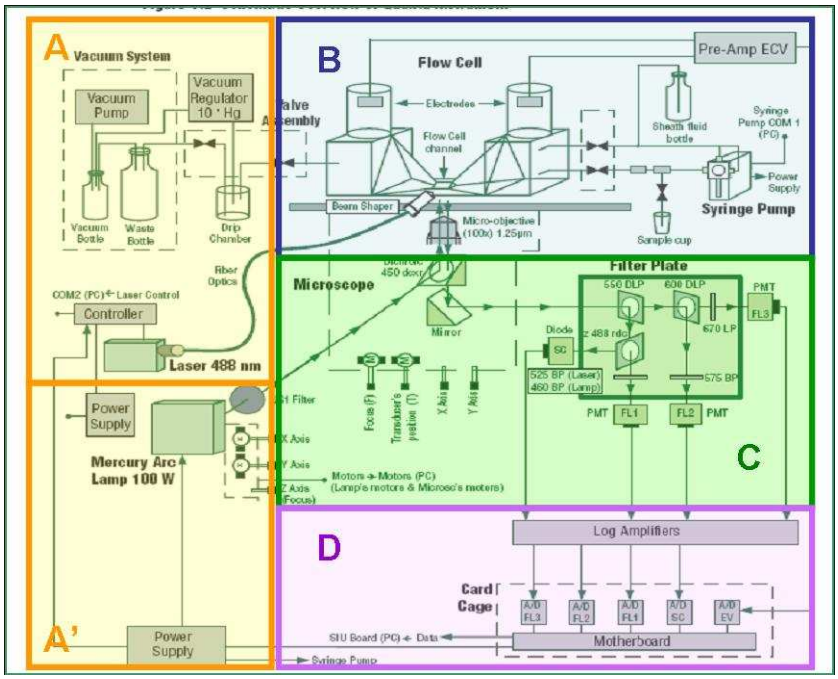


Figure 16. Flow cytometer components. Beckman coulter Cell Lab Quanta SC cytometer components: A) laser and A') arc lamp light sources, B) fluidic system, optical flow chamber and flow cellular system, C) optical components and, D) electronic elements.

Typical **light sources** in flow cytometry are lasers (light amplification by stimulated emission of radiation) and arc lamps. Lasers generate a very intense monochromatic beam focalized in a small diameter. Advanced equipments have up to 3 or 4 lasers; however, more common equipments include a

single argon gas laser which emits at 488 nm (blue spectrum). Moreover, arc lamps do not produce a monochromatic beam of light, so they need a complex optic system to focalize the light in the same point of the flux.

Fluidic system is based in a hydraulic system which allows the individual pass and focalization in the centre, in order to avoid obstructions of the cells or particles in suspension in front of the light source. For this reason laminar flux is indispensable as described by Reynolds principle in 1883. Generation of the laminar flux is obtained with a thin diameter of the capillary and with the velocity of the stream liquid. In the optical flow chamber defined by Crosland-Taylor in 1953 a hydrodynamic focalization by decreasing the tube section as described the Bernoulli principle. According to this principle decreasing the tube section increasing of the velocity of the fluid, and decreasing the pressure are produced. Nevertheless, the increase of the velocity is higher in the centre of the flux, and helps the focalization of the cells into the capilar and allows the single pass of the cells in front of the light source (Figure 17).

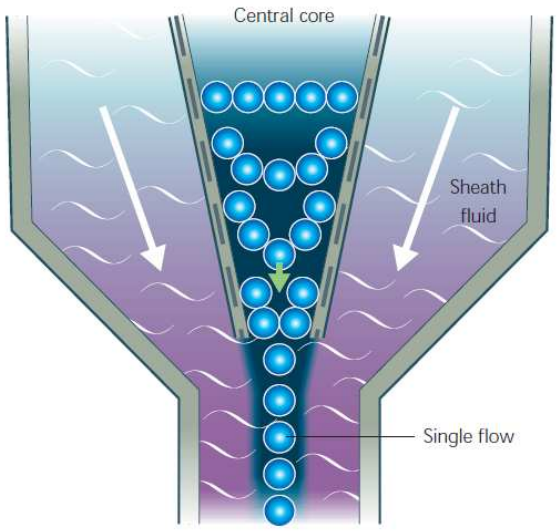


Figure 17. Fluidic system. The hydrodynamic focusing produces a single stream of particles which can be assessed one by one in front of the light source. Figure from.http://www.spacesrl.com/wp-content/uploads/2011/02/FlowCytometry_V3_20101.pdf.

In flow cytometry the light dispersion by each cell gives information about its physical characteristics. The cell size, the nucleus, the plasma membrane, and the granular material inside the cell generate a specific light dispersion when the laser impact onto its surface, inducing changes in the direction but not in its wavelength, allowing the differentiation of different cellular populations or subpopulations. Two types of light dispersion are observed: forward scatter (FSC), which gives estimation of the cellular size, and side scatter (SSC), which depends on cellular density and granularity estimating the cellular complexity (Peña & Rodríguez 2006).

Furthermore, flow cytometry also allows the analysis of several biochemical properties from the light emission of fluorochromes and fluorescent constituents specifically blinded at cells. Fluorochromes are reagents which absorb light at a specific wavelength and emit photons in an inferior energetic level, so in a longer wavelength that can be detected and identified by a set of filters and detectors connected to the flow cytometer.

In order to discriminate and detect light dispersion and different wavelengths generated, all cytometers have different types of optic filters (**optic components**) which classify the signs generated by the cells

when the light source impact onto them. Photodetectors are connected with each one of the filters, and they detect the signal and then transform the light in electric impulses. These impulses constitute the analogical signal that then will be converted in digital values which are processed by the informatic system. Common cytometers present five optical systems of measure: two filters of dispersion (FSC for forward scatter and SSC for side scatter), and three filters for fluorescence (FL1, FL2 and FL3, commonly for green, orange and red spectrum, respectively) (Figure 18).

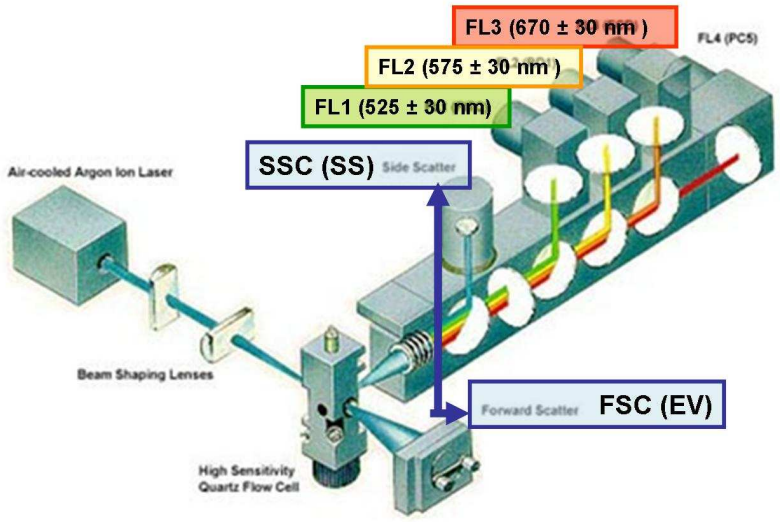


Figure 18. Optic components diagram. Beckman coulter Cell Lab Quanta SC optic components and optical flow chamber disposition. Forward scatter (EV) detector gives estimation information of the cellular size, whereas side scatter (SS) detector estimated the cellular complexity. Three photodectors are also observed connected with each one of the filters (FL1 525 ± 30 nm, FL2 575 ± 30 nm and FL3 670 ± 30 nm).

Objectives

This thesis proposes four main objectives according to challenges that currently concern the changes occurring in boar spermatozoa during epdidymal maturation, ejaculation and *in vitro* capacitation, as exposed in the Introduction. Four papers have been published in response to these four objectives, which are named at the end of each point.

1. Determine the effects of epididymal maturation, ejaculation and *in vitro* capacitation on sperm viability, acrosome integrity, mitochondrial activity, membrane fluidity and calcium influx both as indicators of capacitation status and, sperm motility. Paper I.

2. Investigate and describe the impact of epididymal maturation, ejaculation and *in vitro* capacitation on the phosphotyrosine content of sperm proteins. Paper II.

3. Characterize the molecular modifications on the sperm surface carbohydrate coat, glycocalyx, associated with epididymal maturation. Paper III.

4. Characterize the expression, processing and location changes throughout the boar epididymal duct of a sperm plasma membrane protein complex, fertilin. Paper IV.

Identifying and characterizing the proteins involved in sperm maturation and capacitation processes will serve to design new strategies for increasing the yield of the artificial insemination procedures in several purposes. Sperm protein knowledge is a pre-requisite to address the question of the fertilin role in boar fertility and its potential use as a fertility marker in boars.

Materials and methods

1. Materials

1.1. General reagents

2-mercaptoethanol (161-0710, Bio-Rad Laboratories, Inc, Hercules, CA, United States); 4-chloro-1-napthol (C6788, Sigma-Aldrich Química, S.A., Madrid, Spain); Agarose (BIO-41025, Bioline, London, England); Ammonium Persulfate (161-0700, Bio-Rad Laboratories, Inc, Hercules, CA, United States); Bromophenol blue (131165, Panreac Quimica, S.A.U., Barcelona, Spain); Calcium chloride ($CaCl_2$, C1016, Sigma-Aldrich Química, S.A., Madrid, Spain); Caffeine (142833, Panreac Quimica, S.A.U., Barcelona, Spain); Ethanol (131086, Panreac Quimica, S.A.U., Barcelona, Spain); Ethylenediaminetetraacetic acid (EDTA, ED2SS, Sigma-Aldrich Química, S.A., Madrid, Spain); Fluoromount mounting medium (Electron Microscopy Sciences, Hatfield, PA, United States); Formaldehyde 37 % (344198, Merck, Calbiochem, Nottingham, England); Glucose (G6152, Sigma-Aldrich Química, S.A., Madrid, Spain); Glycin (23390, Serva electrophoresis GmbH, Heidelberg, Germany); Glycerol ($C_3H_8O_3$, 131339, Panreac Quimica, S.A.U., Barcelona, Spain); Glycerol 87 % (104094, Merck, Calbiochem, Darmstadt, Germany); Kanamicine (K4000, Sigma-Aldrich Química, S.A., Madrid, Spain); Lactate (141034, Panreac Quimica, S.A.U., Barcelona, Spain); Methanol (141091, Panreac Quimica, S.A.U., Barcelona, Spain); N-octyl-β-D-gluopyranoside (861936, Sigma-Aldrich Química, S.A., Madrid, Spain); O-phospho-L-tyrosine (P9405, Sigma-Aldrich Química, S.A., Madrid, Spain); Percoll (P1644-100, Sigma-Aldrich Química, S.A. , Madrid, Spain); Pyruvate (Panreac Quimica, S.A.U., Barcelona, Spain); Phenylmethanesulfonyl fluoride (PMSF, P7626, Sigma-Aldrich Química, S.A., Madrid, Spain); Polyvinylpyrrolidone (PVP K30, 81420, Sigma-Aldrich Química, S.A., Madrid, Spain); Potassium dihydrogen phosphate (KH_2PO_4, 131509, Panreac Quimica, S.A.U., Barcelona, Spain); Potassium chloride (KCl, 141494, Panreac Quimica, S.A.U., Barcelona, Spain); Potassium hydrogen carbonate ($KHCO_3$, 201480, Panreac Quimica, S.A.U., Barcelona, Spain); Protease Inhibitor Cocktail (P8340, Sigma-Aldrich Química, S.A., Madrid, Spain); ProtoGel ready-to-use 30% (w/v) acrylamide/methylene bisacrylamide solution (37.5:1 ratio) (National Diagnostics, Atlanta, GA, United States); Sodium bicarbonate ($NaHCO_3$, S6014, Sigma-Aldrich Química, S.A., Madrid, Spain); Sodium chloride (NaCl, 131659, Panreac Quimica, S.A.U., Barcelona, Spain); Sodium citrate tribasic dehydrate ($Na_3C_6H_5O_7{\cdot}2H_2O$, S4641, Sigma-Aldrich Química, S.A., Madrid, Spain); Sodium dodecyl sulfate (20783, SDS, Serva electrophoresis GmbH, Heidelberg, Germany); Sodium hydrogen phosphate (Na_2HPO_4, 141679, Panreac Quimica, S.A.U., Barcelona, Spain); Sodium lactate (143306, Panreac Quimica, S.A.U., Barcelona, Spain); Sodium vanadate (Na_3VO_4, S6508, Sigma-Aldrich Química, S.A., Madrid, Spain); Streptavidin peroxidase conjugated (UP-385220, Uptima, Interchim, Montlucon-Cedex, France) TEMED (161-0801, Bio-Rad, Hercules, CA, United States); Tergitol®Solution (NP40S, Sigma-Aldrich Química, S.A., Madrid, Spain); Thiourea (T7875, Sigma-Aldrich Química, S.A., Madrid, Spain); Tris(hydroxymethyl)aminomethan (37190, Serva electrophoresis GmbH, Heidelberg, Germany); Triton

X100 (T8787, Sigma-Aldrich Química, S.A., Madrid, Spain); Tween 20 (162312, Panreac Quimica, S.A.U., Barcelona, Spain); Urea (161-0731, Bio-Rad Laboratories, Inc, Hercules, CA, United States).

Other chemicals used sporadically in this thesis were obtained from Sigma-Aldrich Química, S.A. (Madrid, Spain).

1.2. Antibodies

Primary antibodies

Anti-phosphotyrosine mouse monoclonal IgG2b antibody (05-1050, 4G10® Platinum, Anti-Phosphotyrosine, 05-1050, Milliopore, Temecula, CA, United States); Anti-phosphotyrosine mouse IgG1 antibody conjugate with Alexa Fluor®488(P-Tyr-100, PN A24072, Cell Lab, Beckman Coulter, Marseille Cedex, France); Anti-α-tubulin mouse monoclonal IgG1 antibody clone DM1A; (05-829, Millipore, Temecula, CA, United States).

Four different rabbit polyclonal antibodies against ADAM-1 and ADAM-2 were prepared from defined peptides by "l'*Equipe Gamètes Mâles et Fertilié*" of the *Institute National de la Recherche Agronomique* de Tours (Nouzilly, France). Two peptides, one in the N-terminal metalloprotease domain and one in the C-terminal near the trans-membrane domain of each ADAM were chosen (ADAM-1-NT : LTEVPVDLQVALRC ; ADAM-1-CT : CSSPGSGGSVDSGP ; ADAM-2-NT : GLTNAIFTSFNITC ; ADAM2-CT : NATITYSNINGKIC). A cysteine was added in C-terminal position of the peptides that did not have one for the coupling with keyhole limplet hemocyanin (Sigma, Lyon, France) using a bi-functional coupling agent (MBS Pierce). The coupled peptides were injected to rabbits with the first injection of 250 micrograms of peptide using Freund complete adjuvant followed by three injections of 250 micrograms with Freund incomplete adjuvant. Preimmune serums were tested prior immunization and showed no reactivity versus the extracts (not shown). Another rabbit polyclonal anti-germinal angiotensin-converting enzyme (anti-ACE) has been produced by this group (Gatti et al. 1999) and used in this thesis.

Secundary antibodies

AlexaFluor® 488 goat-anti-mouse IgG_1 (γ1) (A21121, Molecular ProbesTM, Invitrogen, Eugene, OR, United States); Polyclonal goat anti-rabbit-HRP (P0448, DakoCytomation, Glostrup, Denmark); Polyclonal rabbit anti-goat IgG, HRP conjugate (AP106P, Millipore, Temecula, CA, United States); Polyclonal rabbit anti-mouse-HRP (P0260, DakoCytomation, Glostrup, Denmark).

1.3. Biotin and lectin labeling

Biotin EZ-Link Sulfo-NHS-SS-Biotin (21331, Pierce, Thermo Scientific, Rockford, United States); Biotinylated lectin Kit I (BK-1000, Vector laboratories, Burlingame, CA, United States); Streptavidin beads (Dynabeads®M-280 Streptavidin, Invitrogen Dynal AS, Oslo, Norway).

1.4. Electrophoresis 1D

DC Protein Assay (1-800-424-6723, Bio-Rad Laboratories, Inc, Hercules, CA, United States); Precision Plus Protein™ Standards All Blue (161-0373, Bio-Rad Laboratories, Inc, Hercules, CA, United States); Prestained Protein Ladder, Broad Range (P7710S, New England BioLabs, Ipswich, MA, United States).

1.5. Electrophoresis 2D

Dithiotreitol (DTT, 17-1318-01, GE Healthcare Bio-Science GmbH, Barcelona, Spain); IPG Buffer pH 3-10 ampholites (17-6000-87, GE Healthcare Bio-Science GmbH, Barcelona, Spain); Immobiline™ DryStrip Cover Fluid (17-1335-01, GE Healthcare Bio-Science GmbH, Barcelona, Spain); Iodoacetamide (RPN6302, GE Healthcare Bio-Science GmbH, Barcelona, Spain); Ready Strip IPG pH 3-10 NL (7 and 11 cm, 163-2000/163-2014, Bio-Rad Laboratories, Inc, Hercules, CA, United States).

1.6. Flow cytometry

Fluo-3-acetomethoxy ester (Fluo-3 AM, F-1241, Molecular proves, Eugene, OR, United States); Fluorescein isothiocyanate-labelled Arachis hypogoea peanut agglutinin (FITC-PNA, L7381, Sigma, Madrid, Spain); JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, T-3168, Molecular Probes, Eugene, OR, United States); LIVE/DEAD®Sperm Viability kit (L-7011, Molecular Probes, Eugene, OR, United States); Merocyanine 540 (M540, 63876, Fluka, Sigma, Madrid, Spain); MitoTracker®GreenFM (M7514, Molecular-Proves, Eugene, OR, United States); Propidium iodide (PI, P4170, Sigma, Madrid, Spain); Yo-Pro®-1 (Y-3603, Molecular proves, Eugene, OR, United States).

1.7. Protein blotting

Bovine serum albumin (BSA, Roche Diagnositcs, S.L., Mannheim, Germany); Hyperfilm ECL (28-9068-36, GE Healthcare Ltd, Buckinghamshire, England), Immobilon™ Western Detection Reagents (WBKLS0500, Millipore, Molsheim Cedex, France); Immobilon-P Transfer Membrane of polyvinylidene fluoride (PVDF) (IPVH00010, Millipore, Bedford, MA, United States); Protan®Nitrocellulose membranes (Watmann Inc, Dassel, Germany).

1.8. Solutions

Phosphate buffered saline (PBS)

PBS 10x (volum 1l)

Products	Molarity (M)	Quantity (g)
NaCl	1.400	80.0
KCl	0.150	11.5
Na_2HPO_4	0.070	9.9
KH_2PO_4	0.015	2.0
Final volume with bidestilated H_2O		1 l

PBS supplemented 1x (volum 0.5 l)

Products	Molarity (mM)	Quantity (g)
NaCl	140.0	4.00
KCl	15.0	0.58
Na_2HPO_4	7.0	0.49
KH_2PO_4	1.5	0.10
		0.5 l
Piruvate	2.0	0.11
Lactate	2.0	0.09
Glucose	2.0	0.15
$KHCO_3$	2.0	0.10

Filtration with 0.45 µm and check pH 7.2-7.4 and osmolarity 295-330 mOsm before adding under line reagents

Belstville Thawing Solution (BTS)

BTS (volum 1l)

(Pursel & Johnson 1976)

Products	Molarity	Quantity (g)
Glucose	0.205 M	37.00
$Na_3C_6H_5O_7{\cdot}2H_2O$	0.020 M	5,88
$NaHCO_3$	0.015 M	1.26
EDTA	0.004 M	1.25
KCl	0.010 M	0.75
Kanamicine	70 mg/ml	0.05
Final volume with bidestilated H_2O		1 l

Check pH 7.2-7.4 and osmolarity 295-330 mOsm. Conservation a week at 4 ºC

BTS capacitating medium (BTS+) (volum 250 ml)

(Saravia *et al.* 2007)

Products	Molarity	Quantity (g)
Glucose	0.205 M	9.24
$Na_3C_6H_5O_7{\cdot}2H_2O$	0.020 M	1.50
$NaHCO_3$	0.015 M	0.32
EDTA	0.004 M	0.31
KCl	0.010 M	0.19
Kanamicine	70 mg/ml	0.01
		0.25 l
$NaHCO_3$	37.00 mM	0.77
CaCl2	2.25 mM	0.06
Caffeine	2.00 mM	0.10
BSA	0.5 %	1.25

Check pH 7.2-7.4 and osmolarity 295-330 mOsm. Temper at 5% CO_2 and 37 ºC 2-24h before use.

Formaldehyde saline solution

Products	Molarity	Quantity
NaCl		9 g
Formaldehyde		30 ml
Final volume with bidestilated H_2O		1 l

SDS-PAGE electrophoresis and western blot

1D-Lysis buffer (volum 10 ml)

Products	Molarity	Quantity
SDS	2%	0.20 g
Protease inhibitor cocktail*		0.10 ml
Na_3VO_4 0.7 M	1 mM	0.02 ml
PMSF 0.1 M	1 mM	0.10 ml
EDTA	15 mM	0.06 g
Final volume with bidestilated H_2O		10 ml

Adjust pH 7

*Protease inhibitor cocktail contains: AEBSF – [4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride] – serine proteases; Aprotinin – serine proteases; Bestatin hydrochloride– aminopeptidases; E-64 – [N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide] – cysteine proteases; Leupeptin hemisulfate salt– both serine and cysteine proteases; Pepstatin A – acid proteases.

Stacking gel solution (volum 100 ml)

Products	Concentration	Quantity (g)
Tris	0.500 M	12.0
SDS (v/w)	0.400 %	0.4
Final volume with bidestilated H_2O		0.1 l

Adjust pH at 6.8 with HCl and filtration with 0.45 µm. Conservation for 15 days at 4 ºC

Resolving gel solution 2x (volum 100 ml)

Products	Concentration	Quantity (g)
Tris	3.000 M	72.6
SDS (v/w)	0.800 %	1.6
Final volume with bidestilated H_2O		0.1 l

Adjust pH at 8.8 with HCl and filtration with 0.45 µm. Conservation for 15 days at 4 ºC

Electrophoresis buffer 10x (volum 2l)

Products	Concentration	Quantity (g)
Tris	0.25 M	60.4
Glycine	1.90 M	288.0
SDS (v/w)	1.00 %	20.0
Final volume with bidestilated H_2O		2 l

Filtration with 0.45 µm. Diluted at work solution 1x with H_2O MiliQ. Conservation at 4 ºC.

Transference buffer 3x (volum 2l)

Products	Concentration	quantity (g)
Tris	0.172 M	41.6
Glycine	1.320 M	198.2
SDS (v/w)	0.012 M	6.8
Final volume with bidestilated H_2O		2 l

Check pH 8.3. Filtration with 0.45 µm. Diluted at work solution 1x with H_2O MiliQ and methanol 20% (v/v). Conservation at 4 ºC.

Tris Buffer Saline (TBS)/Tween 20 buffer 10x (volum 2l)

Products	Concentration	Quantity
Tris	0.100 M	24.0 g
NaCl	1.490 M	174.0 g
Tween 20	0.500 %	10.0 ml
Final volume with bidestilated H_2O		2 l

Adjust pH 7.3 with HCl. Conservation at 4 ºC.

Ammonium persulfate (APS) 10%(volum 1ml)

Products	Concentration	Quantity
APS	10%	0.1 g
Bidestilated H_2O		1 ml

Long term conservation at -20 ºC.

Laemmli reducer buffer 2x (volum 23.5 ml)

Products	Concentration	Quantity
Tris 0.5M pH=6.8	0.13 M	6.00 ml
Glycerol	21.00 %	4.80 ml
SDS	4.30 %	0.96 g
H_2O MiliQ		16.55 ml
Bromophenol blue		Trace
Beta-met	5.00 %	1.13 ml

Filtration with 0.22 µm previously to beta-mercapto-ethanol addition. Long term conservation at -20 ºC

Stripping solution (volum 200 ml)

Products	Concentration	Quantity
Glycine	0.20 M	3.0 g
Tween 20	0.05 %	0.1 ml

Adjust pH 2.5 with HCl. Conservation for a week at 4 ºC.

Bidimensional electrophoresis (IEF)

2D Rehidratation buffer (volum 5 ml)

Products	Concentration	Quantity
Urea	7 M	2.10 g
Thiourea	2 M	0.76 g
Triton X100	1 %	0.05 ml
N-Octyl-beta-D-glucopyramidose	2 %	0.10 g
Final volume with bidestilated H_2O		5.00 ml
Filtration with 0.22 µm		
Ampholines (IPG)	0.4 %	0.02 ml
Bromophenol blue		trace

Aliquot in 200 µl. Long term conservation at -20 ºC. Add 20 µl of DTT 0.3M in each aliquot previously to use.

2D Equilibration buffer (EB) I and II

2D Equilibration buffer (EB) (volum 200 ml)

Products	Concentration	Quantity
Tris-HCl pH 8.8	0.05 M	6.7 ml
Urea	6 M	72.1 g
Glycerol 87%	30 %	69.0 ml
SDS	2 %	4.0 g
Final volume with bidestilated H_2O		0.2 l

Aliquot in 5ml. Long term conservation at -20 ºC.

2D EB I-reduction (volum 5 ml)

Products	Concentration	Quantity
Equilibration buffer		5 ml
DTT	0.065 M	0.05 g

2D EB II-alkylation (volum 5 ml)

Products	Concentration	Quantity
Equilibration buffer		5 ml
Iodoacetamide	0.135 M	0.125 g

2D Sealing agarose solution (volum 100 ml)

Products	Concentration	Quantity
Agarose	0.5 %	0.5 g
Electrophoresis buffer 1x		100 ml
Bromophenol blue		trace

Aliquot in 5ml. Conservation at room temperature.

DTT 0.3 M (volum 1 ml)

Products	Molarity (M)	Quantity
DTT	0.03	0.046 g
H_2O MiliQ		1 ml

Aliquot in 20 µl. Long term conservation at -20 ºC.

2. Methods

2.1. Epididymal Samples

Several postpuberal boars were slaughtered and their epididymides were removed following the approved guidelines for the ethical treatment of animals. The **epididymides** were dissected out and cleaned of blood. Luminal epididymal samples were obtained from the efferent duct and from four different anatomical regions of the epididymis: regions 2 and 4 from **caput**, region 6 from **corpus** and region 9 from **cauda** (Figure 18). Regions 2, 4 and 6 were microperfused (Druart *et al.* 1994, Syntin *et al.* 1996, Fouchecourt *et al.* 2000, Dacheux *et al.* 2006) with phosphate buffer saline (PBS). Region 9 was obtained by retroperfusion from the deferens duct using PBS supplemented. This technique leads to collect samples from the different epididymal regions and without serum and extracellular fluid contaminations (Dacheux *et al.* 1989).

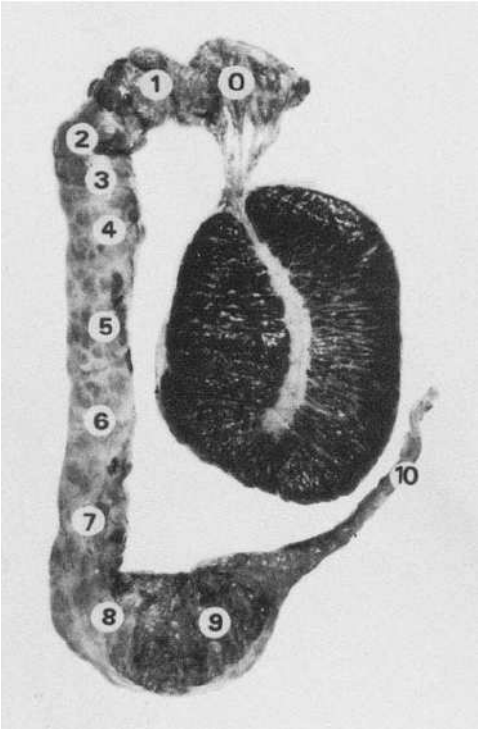


Figure 18. Macroscopic view of boar epididymis. The epididymis is attached to the testis by the efferents ducts and divided into 3 segments and 10 regions: caput (regions 0-4), corpus (regions 5-7) and cauda (regions 8-9) followed by the deferens duct (10). Figure from (Syntin *et al.* 1999).

Luminal samples from each epididymal region were centrifuged at 400 x g for 10 min at 15-20 ºC. The supernatants were centrifuged again at 15000 x g for 20 min at 4 ºC and stored at -20 ºC as **fluid samples**.

Pellets were washed at 400 x g for 10 min at 15-20 ºC with PBS supplemented and a 40-90 % Percoll gradient when it was necessary to obtain **sperm samples** before the total protein extraction or the biotin surface labeling. After washes and before total protein extraction or biotin labeling the sperm viability was assessed using the sperm viability kit of SYBR-14 and PI by flow cytometry (Cell Lab QuantaSC, Beckman Coulter, Weston, FL, United States).

2.2. Ejaculated samples

Ejaculated sperm samples were obtained from healthy postpuberal **Piétrain** boars (Selecció Batallé SA, Girona, Spain). Boars were fed under standard protocols and provided with water *ad libitum* while being submitted to a collection rhythm of twice per week. Approved guidelines for the ethical treatment of animals were followed. Semen was collected with the gloved-hand technique. The **sperm-rich** fraction was filtered to remove the gel and then pre-diluted 2:1 (v/v) in a long-term extender (Vitasem LD, Magapor SL, Zaragosa, Spain) at 37 ºC inside a collecting recipient, and transported to the AI centre

(BioGirona SL, Girona, Spain) to pack into 90 ml commercial doses at a concentration of $3{\cdot}10^9$ spz/dose. One dose per boar was send to our laboratories.

2.3. *In vitro* capacitation procedure

Distal cauda (region 9) and ejaculated sperm samples were centrifuged at 400 x g for 5 min at room temperature. The resultant pellet was divided into two aliquots. One of this was further extended with **BTS**: non-capacitating medium (Pursel & Johnson 1976), while the other one was extended with modified BTS medium (**BTS+**: capacitating medium (Saravia *et al.* 2007)) and incubated during 90 min under 37-38 ºC and 5 % CO_2 conditions (Puigmulé *et al.* 2011).

In vitro capacitation was assessed by flow cytometry by measuring the plasma membrane integrity (viability), plasma membrane fluidity and intracellular calcium levels using, respectively, the sperm viability kit of SYBR-14 and PI, the M540 and YP1 procedure and the Fluo-3- AM procedure.

2.4. Sperm analysis

2.4.1. Sperm motility

Epididymal or ejaculated non-capacitated samples were incubated for 20 min at 37 ºC to recover the sperm motility (this step was omitted when samples were *in vitro* capacitated). After the incubation, 20 µl of sample were dropped on a **Makler®** counting chamber (Self-Medical Instruments, Haifa, Israel) and then rapidly analyzed using the computer assisted sperm analysis (CASA), counting at least three drops per sample and capturing 1000 spermatozoa per drop. Different sperm motility parameters were obtained with the sperm class analyzer (SCA5, 2010, Microptic, S.L., Barcelona, Spain) under a negative phase-contrast objective coupled in a Olympus BX41 microscope (Olympus Europe GmbH, Hamburg, Germany) (Figure 19):

- Total motility percentage (**MOT**)
- Progressive motility percentage: spermatozoa showing more than 45 % of their STR (**PRG**)
- Curvilinear velocity in µm/s (**VCL**)
- Straight-linear velocity in µm/s (**VSL**)
- Average velocity in µm/s (**VAP**)
- Linearity index percentage (**LIN** = VSL/VCL x 100): linearity of the curvilinear trajectory
- Straightness index percentage (**STR** = VSL/VAP x 100): linearity of the mean trajectory
- Oscillation index percentage (**WOB** = VAP/VCL): oscillation of the real trajectory with respect to the mean trajectory
- Amplitude of lateral head displacement in µm (**ALH**): mean width of the head oscillation as the sperm cell swims.
- Beat Cross frequency in Hz (**BCF**): frequency of crossings between the curvilinear trajectory and the mean trajectory

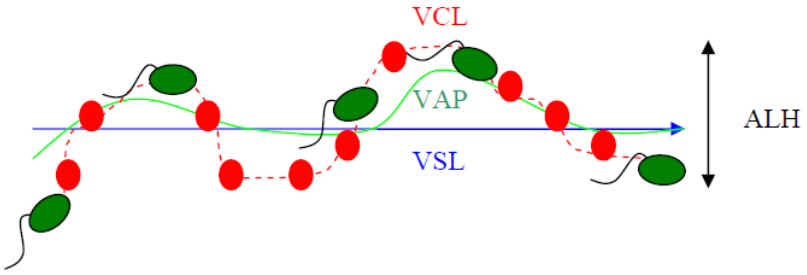


Figure 19. Representation of some motility parameters assessed by a computerized system over the displacement trajectory of a spermatozoon. Motility parameters represented in figure are: amplitude of lateral head displacement in µm (ALH), average velocity in µm/s (VAP), curvilinear velocity in µm/s (VCL) and straight-linear velocity in µm/s (VSL). Red points represent the tracked spermatozoon's trajectory. Figure from (Casas 2010).

2.4.2. Sperm concentration

Sperm counting is essential on the purpose of diluting semen. In boars, the number of spermatozoa per ml of an epididymal or an ejaculated sample is usually calculated with a Makler® counting chamber or by flow cytometry.

The analysis of sperm concentration using a Makler® counting chamber (Makler 1980) was performed as follows: 1) dilution of sperm samples in a formaldehyde saline solution, normally at 1/100 (v/v), 2) deposition of 20 µl of sperm dilution in a Makler® counting chamber (10 µm deep) and 3) counting three rows (10 squares per row). Only, those spermatozoa located inside the squares were counted and those located over each line converging in one angle of the square (only this angle). This count was repeated with new drops following the same criterion in the different squares counted. The number of spermatozoa counted in one row of 10 squares indicates their concentration in $\cdot 10^6$ spz/ml, retrieving the mean from different strips and drops (Figure 20). The concentration is calculated from the volume under one row of the chamber (0.001 mm^3) and the dilution factor performed.

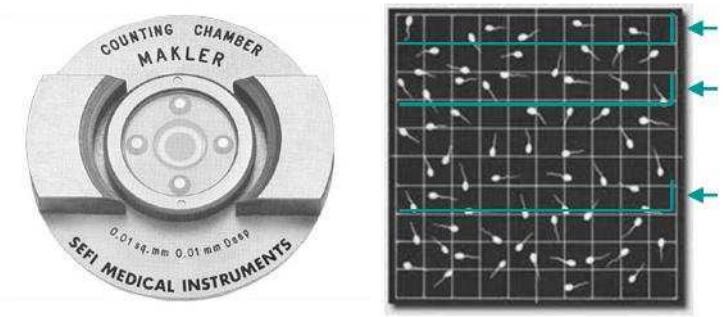


Figure 20. Makler® counting chamber. Sperm concentration was calculated in triplicate as the mean of the three rows counted. For each row only those spermatozoa located inside the squares were counted and those located over each line converging in one angle of the square (market with a green line in the picture).

The sperm concentration could also be analysed by flow cytometry according to the following proceed: 1) sperm samples were diluted in BTS, normally at 1/100 (v/v). Then, 2) aliquots of 500 µl of sperm dilution were analyzed without staining by flow cytometry. 3) A total of 20,000 events were analyzed, being the sperm concentration determinated after gathering the sperm population among the overall events captured (Figure 21).

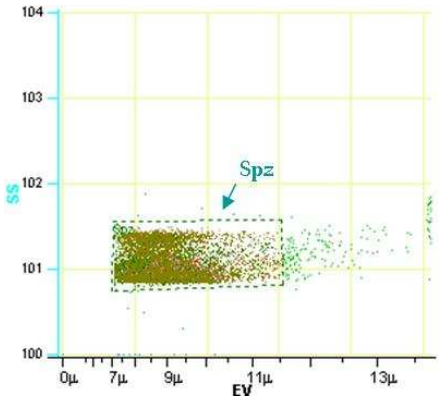


Figure 21. Flow cytometry gated sperm population. Sperm concentration was calculated as the mean of the three counts. Sperm concentration was obtained as the concentration of events present in the gathered region called as sperm population (Spz).

2.4.3. Sperm status

Sperm viability, acrosomal status, evaluation of sperm capacitation status, assessment of mitochondrial activity and, detection of apoptosis-like changes could be assessed by flow cytometry. Routinely, for each assay, the measures were repeated three times per sample and male. Analyses were done on a Cell Lab Quanta flow cytometer (Figure 22). Forward-scatter (FSC) and side-scatter (SSC) dot plots gave the cellular physical properties of size and granularity and allowed us to gate single oriented sperm. After fluorescence excitation by Argon ion 488 nm, the fluorescence from SYBR-14-stained, FITC-PNA-stained, Fluo-3 AM-stained, JC-1 monomers-stained, MitoTracker® GreenFM-stained and YP1-stained were collected in FL1 (525±30 nm), JC-1 aggregation-stained was also collected in FL2 (575±30 nm) and PI-stained and M540-stained were collected in FL3 (670±30 nm) fluorescence detectors. Through inspection of FSC data indicative of size, small non-sperm events and large events originated from cell agglutination were excluded. A total of 10,000-20,000 singlet cells were acquired and analyzed by Cell Lab QuantaTM SC Software v 1.0 (Beckman Coulter, Fullerton, CA, United States). Flow cytometry combines benefits of fluorescence microscopy and biochemical analysis to provide fast and quantitative measurements on individual cells (Taitzoglou *et al.* 2007).

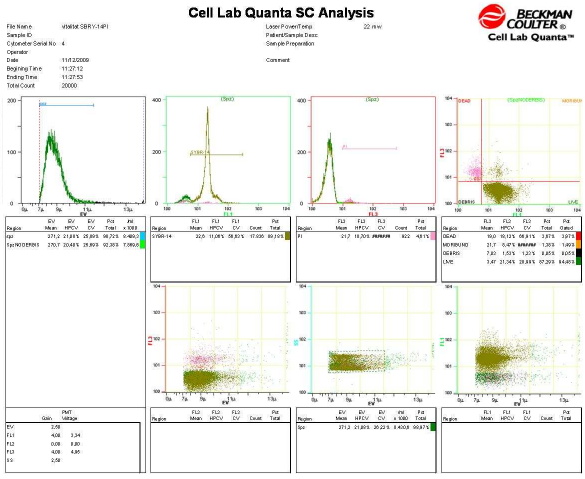


Figure 22. Flow cytometry excel report. Stained samples analysed by flow cytometry are reported in an excel data file where the protocol parameters are observed as well as the cytogrames, dot plots and statistical data.

2.4.4. Sperm membrane integrity

Membrane integrity of spermatozoa can be evaluated either with the eosin-nigrosin dye or with the **SYBR-14/PI** staining (Garner & Johnson 1995, Christensen *et al.* 2004). Currently, one of the most frequently used viability probes for staining mammalian cells is SYBR-14 and propidium iodure (PI) procedure. SYBR-14 is membrane permeable and stains all sperm heads in green, whereas PI is membrane impermeable and penetrates in membrane-damaged spermatozoa staining the sperm heads in red. PI displaces or quenches SYBR-14 fluorescence.

Working solutions of SYBR-14 and PI from Sperm Viability kit were prepared: SYBR-14 must be diluted 1/10 (v/v) in DMSO to make a 0.1 mM working solution; PI was used at the concentration of the original solution in the kit (2.4 mM). Both fluorochromes were aliquotated in order to avoid repeated freeze-thawing cycles and conserved at -20 ºC.

Epididymal and ejaculated sperm samples are diluted to $10{\cdot}10^6$ spz/ml in BTS. Three aliquots of 500 µl of diluted sperm samples were prepared for each sample (triplicates) and stained with 0.5 µl of SYBR-14 0.1 M and incubated during 10 min at 38 ºC. Then 2.5 µl of PI 2.4 mM were added and incubated for 5 min at 38 ºC. Finally 20,000 events were analyzed by flow cytometry for each sample and replicate (Figure 23).

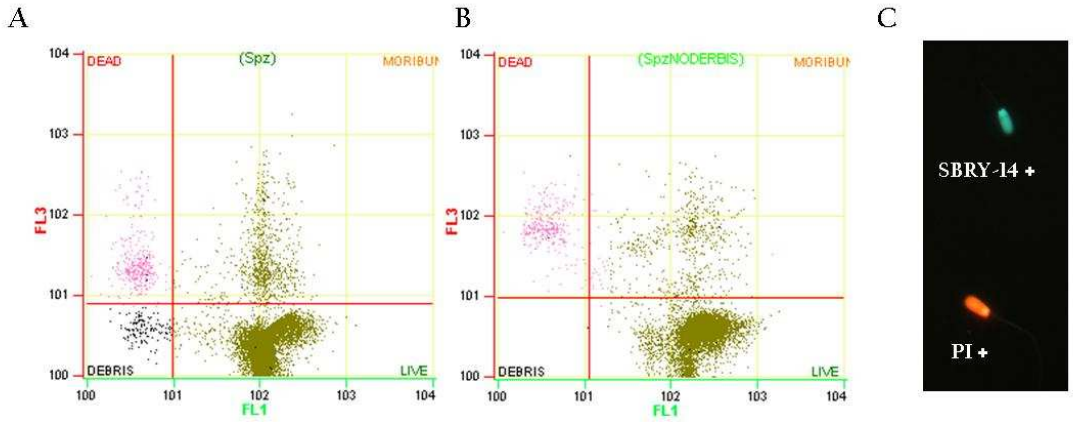


Figure 23. Flow cytometry dot blots of a SYBR-14/PI stain. Four populations can be identified (A): non-viable spermatozoa (red stained; upper left), recently membrane-damage, "moribund", spermatozoa (double staining red and green; upper right), viable spermatozoa (green stained; lower right) and debris (unstained; lower left). Unstained debris can be easily discarded gathering this population in order to obtain accurate sperm percentage data (B). (C) Digital images of sperm cell stained with SYBR-14 (green fluorescence) and with PI (red fluorescence) captured at x400 magnification under a fluorescence microscope with AxioVs40 v4.6.3.0 software (Zeiss Axio Imager.Z1).

2.4.5. Sperm acrosome integrity

For the assessment of acrosomal status, two different lectins are currently used: PNA and PSA. These lectins are generally conjugated with FITC, which emits green fluorescence, and combined with a viability dye such as PI (Nagy *et al.* 2003, Matás *et al.* 2010). In this project acrosomal integrity was assessed using fluorescein isothiocyanate-labelled Arachis hypogoea peanut agglutinin (**FITC-PNA**).

Working solutions of FITC-PNA 1 mg/ml were prepared by adding 1 ml of PBS in 1 mg of FITC-PNA reagent. Working solution of propidium iodide 2.4 mM was prepared dissolving 3.2 mg of PI in 2 ml of

PBS. Both fluorochromes were aliquotated in order to avoid repeated freeze-thawing cycles and conserved at -20 ºC.

From each epididymal and ejaculated sperm samples, aliquots of 500 µl of $10{\cdot}10^6$ spz/ml diluted in BTS were prepared and stained with 1.5 µl of FITC-PNA working solution for 5 minutes at 38 ºC in dark. Following incubation, 2.5 µl of PI (2.4 mM) was added in each sample and incubated for 5 minutes in dark, previous to cytometric analysis. 20,000 events were analyzed by flow cytometry for each sample and replica (Figure 24).

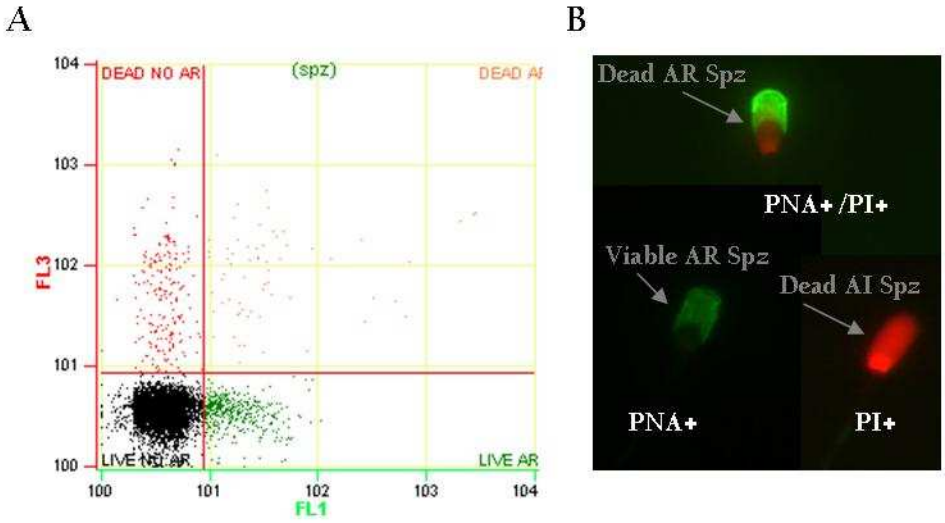


Figure 24. Flow cytometry dot blot of a FITC-PNA/PI stain. Four populations can be identified (A): non-viable spermatozoa with intact acrosome (red stained; upper left), non-viable spermatozoa with reacted acrosome (double staining red and green; upper right), viable spermatozoa with intact acrosome (unstained; lower left) and viable spermatozoa with reacted acrosome (green stained; lower right). (B) Digital images of sperm cells stained with PNA (green fluorescence) and with PI (red fluorescence) captured at x400 magnification under a fluorescence microscope with AxioVs40 v4.6.3.0 software (Zeiss Axio Imager.Z1). Three sperm typologies could be distinguished by fluorescence microscopy: non-viable spermatozoa with reacted acrosome stained with both PNA and PI (Dead AR spz), viable spermatozoa with reacted acrosome stained with PNA (Viable AR spz) and non-viable spermatozoa with intact acrosome (Dead AI spz).

2.4.6. Sperm mitochondrial function

Mitochondrial status is another important trail of sperm physiology (Peña *et al.* 2009). **JC-1** (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) and **Mitotacker®GreenFM** are extensively used in the assessment of mitochondrial function. JC-1 accumulates in the mitochondria as green-fluorescence monomers. In the presence of high mitochondrial membrane potential ($\Delta\Psi m$), the monomers aggregate, shifting the fluorescence to orange (Garner & Thomas 1999, Love *et al.* 2003, Marchetti *et al.* 2004, Martinez-Pastor *et al.* 2004). MitoTracker® dyes are a group of recently developed dyes that accumulate and stain active mitochondria. **MitoTracker®Green FM** is currently used in combination with **PI** to distinguish between the viable spermatozoa with membrane active or inactive mitochondria (Bussalleu *et al.* 2005, Martínez-Pastor *et al.* 2010).

Briefly, for JC-1 procedure diluted sperm samples in aliquots of 500 µl were stained with 0.5 µl of JC-1 and incubated during 30 min at 38 ºC in the dark, prior to the flow cytometric analysis (Figure 25).

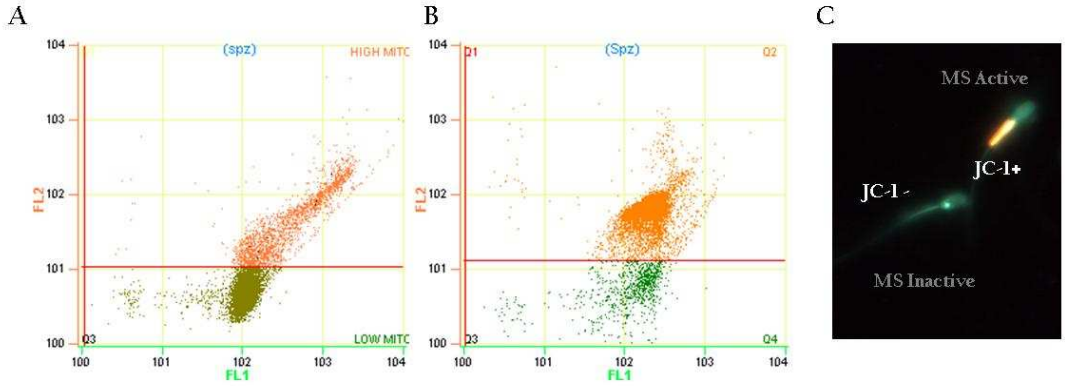


Figure 25. Evaluation of sperm mitochondrial function by JC-1. (A-B) Dot blot representations of the flow cytometric assessment of non-capacitated (A) and capacitated (B) sperm samples by JC-1 stainning. Two populations can be identified in both cytograms: spermatozoa with high mitochondrial membrane potential (ΔΨm) (orange stained; upper) and spermatozoa with low mitochondrial membrane potential (ΔΨm) spermatozoa (green stained; lower). (C) Digital images of sperm cells stained with JC-1 captured at x400 magnification under a fluorescence microscope with AxioVs40 v4.6.3.0 software (Zeiss Axio Imager.Z1). Two sperm typologies could be also distinguished under the fluorescence microscope: spermatozoa with high mitochondrial membrane potencial with orange fluorescence (MS active) and spermatozoa with low mitochondrial membrane potencial with green fluorescence (MS inactive).

The percentage of spermatozoa with functional mitochondria was estimated by combining fluorescent stains of **MitoTracker®GreenFM** (MTG) and **PI** adapting the protocol of Bussalleu et al., (Bussalleu *et al.* 2005). Working solutions of MTG were prepared diluting 50 µl of commercial solution into 74.4 µl of DMSO in order to obtain a stock solution of MTG 1 mM. Stock solution 1 mM was conserved at -20 ºC in 1 µl aliquots. Previously, 999 µl of DMSO were added into 1 µl of MTG 1 mM in order to obtain a working solution of MTG 1 µM. Then, 50 µl of MTG working solution (1 µM) were added in 500 µl sperm aliquots ($10{\cdot}10^6$ spz/ml) and incubated for 5 min at 38 ºC in dark. And finally, 2.5 µl of PI (2.4 mM) were added and incubated during 5 minutes before cytometric analysis (Figure 26).

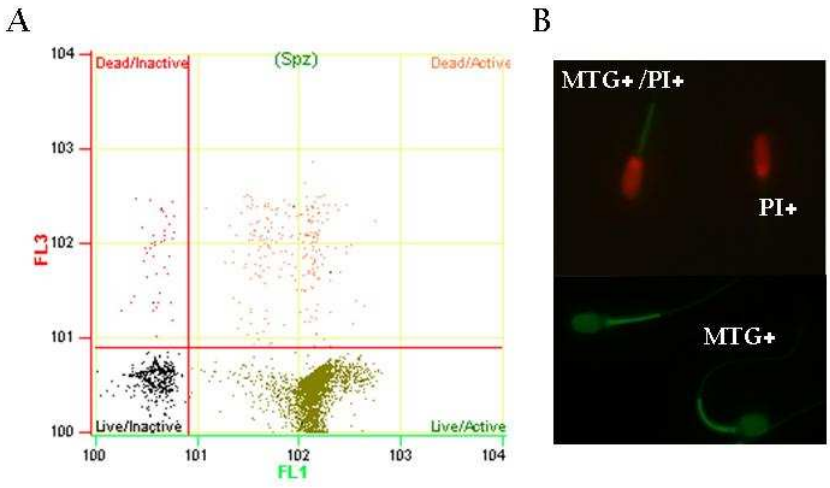


Figure 26. Flow cytometry dot blot of a MitoTrackerGreen/PI staining. Four populations can be identified (A): non-viable spermatozoa with damaged mitochondrial sheath (red stained; upper left), non-viable spermatozoa with intact mitochondrial sheath (double staining red and green; upper right), viable spermatozoa with damaged mitochondrial sheath (unstained; lower left) and viable spermatozoa with intact mitochondrial sheath (green stained; lower right). (B) Digital images of sperm cell stained with MTG (green fluorescence) and with PI (red fluorescence) captured at x400 magnification under a fluorescence microscope with AxioVs40 v4.6.3.0 software (Zeiss Axio Imager.Z1). Three sperm typologies could be distinguished under the fluorescence microscope: non-viable spermatozoa with intact mitochondrial sheath showing red head and green mitochondrial sheath, non-viable spermatozoa with damaged mitochondrial sheath showing red fluorescence in the head and lack of fluorescence in the mitochondrial sheath and, viable spermatozoa with intact mitochondrial sheath showing green fluorescence.

2.4.7. Sperm capacitation status

Capacitation is a sequence of events that results in changes in the plasma membrane composition and structure, in an increase of the influx of calcium inside of the spermatozoon, and in the activation of both the hyperactivated movement and the second messenger pathways. Different fluorometric procedures have been developed to study the increases of sperm membrane fluidity and the calcium influx. Two main procedures are currently used in order to evaluate the sperm capacitation status: **Merocyanine 540** (M540) combined with **YO-PRO®-1** (YP1) staining and **Fluo-3-acetomethoxy ester** (Fluo-3 AM) combined with **PI**, which lead to assess the plasma membrane fluidity and the calcium influx, respectively.

M540 is a lipophilic molecule that binds to the plasmalemma, and its orange fluorescence increases with decreasing phospholipid membrane packaging, as occurs during capacitation (Gadella & Harrison 2002, Spjuth *et al.* 2007).

Stock solutions of **M540** 88 mM were prepared adding 1 ml of DMSO to 50 mg of M540. Previously, 11.4 µl of stock solution M540 88 mM were diluted in 988.6 µl of DMSO to obtain a working solution of M-540 1 mM. For **YP1** 2.5 µM working solutions were prepared diluting 2.5 µl of commercial YP1 1 mM in 97.5 µl of DMSO. M-540/YP1 staining was performed as described by Januskauskas et al., (Januskauskas *et al.* 2005). Sperm samples were incubated during 10 min at 38 ºC with 1.3 µl of M-540 working solution (1 mM) and 0.5 µl of YP1 working solution (25 µM). For each sample 20,000 events were analyzed by flow cytometry (Figure 27).

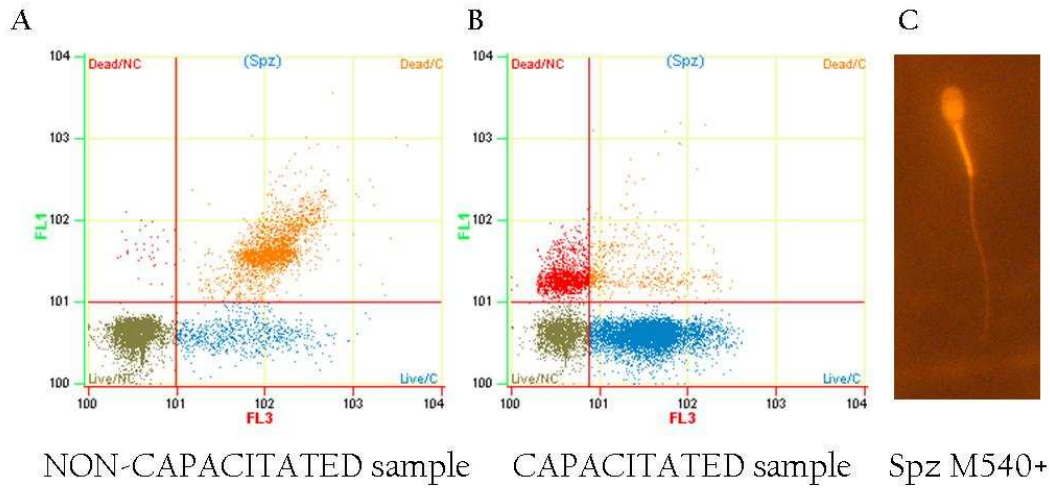


Figure 27. Evaluation of sperm capacitation status by Merocyanine 540 (M540). (A-B) Dot blot representations of the flow cytometry assessment of non-capacitated (A) and capacitated (B) sperm samples by merocyanine 540 and YO-PRO®-1 stainning. Four populations can be identified in both cytograms: non-viable and non-capacitated spermatozoa (green stained; upper left), non-viable and capacitated spermatozoa (double stained green and red; upper right), viable and non-capacitated spermatozoa (unstained; lower left) and, viable and capacitated spermatozoa (red stained; lower right). (C) Digital image of a viable and capacitated spermatozoon stained with M-540 were captured at x630 magnification under a fluorescence microscope with AxioVs40 v4.6.3.0 software (Zeiss Axio Imager.Z1).

Fluo-3 AM is a green fluorochrome whose fluorescence is increased when bound to Ca^{2+}(Kadirvel *et al.* 2009); it is currently used in combination with PI. Working solutions of 1 mM of Fluo-3 AM were prepared adding 885 µl of DMSO in 1 mg of Fluo-3 AM commercial powder. The mean of Fluo-3 AM fluorescence intensity was analyzed according to the following proceed: 1) 0.5 µl of Fluo-3 AM was

added in 500 µl of diluted sperm aliquots during 5 min at 38ºC and dark. Then 2) 2.5 µl of PI was added and incubated for 5 min at the same conditions and, then 3) assessment the mean FI of the viable Fluo-3 AM positives singlet cells (Figure 28).

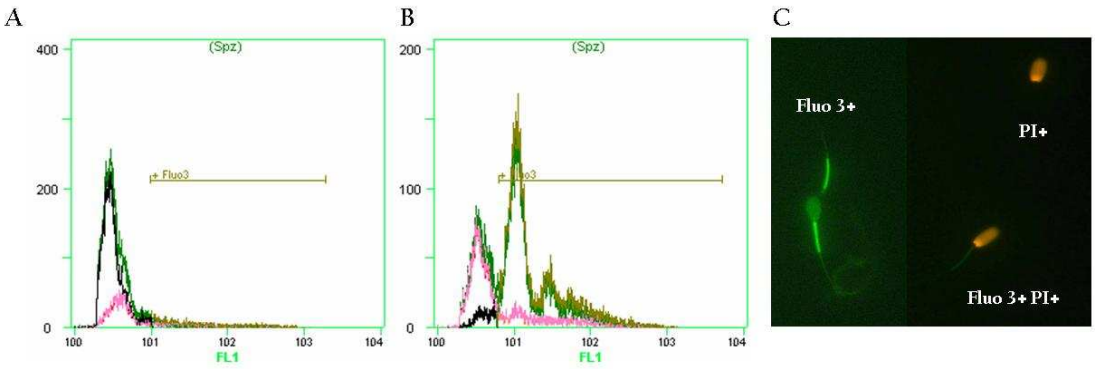


Figure 28. Fluo-3AM fluorescent intensity cytogram analysed by flow cytometry. Intracellular calcium levels (Fluo-3AM fluorescent intensity) measured by flow cytometry in non-capacitated (A) and capacitated (B) sperm samples. A significant increase of FL1 fluorescent signal (Fluo-3 AM) can be observed in capacitated samples (B). Digital images of sperm cell stained with Fluo 3 and PI were captured at x630magnification under a fluorescence microscope with AxioVs40 v4.6.3.0 software (Zeiss Axio Imager.Z1) (C).

2.4.8. Sperm apoptotic status

Sperm apoptosis manifests in an increase of membrane permeability. YO-PRO®-1 iodide (YP1) is able to stain the nucleus of apoptotic cells (Peña *et al.* 2005). YP1/PI combination allows non-viable (membrane-damaged) spermatozoa to be discriminated, because it cannot enter cells with increased membrane permeability, and apoptotic feature. Therefore, YP1 stain such spermatozoa, separating those from the unstained non-apoptotic spermatozoa (Martínez-Pastor *et al.* 2010).

For YP1/PI staining aliquots of 500 µl diluted sperm were stained with 0.5 µl of YP1 working solution 25 µm and 2.5 µl of PI 2.4 mM during 5 min at 38 ºC at dark and then evaluated by flow cytometry (Figure 29). Three sperm populations were observed: a non-viable population (+ PI membrane-damaged), an apoptotic population (+ YP1; -PI) and a viable population (- YP1; -PI)

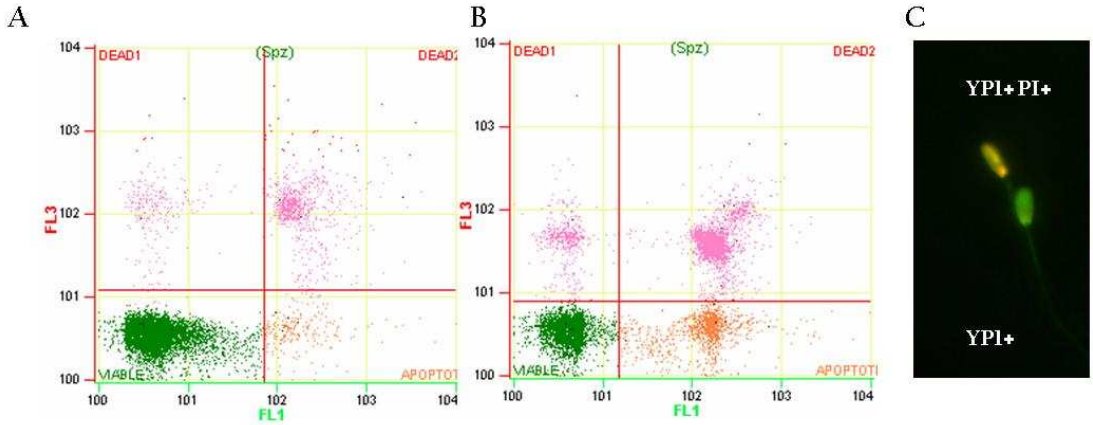


Figure 29. Flow cytometry dot blots of Yo-Pro-1/PI staining. Dot blot graphics obtained in the flow cytometric assessment of non-capacitated (A) and capacitated (B) sperm samples by YP1/PI staining. Three main sperm populations could be observed: non-viable spermatozoa (red stained; upper), viable spermatozoa (unstained; lower left) and apoptotic spermatozoa (green stained; lower right). Digital images of sperm cells stained with Yo-Pro-1® and PI were captured at x630 magnification under a fluorescence microscope with AxioVs40 v4.6.3.0 software (Zeiss Axio Imager.Z1) (C).

2.5. Total protein extraction

Sperm samples were divided in two aliquots; one of them was used for total protein extraction and the other for biotin labeling. Total protein extraction was performed with 2% N-octyl-beta-D-glucopyranoside or SDS with a protease inhibitor cocktail, sodium vanadate 1mM and PMSF 1mM. The samples were incubated with the extraction buffer for 30 min at 4ºC and agitation, and then centrifuged at 15000 x g for 15 min at 4ºC. The supernatant was carefully removed and stored at -20ºC.

2.6. Protein surface labeling with biotin

Sperm samples were resupended in PBS supplemented at a final concentration of $3{\cdot}10^9$ spz/ml. The labeling was performed with 3 mg/ml (~ 6 mM) of NHS-SS-Biotin during 30 min at room temperature and agitation. The biotin labeling was stopped with Tris 50 mM or glycine 300 mM. Biotin excess was eliminated by successive washes with PBS supplemented and a 40-90% Percoll gradient.

Proteins were extracted by mixing the pellet with a twice volume of PBS tergitol Nonidet P-40 solution (NP40S) 2% (v/v), a protease inhibitor cocktail, sodium vanadate 1 mM and PMSF 1 mM during 30 min at 4ºC and agitation. Then, the samples were centrifuged at 15,000 x g for 15 min at 4ºC and the supernatant was carefully removed and stored at -20ºC.

Biotin is a small vitamin of 244,3 Da (vitamin H) with high affinity to avidin and streptavidin; it bonds covalenty to lysine groups of many proteins without altering their biological activities (Rosenberg 2005).

A number of biotin derivates are available for conjugation with a variety of functional groups. One commonly used derivative is biotinyl-N-hydroxysuccinimide ester (**NHS-Biotin**). In pH 7-9 buffers, NHS-actived biotins react efficiently with primary amino groups (-NH_2), to form stable amide bonds. **Primary amines** of several proteins are located in the side **chain** of **lysine** (K) residues and in the **N-terminus** of each polypeptide, being available as targets for labeling with NHS-activated biotin reagents. Several different NHS esters of biotin are available, differing in properties and spacer arm lengths (Figura 30).

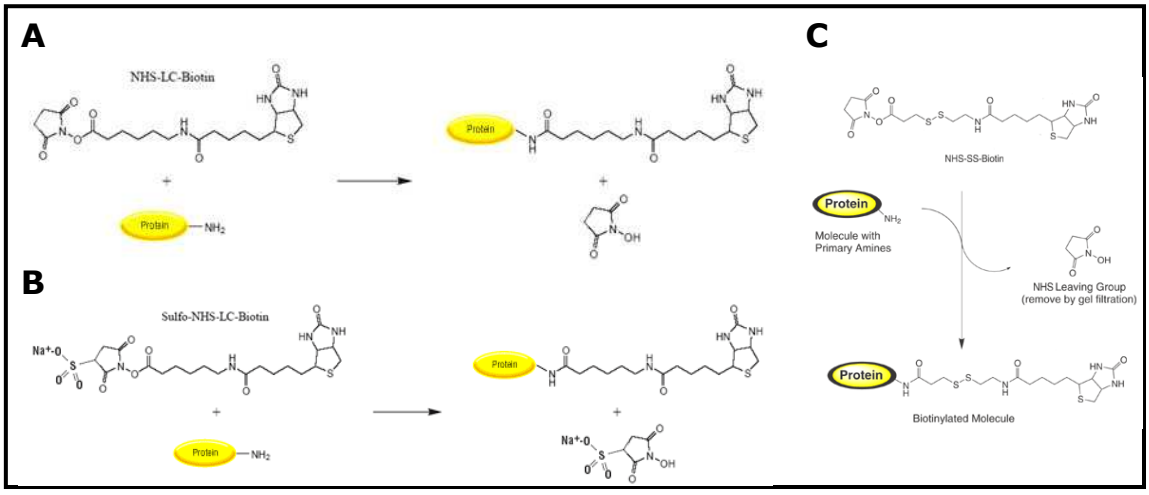


Figura 30. Schematic representations of NHS-Biotin reaction with a primary amine-containing protein. A. In non-sulfonated-NHS-biotins. B. In sulfonated-NHS-biotins. C. In NHS-SS-biotin. Figures from Thermo Scientific®.

One type of NHS-biotin conjugation available reagents are **non-sulfonated** NHS-biotins. These reagents require an organic solvent such as DMSO or DMF to be dissolved. The other type, the **sulfo-**NHS ester biotins reagents are water soluble, enabling reactions to be performed in the absence of organic solvents. There are different structures of NHS-Biotin labeling reagents for each type differing among them in properties, spacer arm lengths and structures (Table 2).

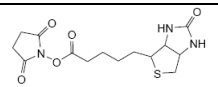
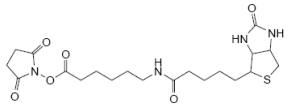
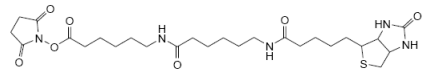
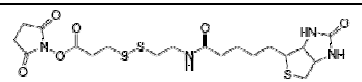
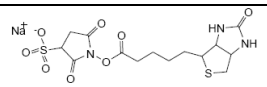
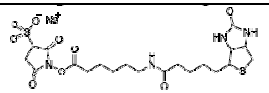
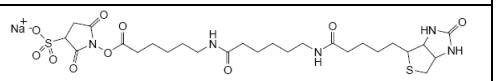
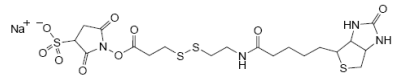
N-hydroxysulfosuccinimide reagent (sulfo-NHS) is membrane impermeable and confers a unique property that it is useful for specific labeling of cell surface proteins. As long as the cell remains intact, only primary amines exposed on the surface will be biotinylated with these sulfo-NHS-biotin reagents labeling selectively the cell surface.

Others variants of biotin reagents also exist (sulfo/non-sulfo), NHS-SS-biotin, which present a disulfide bond (-S-S-) in its spacer arm, enabling labelled proteins to be cleaved from the biotin group by treatment with DTT or other reducing agents. This feature is especially useful in experiments of affinity purification where it is necessary to elute the biotinylated protein from its bound state to avidin or streptavidin.

Several NHS-biotin reagents are used for the surface protein labeling of both spermatozoa (Koch *et al.* 1994, Grace *et al.* 2002, Kathryn *et al.* 2006, Nixon *et al.* 2006, Kim *et al.* 2009) and oocytes (Flaherty & Swann 1993).

Independently of the variant of commercial biotin reagent, labeled proteins with biotin can be used to **purify** from unlabeled ones using immobilized streptavidin and avidin affinity gels and, they also can be **detected** by ELISA, dotblot or Western blot applications using streptavidin or avidin-conjugated probes. Avidin and streptavidin are avaible commercially conjugated to either Horseradish Peroxidase (HRP) or alkaline phosphatase (AP), being detected the bands of interest colorometrically. Due to avidin (67 KDa) is a glycoprotein, it can produce unwanted binding to other molecules like lectins. Streptavidine (60 KDa) has similar properties but it has two important advantges that make it replace avidin as the preferred ligand: 1) as streptavidin is not a glycoprotein, non-specific junctions are avoided, and 2) its isoelectric point is close to neutrality so that it bears few strongly charged groups at neutral or the slightly alkaline pH of most detection systems (Rosenberg 2005).

Table 2. Commercial variants of biotin reagents with its proprieties and structure. Structures from Thermo Scientific®.

	Abreviation	Molecular formula	Molecular weight (kDa)	Spacer arm length	Solubility	Membrane permeability	Structure
Succinimidobiotin	NHS-Biotin	$C_{14}H_{18}O_5N_3S$	341,38	13,5 Å	Organic solvent	yes	
Succinimidyl-6-[biotinamido]hexanoate	NHS-LC-Biotin	$C_{20}H_{29}O_6N_4S$	454,54	22,4 Å	Organic solvent	yes	
Succinimidyl-6-[biotinamido]-6-hexanamidohexanoate	NHS-LC-LC-Biotin	$C_{26}H_{40}O_6N_5S$	567,7	30,5 Å	Organic solvent	yes	
Succinimidyl 2-(biotinamido)-ethyl-1,3'–dithiopropionate	NHS-SS-Biotin	$C_{19}H_{28}O_6N_4S_3$	504,65	24,3 Å	Organic solvent	Yes	
Sulfosuccinimidobiotin	Sulfo-NHS-Biotin	$C_{14}H_{18}O_8N_3S_2Na$	443,43	13,5 Å	Water	No	
Sulfosuccinimidyl-6-[biotinamido]hexanoate	Sulfo-NHS-LC-Biotin	$C_{20}H_{29}O_9N_4S_2Na$	556,59	22,4 Å	Water	No	
Sulfosuccinimidyl-6-[biotinamido]-6-hexanamidohexanoate	Sulfo-NHS-LC-LC-Biotin	$C_{26}H_{40}O_9N_5S_2Na$	669,75	30,5 Å	Water	No	
Sulfosuccinimidyl 2-(biotinamido)-ethyl-1, 3-dithiopropionate	Sulfo-NHS-SS-Biotin	$C_{19}H_{27}O_9N_4S_4Na$	606,69	24,3 Å	Water	no	

2.7. Purification of surface proteins

After biotin labeling the surface proteins were purified using streptavidin beads. The beads were pelleted and washed with PBS NP40 1%, NaCl 2 M, Na_2CO_3 0.1 M pH 11.5 and PBS NP40 1% and incubated for 1 h at 4ºC and agitation with the protein extract obtained after the biotin labeling. After incubation the beads were pelleted and washed with PBS NP40 1%, NaCl 2M, Na_2CO_3 0,1 M pH 11.5 and PBS NP40 1% and then the surface proteins were eluted from beads by boiling for 5 min in sample buffer containing 2% SDS, 2% 2-mercaptoethanol, 0.375 M tris pH 6.8 and bromophenol blue. The 2-mercaptoethanol treatment breaks the disulfide bonds and the surface membrane proteins can be recovered and eluted from the biotin-streptavidin microbead supports.

2.8. Gel electrophoresis and protein blotting

One-dimensional electrophoresis

The electrophoresis in 8-16% gradient SDS-PAGE gels. The samples were diluted in Laemmli reductor buffer and boiled during 3 min at 95 ºC.

SDS-PAGE Gradient 8-16% resolving gels (12 gels)

%Acrilamide/ Bisacrilamide	MiliQ (ml)	Acrilamide/ Bisacrilamide 37.5:1 ratio (ml)	resolving solution 2X (ml)	Glycerol (ml)	APS (ml)	TEMED (ml)	Total (ml)
16%	6,650	20,800	4,850	7,700	0,070	0,013	40,083
8%	22,350	10,400	4,850	2,400	0,070	0,013	40,083
Top solution	17,675	0,000	2,360	0,000	0,035	0,013	20,083

SDS-PAGE 5% stacking gels (2 gels)

%Acrilamide/ Bisacrilamide	MiliQ (ml)	Acrilamide/ Bisacrilamide 37.5:1 ratio (ml)	stacking solution (ml)	Glycerol (ml)	APS (ml)	TEMED (ml)	Final Volume (ml)
5%	3,000	0,750	1,250	0,000	0,050	0,005	5,055

Bi-dimensional electrophoresis

Protein extracts were rehydrated overnight at 20ºC in IPG strip precast gels with immobilized pH gradient of 7 or 11 cm long with the rehidratation buffer. For 7 and 11 cm strips, a different isofocalization programmer (IEF Cell Protean system, Bio-rad) was performed (Table 3).

Until the second dimension, SDS-PAGE electrophoresis, the strips were rinsed with two equilibration solutions, equilibration solution I and then with equilibration solution II, during 15 min at room temperature and agitation for each one, in order to reduce and alcalynizate the proteins, respectively. The strips were deposited in 8-16% gradient mini (7 cm) or grand (11 cm) gels and steeled with an agarose 0.5% gel dissolved in electrophoresis buffer with bromophenol blue. The electrophoresis was run at 30 mA for gel (IEF Cell Protean system (Bio-rad).

Table 3. Focalization programmers performed for the isofocalization of the strips of 7 and 11 cm.

	7 cm strip (52000 V·h total)			11 cm strip (95000 V·h total)		
Step	Voltage (V)	Time (hh:mm)	gradient	voltage (V)	Time (min)	gradient
1	100	10	Fast	250	15	Fast
2	4000	7:30	Lineal	8000	12:00	Lineal
3	4000	9:00	Constant	8000	16:00	Constant
4	8000	1:30	Fast	500	-	Fast
5	500	∞	Fast			

Protein blotting

After one or bi-dimensional electrophoresis the gels were transferred in nitrocellulose or PVDF membranes 0.2 µm with a transfer system (Mini-Trans Blot®, Bio-Rad Laboratories, Inc, Hercules, CA, United States or with Amersham ECL SemiDry Blotter system, GE Healthcare Ltd, Buckinghamshire, England). Membrane transfers were dyed with red Ponceau solution to check an homogeneous transfer and then blocked overnight at 4ºC or during 2 hours at room temperature with TBS/Tween 20 with blocking solution (BSA or low fate milk 3-5%).

Western blot assays

Blocked membranes were incubated with the first antibody at different dilutions with blocking solution during 1 h at room temperature and agitation, according with previous tests (data not showed). After incubation, the membranes were washed for 10 min and agitated twice with blocking solution and once with TBS/Tween 20. Secondary antibodies diluted 1/5000 with blocking solution were incubated during 1h at room temperature and then washed as the first antibody.

Membrane developing was performed by incubating the membranes with a chemiluminescent substrate and exposed in photographic films. The membranes were also developed with a chromogenic system with 4-chloro-1-naphtol.

Protein patterns were scanned and quantified using a gel documentation system (Gel Doc XR; Bio-Rad; Hercules, CA, USA) and Quantity One Version 4.6.2. Software (Bio-Rad Laboratories Inc., Hercules, CA, United States). Protein levels were expressed as normalized quantity means (± SEM) using alpha-tubulin antibody as an internal standard to normalize the protein quantity of each protein band, after stripping with glycine stripping solution and reproving membranes.

Stripping and reusing membranes

The stripping procedure works with blotted membranes from one- and two-dimensional gels and it allow the reusing of PVDF membranes developed with chemiluminescent reagents.

Blots are washed in distilled water for 5 minutes and then transfered into stripping solution during 15-45 minutes at room temperature. Blots were washed in distilled water during 5 minutes and then were

successively washed with TBS-Tween 20 for 30 minutes at least. Re-block membranes with blocking solution before processing to new immunoblotting assays.

2.9. Lectin assays

Western blot assays

To study the glycoprotein composition of glycocalyx in sperm epididymal samples, electrophoretic and Western blot assays were developed. Membranes were blocked with TBS/Tween20 with polyvinylpyrrolidone K30 2% (v/w) overnight at 4ºC or for 2 hours at room temperature. After blocking, membranes were rinsed for 10 minutes with TBS/Tween20 with 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 mM $ZnSO_4$. After dilution at 1 µg/ml with rinse solution, lectins were incubated during 1 h at room temperature and agitation. After incubation, the membranes were washed for 10 min twice with blocking solution and once with TBS/Tween 20. Streptavidin diluted 1/10,000 with blocking solution was incubated during 1h at room temperature and then washed.

Membrane developing was performed incubating them with chemiluminescent reagents and exposing in photographic films.

Flow cytometry assays

In flow cytometry assays $10{\cdot}10^6$ spz/ml sperm samples were labelled with 5 µg/ml of lectin in BTS supplemented with 1 mM Ca^{2+}, 1 mM Zn^{2+}, 1 mM Mn^{2+}, 1 mM Mg^{2+} pre-warmed at 38 ºC BTS. Samples were incubated for 5 minutes at 38 ºC and protected from light, and then counterstained with an impermeable nuclear stain, such as PI or YP1, in order to avoid false positive lectin binding of intracellular structures of non-viable sperm cells (Ashworth *et al.* 1995). Counterstaining was performed by addition of either, 5 µl of PI 2.4 mM or 1 µl of YP1 25 µM, and incubation at the same conditions for 5 minutes more. For each lectin, measures of 20,000 events were repeated in three different replicates on a Cell Lab Quanta flow cytometer. The mean of FITC/AF488/TRIC fluorescence intensity (FI) of the viable sperm population was recorded from the detector output to determine lectin binding-related changes in the different sperm samples. Specific inhibitory sugars were preincubated with lectin labeling solutions at least for 10 min prior to the sperm addition in order to assess lectin specificity with the lack of fluorescence emission.

Immunocytochemestry assays

Boar sperm samples were labeled "*in vivo*" with 5 µg/ml of each lectin diluted and pre-warmed at 38 ºC in BTS supplemented with 1 mM Ca^{2+}, 1 mM Zn^{2+}, 1 mM Mn^{2+}, 1 mM Mg^{2+}. Then pre-warmed lectin solutions were incubated with sperm samples during 5 min at 38 ºC and protected form light. A simultaneous counterstaining with an impermeable nuclear dye, such as PI or YP1, was performed. Labelled sperm samples were observed under a fluorescent microscope (Axio Imager.Z1, Carl Zeiss AG,

Göttingen, Germany) and digital images were captured at x 630 and x 1000 magnifications under a fluorescence microscope with AxioVs40 v4.6.3.0 software (Carl Zeiss AG, Göttingen, Germany).

2.10. Immunocytochemistry

In the present thesis three different protocols of sperm immunocytochemistry were used: 1) immunolabeling with dropped fixed samples, 2) immunolabeling in suspension of fixed sperm samples and 3) *in vivo* immunolabelling (described in section 2.9 of lectin immunochemistry assay).

Immunolabeling with dropped fixed sperm samples

Boar epididymal and ejaculated sperm samples were washed with PBS and fixed with 3% para-formaldehyde for 30 min at room temperature. After three washes in PBS at 600 x g for 10 min, samples were diluted to ~10^7 spz/ml in PBS and dropped onto poly-lysine-coated slides (Electron Microscopy Sciences, Harfield, PA, United States), rinsed with PBS, and blocked in PBS-1% BSA for 10 min. Primary antibody was incubated for 1 h diluted in PBS at a previous tested concentration. After washing in PBS, samples were incubated for 1h more with the secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, United States), diluted 1/1000 in PBS-1% BSA. After extensive washings with PBS, the mounting medium was applied.

Autofluorescence controls were performed incubating the sperm samples with PBS-1% BSA without antibodies, primary antibody (not incubated with secondary antibody) and secondary antibody (not incubated with primary antibody).

Different patterns of protein localization were observed at x630 and x1000 magnifications under a fluorescence microscope and digital images were captured with AxioVs40 v4.6.3.0 software.

Immunolabeling of fixed sperm cells in supension

In phosphotyrosine assays, boar epididymal and ejaculated sperm samples were washed in PBS at 600 x g for 10 min and fixed with 2% formaldehyde for 10 min at 37ºC. After fixation, samples were permeabilized with ice methanol 90% for 30 min at 4 ºC and then preceded with staining or stored at -20ºC in methanol 90%. Three replicates and the inhibitory control (preincubation of anti-PTyr antibody with O-phospho-L-tyrosine) were prepared for each sample. After rinsed, the samples were diluted at ~10^6 spz/ml and then blocked with 90 µl of incubation buffer (PBS 1% BSA) during 10 min at room temperature. After blocking, 10 µl of conjugated anti-PTyr antibody were added in each sample and incubated for 45 min in dark and room temperature. Samples were rinsed with incubation buffer by centrifugation and resuspended in 0.5 ml of PBS. Stained samples were dropped onto poly-lysine-coated slides and mounting medium was applied. Digital images were captured at x630 and x1000 magnification under a fluorescence microscope with AxioVs40 v4.6.3.0 software.

In vivo immunolabeling of sperm cells

In lectins assays, boar epididymal sperm samples were washed in PBS at 600 x g for 10 min and directly labelled with the lectin labelling solutions and with PI in order to distinguished viable and non-viable labelled sperm cells.

2.11. Analysis of phosphotyrosine fluorescence intensity by flow cytometry

For flow cytometry assays of phosphotyrosine fluorescence intensity, stained samples (following the immunolabeling of sperm cell in suspension procedure) were evaluated on a Cell Lab Quanta flow cytometer. FSC and SSC dot plots gave the cellular physical properties of size and granularity and allowed us to gate single oriented sperm. After fluorescence excitation at 488 nm, the fluorescence from Alexa Fluor® 488 conjugated was collected in FL1 (525±30 nm) fluorescence detector. Through inspection of FSC data indicative of size, small non-sperm events and large events originated from cell agglutination were excluded. A total of 20,000 singlet cells were counted per replicate. The mean of AF488 fluorescence intensity of the sperm population was recorded from the detector output to determine P-Tyr intensity changes in the sperm population over the different experimental conditions (Figure 31).

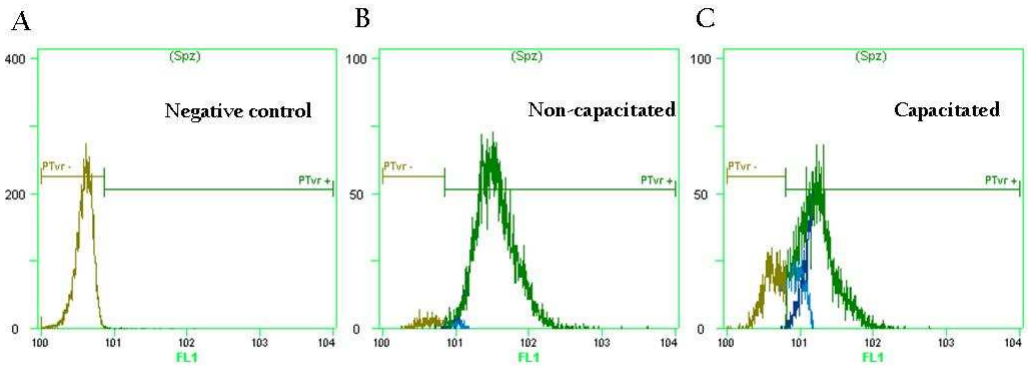


Figure 31. Flow cytometry cytograms of phosphotyrosine fluorescence intensity. Examples of phosphotyrosine fluorescence intensity (PTyr-FI) in boar sperm samples pre-incubated with PTyr inhibitor (A), in non-capacitated boar sperm samples (B) and in capacitated boar sperm samples (C).

Paper I

Epididymal maturation and ejaculation are key events for further in vitro capacitation of boar spermatozoa.

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First revision submitted in Theriogenology

Epididymal maturation and ejaculation are key events for further *in vitro* capacitation of boar spermatozoa.

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§ This study was provided by grants from the University of Girona BR07/12.

ABSTRACT

Mammalian spermatozoa acquire functionality during epididymal maturation, and the ability to penetrate and fertilize the oocyte during capacitation. The aim of this study was to assess the effects of epididymal maturation, ejaculation and *in vitro* capacitation on sperm viability, acrosome integrity, mitochondrial activity, membrane fluidity, and calcium influx, both as indicators of capacitation status and sperm motility. Results indicated that boar spermatozoa acquired the ability to move in the epididymal corpus; however, their motility was not progressive until the corpus and not linear until the ejaculation. Epididymal spermatozoa showed low membrane fluidity and intracellular calcium content; ejaculation lead to an increased calcium content, whilst membrane fluidity showed no changes. Acrosome integrity remained constant throughout the epididymal duct and after ejaculation and *in vitro* capacitation. The frequency of viable spermatozoa with intact mitochondrial sheath was higher in caput and ejaculated samples than in corpus and cauda samples, whereas the frequency of spermatozoa with high membrane potential was significantly lower in cauda samples. *In vitro* capacitation resulted in a decreased frequency of viable spermatozoa with intact mitochondrial sheath and an increased frequency of spermatozoa with high membrane potential in ejaculated samples. These results indicated that both epididymal maturation and ejaculation are key events for further capacitation, because only ejaculated spermatozoa are capable of undergoing the set of changes leading to capacitation.

Key words: boar, capacitation, epididymal maturation, flow cytometry.

INTRODUCTION

Studies on sperm quality are traditionally performed on ejaculated samples. Nevertheless, in the last few years there have been few studies that have focused on the analysis of the sperm quality, freezability and fertility after artificial insemination of cauda epididymal spermatozoa in both wild and livestock species [1-5]. The changes in sperm kinetics and properties throughout the epididymal maturation are crucial for further sperm-egg interaction and fertilization [6]. Furthermore, another essential process for fertilization is sperm capacitation, which includes a set of events that prepare the spermatozoa for the acrosome reaction and further penetration towards the zona pellucida. Sperm capacitation can be defined as the physiological changes that spermatozoa undergo *in vivo* in the female tract to obtain the ability to penetrate and fertilize the oocyte (Review in [7]). Due to difficulties to study capacitation *in vivo*, several procedures have been developed to induce capacitation *in vitro* in defined conditions [8] and media [9].

In order to obtain robust and reliable results, the analysis of sperm quality in either epididymal samples or ejaculated samples must be performed using objective and accurate procedures [10]. It is widely known that the analysis of sperm motility using assisted procedures leads to the obtention of information not only on the types of movement, but also on sperm velocity and kinetics [11,12]. Flow cytometry is widely used in the assessment of sperm quality in several mammalian species, since it makes an objective and accurate analysis of a large number of spermatozoa in a small volume of sample in a short time possible. Besides its objectivity and accuracy, cytometric analyses have a high repeatability and sensitivity [13]. Flow cytometry confers new approaches in the analysis of sperm quality, which pursue the integration of different tests to achieve a better understanding of the sperm functionality [14].
Flow cytometry analysis of sperm viability is currently based on the analysis of plasma membrane integrity by using the dual staining with SYBR-14 and propidium iodide (PI). The assessment of the acrosomal status combines PNA lectin conjugated with the fluorochrome fluorescein isothiocyanate (FITC) and PI [3,15]; this procedure leads to the distinction between viable and non-viable spermatozoa with either intact or altered acrosome. Other procedures, such as the combination of YO-PRO®-1/PI

Paper II

Impact of Epididymal Maturation, Ejaculation and In Vitro Capacitation on the Tyrosine Phosphorylation Patterns Exhibited in Boar (*Sus domesticus*) Spermatozoa.

Anna Fàbrega, Marta Puigmulé, Marc Yeste, Isabel Casas, Sergi Bonet, Elisabeth Pinart

Article Published in Theriogenology

Theriogenology 76 (2011) 1356–1366

A. Fàbrega, M. Puigmulé, M. Yeste, I. Casas, S. Bonet, E. Pinart. “Impact of epididymal maturation, ejaculation and *in vitro* capacitation on tyrosine phosphorylation patterns exhibited of boar (*Sus domesticus*) spermatozoa”. *Theriogenology*. Vol. 76, issue 7 (October 15, 2011) : p. 1356-1366

http://www.sciencedirect.com/science/article/pii/S0093691X11002792

http://dx.doi.org/10.1016/j.theriogenology.2011.06.007

Abstract

Mammalian spermatozoa acquire functionality during epididymal maturation and ability to penetrate and fertilize the oocyte during capacitation. The aim of this study was to investigate the impact of epididymal maturation, ejaculation and capacitation on phosphotyrosine content of sperm proteins. Western blot, immunocytochemical and flow cytometry analyses demonstrated that epididymal maturation *in vivo* is associated with a progressive loss of phosphotyrosine residues of the sperm head followed by a subtle increase after *in vitro* capacitation. As cells pass from caput to cauda epididymis, tyrosine phosphorylation becomes confined to a triangular band over the posterior part of midacrosome region, whereas *in vitro* capacitation causes a spread labeling over the whole head. Different bands with phosphotyrosine residues were detected during epididymal maturation and after *in vitro* capacitation: 1) 93, 66 and 45 kDa bands with specific phosphotyrosine expression in immature spermatozoa; 2) 76, 23 and 12 kDa bands with specific phosphotyrosine expression in mature spermatozoa, being significantly increased in their expression after *in vitro* capacitation; 3) 49, 40, 37, 30, 26 and 25 kDa constitutive bands that increased their phosphotyrosine expression after maturation and/or *in vitro* capacitation; and 4) 28 and 20 kDa bands with a specific phosphotyrosine expression in *in vitro* capacitated spermatozoa. These results provided integral novel data of expression and location of phosphotyrosine residues during epididymal maturation, ejaculation and *in vitro* capacitation of boar spermatozoa. Two new constitutive proteins bands of 26 and 25 kDa with phosphotyrosine residues were also identified.

Keywords

capacitation; epididymis; phosphotyrosine proteins; protein processing

Paper III

Glycocalyx Characterization and Glycoprotein expression of *Sus domesticus* Epididymal Sperm Surface Samples

Anna Fàbrega, Marta Puigmulé, Jean-Louis Dacheux, Sergi Bonet, Elisabeth Pinart

Article Published in Reproduction, Fertility and Development (RFD)

http://dx.doi.org/10.1071/RD11064

A. Fábrega, M. Puigmulé, Jean-Louis Dacheux, S. Bonet i E. Pinart. " Glycocalyx characterisation and glycoprotein expression of *Sus domesticus* epididymal sperm surface samples". *Reproduction, Fertility and Development*. Vol. 24 (4) : p. 619-630

http://www.publish.csiro.au/?paper=RD11064

http://dx.doi.org/10.1071/RD11064

Submitted: 10 March 2011 Accepted: 27 September 2011 Published: 25 November 2011

Abstract

The sperm surface is covered with a dense coating of carbohydrate-rich molecules. Many of these molecules are involved in the acquisition of fertilising ability. In the present study, eight lectins (i.e. *Arachis hypogae* (peanut) agglutinin (PNA), *Lens culimaris* (lentil) agglutinin-A (LCA), *Pisum sativum* (pea) agglutin (PSA), *Triticum vulgari* (wheat) germ agglutinin (WGA), *Helix pomatia* agglutinin (HPA), *Phaseolus vulgaris* (red kidney bean) leucoagglutinin (PHA-L), *Glycine max* (soybean) agglutinin (SBA) and *Ulex europaeus* agglutinin I (UEA-I)) were investigated to identify changes in the nature and localisation of glycoproteins in boar spermatozoa migrating along the epididymal duct. Complementary procedures included measurement of global lectin binding over the surface of the viable sperm population by flow cytometry, analysis of lectin localisation on the membrane of individual spermatozoa using fluorescence microscopy and the electrophoretic characterisation of the major sperm surface glycoprotein receptors involved in lectin binding. A significant increase was found in sperm galactose, glucose/mannose and *N*-acetyl-d-glucosamine residues distally in the epididymis. Moreover, the sperm head, cytoplasmic droplet and midpiece were recognised by most of the lectins tested, whereas only HPA and WGA bound to the principal piece and end piece of the sperm tail. Fourteen sperm surface proteins were observed with different patterns of lectin expression between epididymal regions. The sperm glycocalyx modifications observed in the present study provide an insight into the molecular modifications associated with epididymal maturation, which may be correlated with the degree of maturation of ejaculated spermatozoa.

Additional keywords: boar, epididymis, flow cytometry, immunocytochemistry.

Paper IV

Expression, immunolocalization and processing of fertilins ADAM-1 and ADAM-2 in the boar (*sus domesticus*) spermatozoa during epididymal maturation

Anna Fàbrega, Benoît Guyonnet, Jean-Louis Dacheux, Jean-Luc Gatti, Marta Puigmulé, Sergi Bonet, Elisabeth Pinart

Article in Published in Reproductive Biology and Endocrionology (RB&E)

Reproductive Biology and Endocrinology 2011, 9:96

http://www.rbej.com/content/9/1/96

Fàbrega et al. Reproductive Biology and Endocrinology 2011, 9:96
http://www.rbej.com/content/9/1/96

REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY

RESEARCH Open Access

Expression, immunolocalization and processing of fertilins ADAM-1 and ADAM-2 in the boar (*sus domesticus*) spermatozoa during epididymal maturation

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Abstract

Fertilin alpha (ADAM-1) and beta (ADAM-2) are integral membrane proteins of the ADAM family that form a fertilin complex involved in key steps of the sperm-oocyte membrane interaction. In the present work, we analyzed the presence of ADAM-1 and ADAM-2 mRNAs, the spermatozoa proteins' processing and their sub-cellular localization in epididymal samples from adult boars. ADAM-1 and ADAM-2 mRNAs were highly produced in the testis, but also in the *vas efferens* and the epididymis. On immunoblots of sperm extracts, ADAM-1 subunit appeared as a main reactive band of ~50-55 kDa corresponding to occurrence of different isoforms throughout the epididymal duct, especially in the corpus region where isoforms ranged from acidic to basic pI. In contrast, ADAM-2 was detected as several bands of ~90 kDa, ~75 kDa, ~50-55 kDa and ~40 kDa. The intensity of high molecular mass bands decreased progressively in the distal corpus where lower bands were also transiently observed, and only the ~40 kDa was observed in the cauda. The presence of bands of different molecular weights likely results from a proteolytic processing occurring mainly in the testis for ADAM-1, and also throughout the caput epididymis for ADAM-2. Immunolocalization showed that fertilin migrates from the acrosomal region to the acrosomal ridge during the sperm transit from the distal corpus to the proximal cauda. This migration is accompanied by an important change in the extractability of a part of ADAM-1 from the sperm membrane. This suggests that the fertilin surface migration may be triggered by the biochemical changes induced by the epididymal post-translational processing of both ADAM1 and ADAM-2. Different patterns of fertilin immunolocalization then define several populations of spermatozoa in the cauda epididymis. Characterization of such fertilin complex maturation patterns is an important step to develop fertility markers based on epididymal maturation of surface membrane proteins in domestic mammals.

Background

Modern breeding programs use artificial insemination with a low number of males for improving the livestock genetics of economically important traits. However, some of the interesting males have a low fertility even when classical semen parameters (i.e. viability, motility, abnormal forms...) are normal. It is thus important to develop new molecular tools to accurately estimate fertility levels. As the quality of the epididymal maturation strongly influences sperm fertility, sperm protein markers of this maturation are among the most promising tools. However, only few sperm surface maturation proteins with a role in fertility have been described in large mammals since almost all studies were performed on rodents' models. Among the spermatozoa surface proteins, fertilin, an heterodimer complex composed of two integral membrane glycoproteins named alpha-fertilin (ADAM-1) and beta-fertilin (ADAM-2) (also previously named PH-30 alpha and PH-30 beta in guinea pig), as

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well as several other ADAMs have been reported to be involved in sperm-oocyte recognition and in membrane fusion [1-5]. Both proteins are members of the "A Disintegrin And Metalloprotease" domain protein family (ADAMs) [1] whose sequences contain a pro-domain, a metalloprotease, a disintegrin and a cysteine-rich domain, EGF-like repeats, a transmembrane domain and a carboxy-terminal cytosolic tail. The fertility of male mice lacking fertilin alpha or fertilin beta is substantially reduced due to sperm inability to migrate through the oviduct and to bind to the zona pellucida and to the oocyte plasma membrane [4-8]. It has been suggested that the binding between the disintegrin domain of ADAM-2 and the egg plasma membrane integrin(s) is at least partly responsible for the recognition between sperm and eggs.

In rodents, the fertilin complex can be found in testicular germ cells under the form of a 160-190 kDa precursor composed of pro-alpha and pro-beta subunits assembled into a non-covalently bound complex [9]. The pro-alpha subunit is proteolytically processed into a 50 kDa protein by a pro-protein convertase during spermiogenesis, before emerging on the sperm cell surface. Its molecular mass does not change further during epididymal sperm maturation [2,9]. In contrast, the pro-beta subunit is present as a full length protein on the testicular sperm surface and then proteolytically transformed during sperm maturation in the epididymis [2,9]. The pro-beta subunit maturation is characterized by the formation of intermediate forms during the passage of spermatozoa through the caput, that are cleaved into a 35 kDa main form in spermatozoa from proximal and distal cauda [9]. This proteolytic processing results in the removal of the pro- and metalloprotease-like domains, with only the full or part of the disintegrin domain, the cysteine-rich domain, the EGF repeat, the transmembrane and the cytoplasmic domains remaining on the sperm cell. This processing also induces a relocation of the fertilin complex on a different plasma membrane domain of the mature spermatozoa.

Most of the previous studies have been performed with guinea pig and mouse and although a similar pattern of fertilin modifications was suggested in the bull [10], the processing of the fertilin complex during the epididymal maturation in large domestic mammals has never been described in details. The aim of the present work was to characterize the fertilin complex processing throughout the epididymal duct of the boar, an economically important, yet fewly studied, farm animal. This study is a pre-requisite to address the question of the fertilin role in boar fertility and its potential use as a fertility marker in boars.

Methods

Reagents and chemicals

Bovine Serum Albumin (BSA), Nonidet P-40 (NP-40), proteases inhibitor cocktail (consisting of AEBSF, aprotinin, bestatin hydrochloride, E-64, EDTA, leupeptin hemisulfate salt (S8820, Sigma, L'Isle d'Abeau, France)) supplemented by 1 mM phenyl-methane-sulphonyl-fluoride (PMSF) just before use), Percoll, and common laboratory salts and reagents were purchased from Sigma. Ampholytes pH 2-11 (Servalytes) were obtained from Serva (Heidelberg, Germany) and ampholytes pH 3-10 (Ampholytes), Coomassie Brilliant Blue (Phastgel Blue R), and electrophoresis calibration kits (standard proteins) were purchased from GE-Healthcare (Saclay, France).

Epididymal and testicular samples

Ten adult boars (Large White) aged from 8 months to more than 2 years were used throughout the study, following the approved guidelines for the ethical treatment of animals. Testis and epididymides were immediately removed after slaughter of the animals and dissected avoiding blood contamination (less than 15 minutes after death). For RNA extraction, samples were immediately frozen in liquid nitrogen and stored at -80°C until use. For polyclonal antibodies testing, testis tissues (about 100 mg) were grinded in 2 ml Phosphate Buffer Saline (PBS; 50 mM potassium phosphate; 150 mM NaCl; pH 7.2) containing 2% Triton X100 and proteases inhibitor cocktail, and then centrifuged 10 min at 15 000 g. An aliquot of the supernatant was mixed with reducing Laemmli 4X sample buffer, boiled, and stored at -20°C until use.

Testicular fluid and spermatozoa were obtained by puncture from the rete testis. Epididymal spermatozoa and fluids from the different boar epididymal regions (Figure 1) were obtained through microperfusion with supplemented phosphate buffer saline (PBS supplemented with 2 mM pyruvate, 2 mM lactate, 2 mM glucose and 5 mM K_2CO_3) as described in previous studies [11-15]. Fluid and spermatozoa from *vas deferens* and region 9 were obtained by retroperfusion from the *vas deferens* with supplemented PBS. These techniques allow obtaining samples without blood and extracellular fluid contaminations [15].

Samples were then centrifuged at 600 × g for 10 min at 15-20°C. Sperm pellets were resuspended and washed at 600 × g for 10 min at 15-20°C with supplemented PBS and, when necessary, separated on a 40-90% Percoll gradient (Sigma) to obtain very clean sperm samples. Then, sperm viability was assessed using SYBR-14 and propidium iodure (PI) (LIVE/DEAD®Sperm Viability kit, Molecular Probes, Invitrogen, Illkirch, France) in flow cytometry experiments (Cell Lab QuantaSC, Beckman Coulter, Villepinte, France). This ensured that all

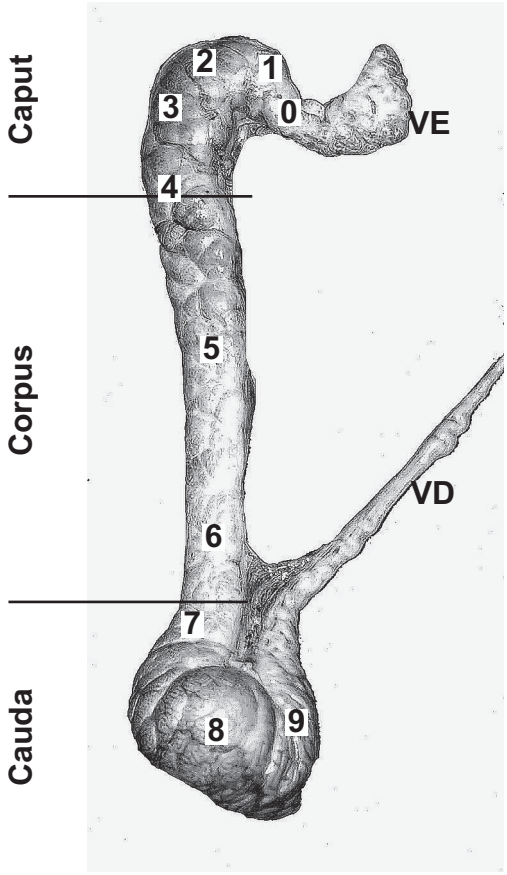

Figure 1 Adult boar epididymis. Picture of boar epididymis indicating the different regions used: VE, *vas efferens*; 0 to 8/9, epididymal zones; VD, *vas deferens*, and the three main classical regions: caput, corpus, cauda.

samples had retained sperm vitality over 80%. For some experiments, sperm samples were divided into two aliquots for total protein extraction and for protein surface labeling, respectively.

Total spermatozoa protein extraction

Total protein extraction was performed by mixing sperm suspension (in average 10^8 to 10^9 spermatozoa) with PBS-2% SDS containing a proteases inhibitor cocktail (v/v), or directly by mixing the washed sperm with an equal volume of Laemmli 4X buffer without β-mercaptoethanol. The samples were incubated with the extraction buffer for 30 min at 4°C under agitation, and then centrifuged at 15 000 × g for 15 min at 4°C. The supernatant was carefully removed and stored for a short period of time at -20°C.

Protein surface labeling with biotin and purification

Labeling was done mainly as described in Belleannèe et al. (2011) [16]. Sperm samples were resuspended in supplemented PBS at a final concentration of 3×10^9 spz/ml. Labeling was performed with 3 mg/ml (~ 6 mM) of NH-SS-Biotin (Thermo Scientific, Pierce, Brebières, France) during 30 min at room temperature under agitation. Biotin labeling was stopped with 50 mM Tris or 300 mM glycine. Biotin excess was eliminated by successive washes with supplemented PBS followed by a 40-90% Percoll gradient. Proteins were extracted by mixing the sperm pellet with an equal volume of PBS with 2% (v/v) Nonidet P-40, a proteases inhibitor cocktail, and 1 mM PMSF during 30 min at 4° C under agitation. Then, the samples were centrifuged at 15 000 × g for 15 min at 4°C and the supernatant was carefully removed and stored at -20°C. The biotin-labeled surface proteins were purified using streptavidin beads (Dynabeads®M-280 Streptavidin, Invitrogen) and extracted from beads by boiling for 5 min in Laemmli buffer.

Gel electrophoresis

For SDS-PAGE separations using 8-16% gradient gels, samples were diluted v/v in Laemmli buffer 4X with 5% β-mercaptoethanol and boiled during 3 min at 95°C. A preliminary gel was run with the extracts and stained with Coomassie blue in order to equilibrate protein amounts for the western blot experiments (not shown).

For isoelectrofocalisation, immobiline strips (7 cm long Ready Strip IPG pH 3-10, Biorad) were first rehydrated overnight at 20°C in presence of the protein extract mixed with the following buffer: urea 7 M, thiourea 2 M, Triton X-100 1%, N-octyl-β-D-glucopyranoside 2%, DTT 30 mM, ampholytes (IPG Buffer pH 3-10, Amersham) 0.4%, and bromophenol blue. For isoelectrofocalization the following program was applied: 100 V 10 min; lineal gradient of 100 V to 4000 V during 7 h 30 min; constant voltage of 4000 V during 9 h; fast gradient from 4000 V to 8000 V during 1 h 30 min; final step at 500 V.

Before SDS-PAGE electrophoresis, the strips were rinsed with two equilibration solutions, equilibration solution I (urea 6 M, Tris 50 mM, glycerol 30%, SDS 2%, DTT 65 mM) and then equilibration solution II (urea 6 M, Tris 50 mM, glycerol 30%, SDS 2%, DTT 135 mM), during 15 min each, at room temperature under agitation, in order to reduce and to alkylate the proteins. The strips were deposited on the top of a 8-16% gradient gel and casted with a 0.5% agarose gel dissolved in electrophoresis buffer with bromophenol blue. The electrophoresis was run at 30 mA per gel.

Antibodies

Four different rabbit polyclonal antibodies against ADAM-1 and ADAM-2 were prepared from defined peptides. Two peptides, one in the N-terminal

metalloprotease domain and one in the C-terminal part near the trans-membrane domain were chosen for each ADAM protein (ADAM-1-NT: LTEVPVDLQVALRC; ADAM-1-CT: CSSPGSGGSVDSGP; ADAM-2-NT: GLTNAIFTSFNITC; ADAM2-CT: NATITYSNINGKIC). A cysteine was added in C-terminal position of the peptides that did not have one for the coupling with keyhole limplet hemocyanin (Sigma) using a bi-functional coupling agent (MBS, Pierce). 250 μg of coupled peptide were used for each injection into rabbit, using Freund complete adjuvant for the first injection and Freund incomplete adjuvant for the three next injections. The preimmune serums were tested prior immunization and showed no reactivity toward the testicular and sperm extracts (not shown). Antibody specificity was also demonstrated by competition assays between the peptide and the antibody on Western blots from testis extract (see example in Additional file 1, Figure S1). The rabbit polyclonal anti-germinal angiotensin-converting enzyme (anti-ACE) has been described previously [17]. Immunized rabbit sera were used throughout the study.

Protein blotting

After one or two-dimensional electrophoresis, gels were transferred to nitrocellulose or polyvinylidene fluoride membranes (Immobilon-P, Millipore, Molsheim, France) during 2 hours at 0.8 mA per cm^2, stained with Ponceau red to check for transfer homogeneity, and then blocked during 1 hour at room temperature with TBS-Tween (Tris HCl 20 mM, NaCl 150 mM, 0.5% Tween 20, pH 7.3) containing 5% low fat milk.

Blocked membranes were incubated with the first antibody diluted 1/5000 (anti-ADAMs) or 1/10000 (anti-ACE) in blocking solution overnight at 4°C, and then, incubated after washing with a goat anti-rabbit-HRP (Sigma) diluted 1/5000 in blocking solution during 1 h at 37°C.

Reactive bands were visualized either with a chemiluminescent substrate (Immobilon™ Western Detection Reagents, Millipore) with exposition of membranes with photographic films (Hyperfilm, GE Healthcare), or directly digitized with an image analysis device or photographed after 4-chloro-1-naphtol staining.

PCR analysis

For PCR, tissues treatment and reverse transcription were performed as described in Guyonnet et al, (2009) [18]. 3 μg total RNA were retro-transcribed using the Superscript Reverse transcriptase H (Invitrogen) and oligo(dT)15 primers. PCRs were performed for 25 and 30 cycles at the temperature specified for the primer sets (ADAM-1 forward GGCCACATTAATGGCAGACT, ADAM-1 reverse ACATGCTTGGCAG GAAAATC; ADAM-2 forward GTGGGAATGGA GAAGTCGAA, ADAM-2 reverse GACATGCTGCA GAAGAACCA). Aliquots of each reaction mixture were analyzed on a 2% ethidium bromide-stained agarose gel. Amplification of RPL19 (Ribosomal protein L19) was used as PCR control (RPL19 forward GGTACTGCCAATGCTCGAAT, RPL19 reverse CCATGAGAATCCGCTTGTTT). Testicular ADAM-1 and ADAM-2 amplicons were sequenced in order to validate the primers (see Additional file 2, Figure S2).

Immunocytochemistry

Boar sperm samples from proximal and distal caput, corpus and distal cauda were washed and fixed with 3% para-formaldehyde for 30 min at room temperature. After three washes in PBS at 600 × g for 10 min, samples were diluted ~10^7 spz/ml in PBS and dropped onto poly-lysine-coated slides, rinsed with PBS, and incubated for 1 h with the anti-C-terminal ADAM-1 polyclonal antibody diluted 1/100 in PBS-1% BSA. After washing in PBS, samples were incubated for 1 h with the secondary antibody goat anti-rabbit conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA), diluted 1/1000 in PBS-1% BSA. After extensive washings with PBS, the mounting medium (Fluoromount; Electron Microscopy Sciences, Hatfield, PA, USA) was applied. Digital images were captured at ×630 and ×1000 magnification under a fluorescence microscope with AxioVs40 v4.6.3.0 software (Zeiss Axio Imager.Z1).

Autofluorescence controls were spermatozoa incubated with PBS-1% BSA without antibodies, with primary antibody only (not incubated with secondary antibody) or with secondary antibody only (not incubated with primary antibody). No labeling was observed when the preimmune serum was used instead of the polyclonal antibody (see Additional file 3, Figure S3). The different patterns of fertilin localization were obtained by analyzing under the microscope 3 replicates of 100 spermatozoa per boar. Results were statistically analyzed with SigmaStat 3.5 statistical software. A one-way analysis of variance with post-hoc multiple comparison procedure (Holm-Sidak method) was performed.

Results

Pig ADAM-1 and ADAM-2 sequence and expression

In order to design peptides for immunization, we searched databases for complete sequences of pig ADAM-1 and ADAM-2 (Figure 2 and 3). At that time, the complete sequence of pig ADAM-2 was available [19], but only a partial C-terminal sequence of ADAM-1 was found in databases. We then used these sequences to design specific N- and C-terminal peptides for ADAM-2 and C-terminal ADAM-1 peptide. The N-terminal ADAM-1 peptide sequence was designed in a highly conserved region using the alignment between

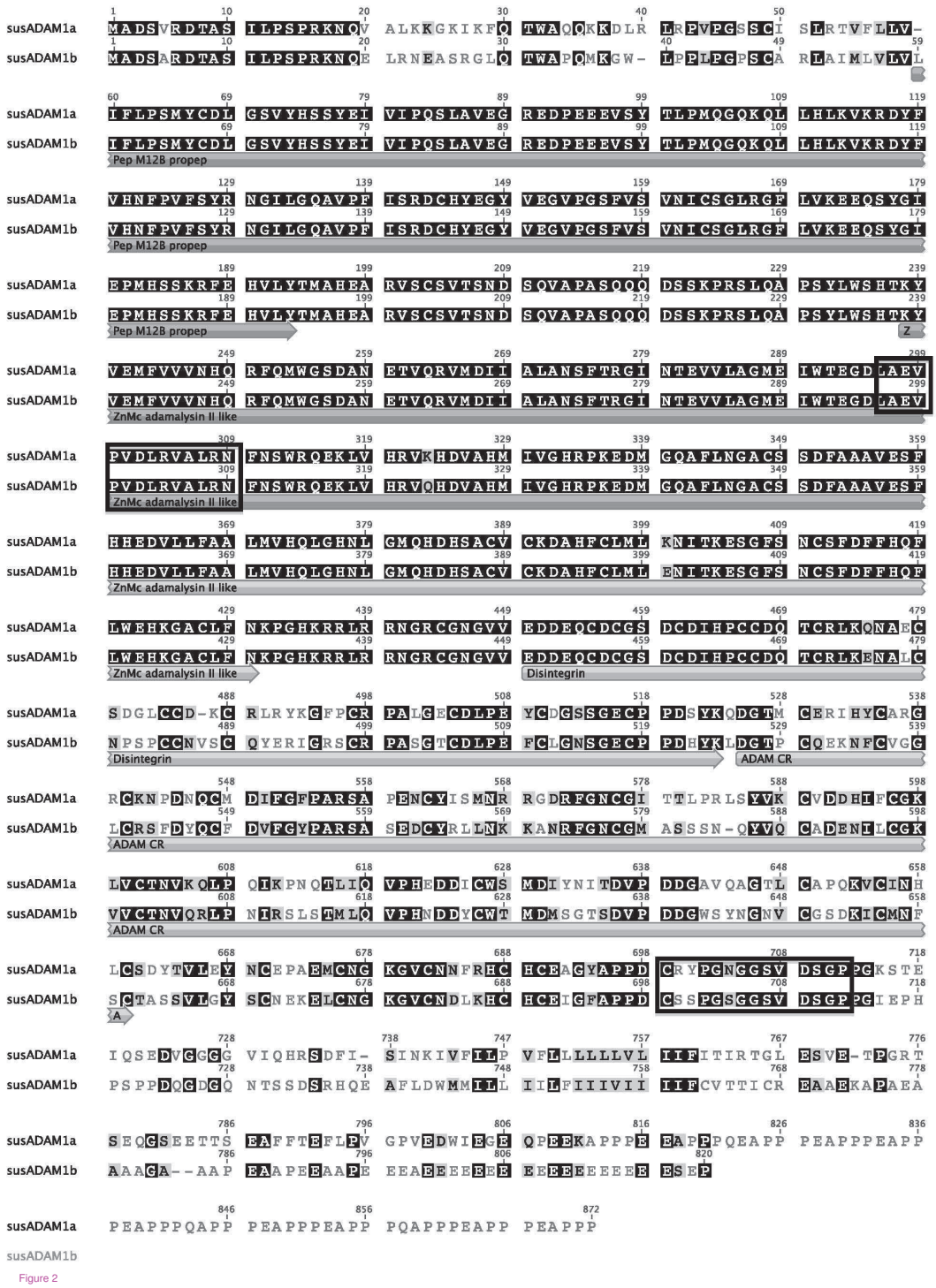

Figure 2 Sequence comparison of the two pig ADAM-1. Sequence alignment between pig ADAM-1a and ADAM-1b showing similarities (black boxes), homologies (grey boxes) and differences in amino acids. The position of the different ADAM domains is indicated. Positions of the N-terminal and C-terminal peptides used for immunization are indicated by boxes.

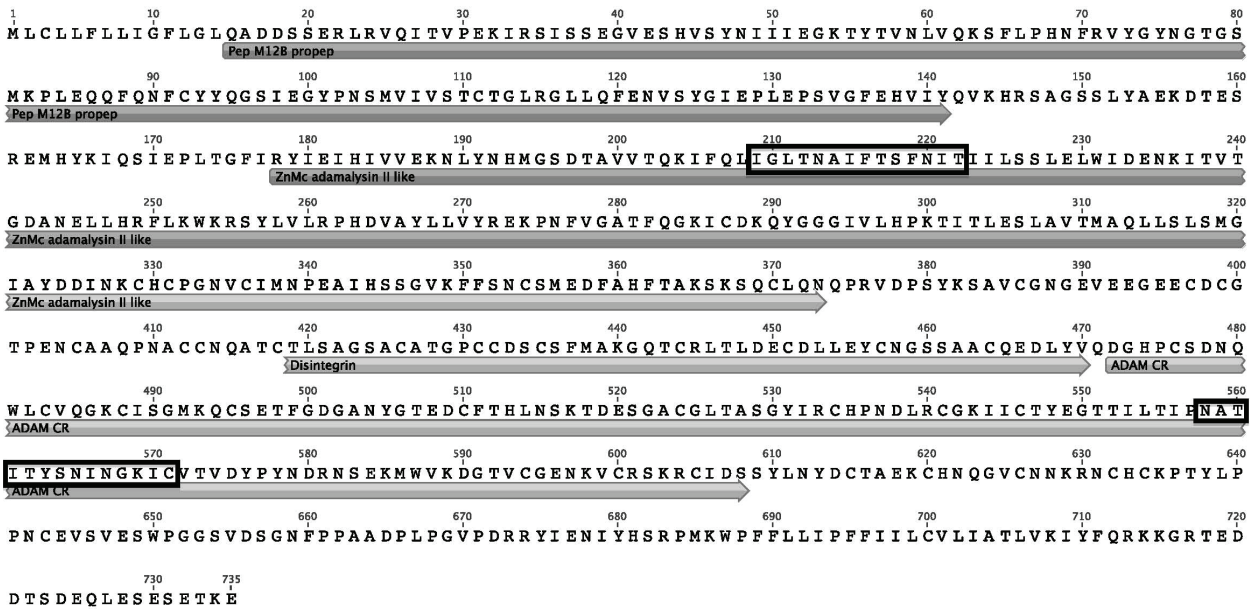

Figure 3 Sequence of the pig ADAM-2. Sequence of the pig ADAM-2 showing the position of the different ADAM domains. Positions of the N-terminal and C-terminal peptides used for immunization are indicated by boxes.

the bull ADAM-1 sequence and rodents' sequences. Except for this peptide, other peptides used for immunization were then specific for the pig ADAMs and did not match any other protein in databases. Since then, an incomplete sequence of the pig ADAM-1 (XP_001924862.1) and an alternative ADAM-1 sequence (XP_001927304.1), named therein ADAM-1a and ADAM-1b respectively, were deposited on the public data bank (Figure 2). We have completed the sequence of the pig ADAM-1a by assembly with the best matching pig ESTs sequences available in public databases (*Sus scrofa* cDNA clone PDUts1113E12 and PDUts2081H12).

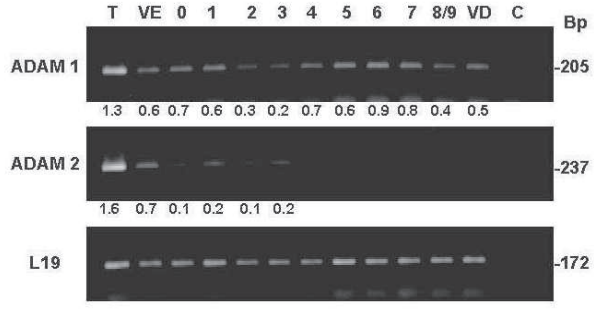

Figure 4 Expression of ADAM-1 and ADAM-2 in testis and epididymis. Agarose gel showing the PCR amplicons obtained for ADAM-1 and ADAM-2 using total RNAs extracted from the testis, *vas efferens*, *vas deferens* and the different epididymal zones, and 30 amplification cycles. The RPL19 gene was used as a control. The intensity ratio between ADAMs and RPL19 amplicons is indicated under each lane.

The pig ADAMs sequences showed the classical architecture of ADAMs family in terms of the presence of the different domains. Both ADAM-1 proteins have a predicted metalloprotease active site and a cleavage site for pro-protein convertase (436-KRRLR-440), which is not present on the ADAM-2 chain. At the protein sequence level, pig ADAM-1a shared only 74% identity with pig ADAM-1b, the main differences being found in the pro-domain, the disintegrin, the cysteine rich and the C terminal domains (Figure 2). Pig ADAM-1a was 71%, 69%, 64%, and 60% identical to horse ADAM-1 (XM_001490830.2), bull ADAM-1 (XM_866254.3), mouse ADAM-1a (NM_172126.2) and guinea pig PH-30 alpha (NM_001173096.1), respectively. Pig ADAM-1b had 68% identity with bull, 66% with horse, 59% with mice alpha-1a and alpha-1b, and 58% with guinea pig PH30 alpha, respectively. Pig ADAM-2 showed 65%, 65%, 57%, and 53% identity with horse (XM_001490821.2), bull (NM_174228.1), mouse (NM_009618.2) and guinea pig ADAM-2 (NM_001172910.1), respectively.

Primers were designed from pig ADAM-1 and ADAM-2 sequences in order to analyze by PCR the presence and expression level of their mRNAs within testicular and epididymal tissues from different regions (Figure 4). After 25 RT-PCR cycles, we observed the presence of both mRNAs only in the testis. After 30 cycles, ADAM-1 mRNAs were also detected at a slightly lower intensity in the *vas deferens* and throughout the

epididymis while ADAM-2 mRNA was detected in the efferent ducts and at very low intensity from the caput to the corpus epididymis.

ADAM-1 and ADAM-2 processing in testicular and epididymal spermatozoa

The four anti-peptide polyclonal antibodies were first tested for specificity on total testis extracts (Figure 5 and Additional file 2, Figure S2). All antibodies reacted with main bands at about 100 kDa, slightly higher than the theoretical masses expected for the pig ADAM-1 (unprocessed expected mass 95 kDa) and ADAM-2 (unprocessed expected mass 82 kDa). This suggested occurrence of post-translational modifications, in agreement with the fact that glycosylation sites are predicted all over the sequence of both ADAM-1 and ADAM-2.

In sperm extracts from the different epididymal regions (Figure 6), the anti-ADAM-1 N-terminal polyclonal antibody recognized one main band at 99 kDa from testicular to the distal caput (zone 3) spermatozoa extracts. Other bands, including a strong 82 kDa band could be transiently visible in zones 0 and 1, as well as several very faint bands such as a 77 kDa band until the cauda, or 50-55 and 40-43 kDa bands in zone 3-4.

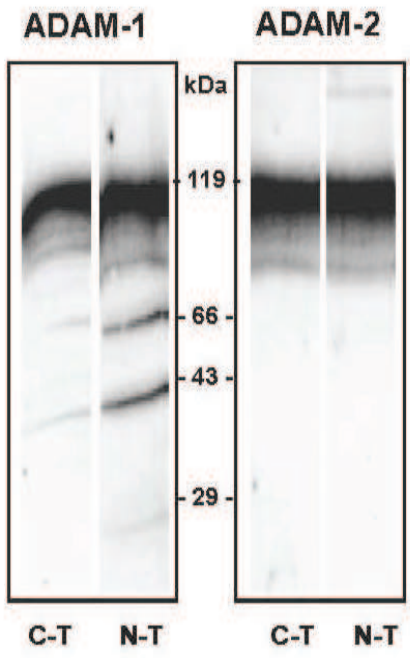

Figure 5 Reactivity of the different anti-ADAMs polyclonal antibodies. Boar testicular tissue extract was separated on a 8-16% SDS PAGE under denaturing conditions, and transferred to nitrocellulose. Individual lanes of the blot were probed with the four different anti-peptide polyclonal antibodies (ADAM-1 and ADAM-2 C-terminal (C-T) and N-terminal (N-T)).

The anti-ADAM-1 C-terminal polyclonal antibody reacts with five bands in testicular sperm with masses at about 118 kDa, 75 kDa, 50-55 kDa, 43 kDa and a faint 40 kDa. For epididymal sperm extracts, the 50-55 kDa band was the most reactive and it increased in intensity and spread largely in the cauda epididymal sperm extracts. Faint 95 kDa and 75 kDa bands that were barely visible in testicular sperm extracts almost disappeared in distal caput and cauda sperm, respectively. The 43 kDa band, present in testicular sperm, disappeared in epididymis while the light 40 kDa band remained present until the cauda.

For the anti-ADAM-2 N-terminal polyclonal antibody, only a very faint reactive band at about 65-70 kDa could be observed for all the epididymal sperm extracts. The anti-ADAM-2 C-terminal polyclonal antibody recognized a 90 kDa band in VE and zone 0 extracts, and a 66 kDa band from testis to the distal caput. Other faint bands at 70-75 and 50 kDa were also transiently observed in the caput while a 40 kDa appeared in zone 1 and remained until the cauda.

Changes in ADAM-1 isoforms during maturation

Detection of ADAM-1 by the anti-C-terminal polyclonal antibody provided the most intense signal on 1D gels, and this antibody was then further used on two-dimensional Western blot (Figure 7). At least three major spots were observed for ADAM-1 in the sperm extracts. The round spot at less than 50 kDa remained almost unchanged in pI and Mr in the different zones analyzed. The highly immunoreactive spot detected at about 55 kDa corresponded to at least 2 isoforms, with a smearing pattern spreading from pI 3 to 10 and a molecular mass varying between 50-55 kDa depending of the epididymal origin of the sperm extract. The less intense isoform at about 60 kDa was also present in corpus (zone 6) with a basic pI, but moved to an acidic pI in distal cauda (zone 9). In the cauda, all isoforms were found around the same acidic pI 5. Because the different isoforms were very close in masses, they were difficult to separate by one-dimensional gel electrophoresis. However, their presence likely explained the spreading of the 55 kDa band observed in immunoblots along the epididymis.

Changes in fertilin localization on spermatozoa membrane domain during maturation

Because the alpha-beta complex is dissociated only in presence of high SDS concentration [9], we assumed that using the ADAM-1 anti C-terminal antibody for immunolocalization studies will allow visualization of the fertilin complex on the sperm surface. Immunolocalization of fertilin complex with the anti ADAM-1-C-terminal polyclonal antibody showed that it was

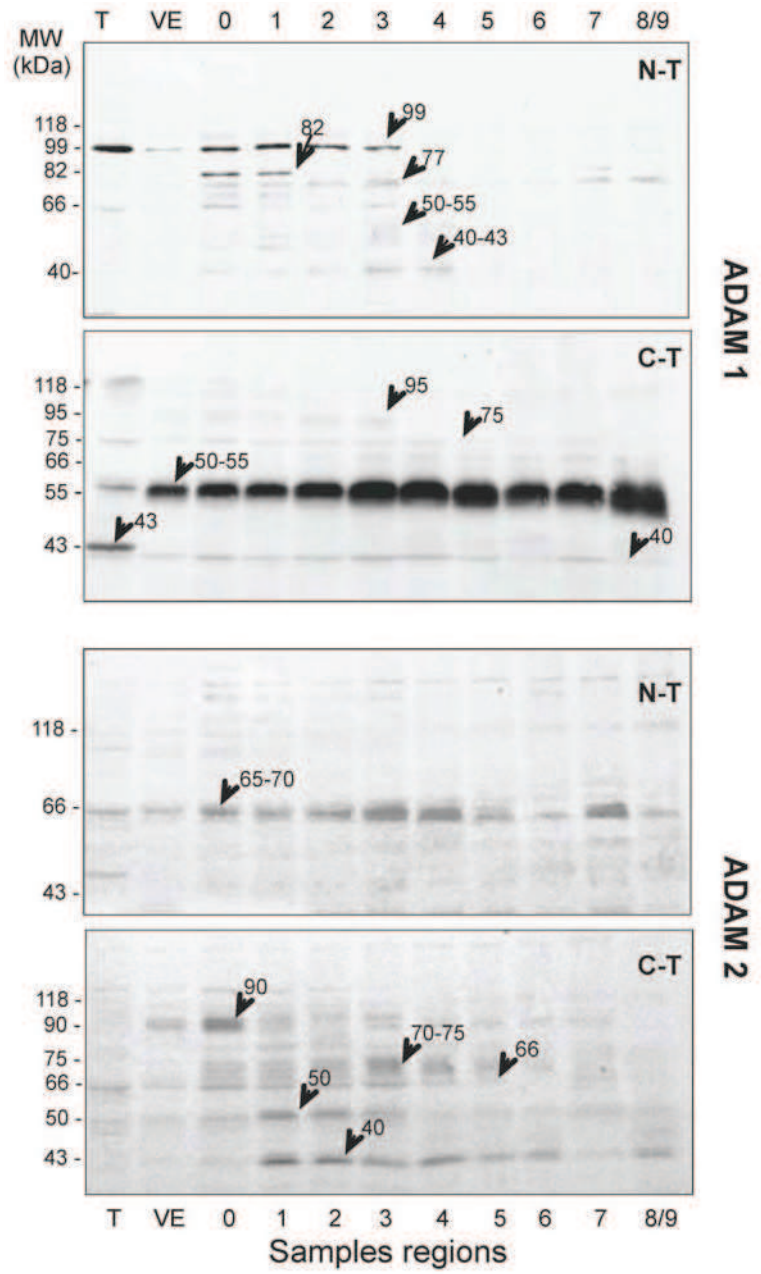

Figure 6 Epididymal processing of ADAM-1 and ADAM-2. Western blots loaded with equivalent total sperm extracts from the different epididymal zones were probed either with the ADAM-1 or ADAM-2 anti-N-terminal or anti-C-terminal polyclonal antibodies as indicated. Arrows and Mr on the figure indicate the main reactive bands (see results). Proteins amounts from about 10^6 sperm were loaded/lane.

distributed uniformly on the acrosomal region of the sperm head throughout the caput and corpus regions, while being only present on the acrosomal ridge of spermatozoa from proximal and distal cauda (Figure 8). From caput to cauda, the sperm population with ADAM-1 on the entire acrosome declined from 94 ± 2% to 12 ± 1%, whereas the sperm population showing ADAM-1 on the acrosomal ridge increased from 6 ± 2% to 88 ± 1% (Table 1). The difference in the patterns observed between caput, corpus and cauda is statistically significant ($p < 0.05$).

Changes in fertilin extractability during relocalization

On total SDS sperm extracts, ADAM-1 was immunodetected as a 50 kDa band in all regions (Z2 to Z9) (Figure 9A). However, when a mild detergent

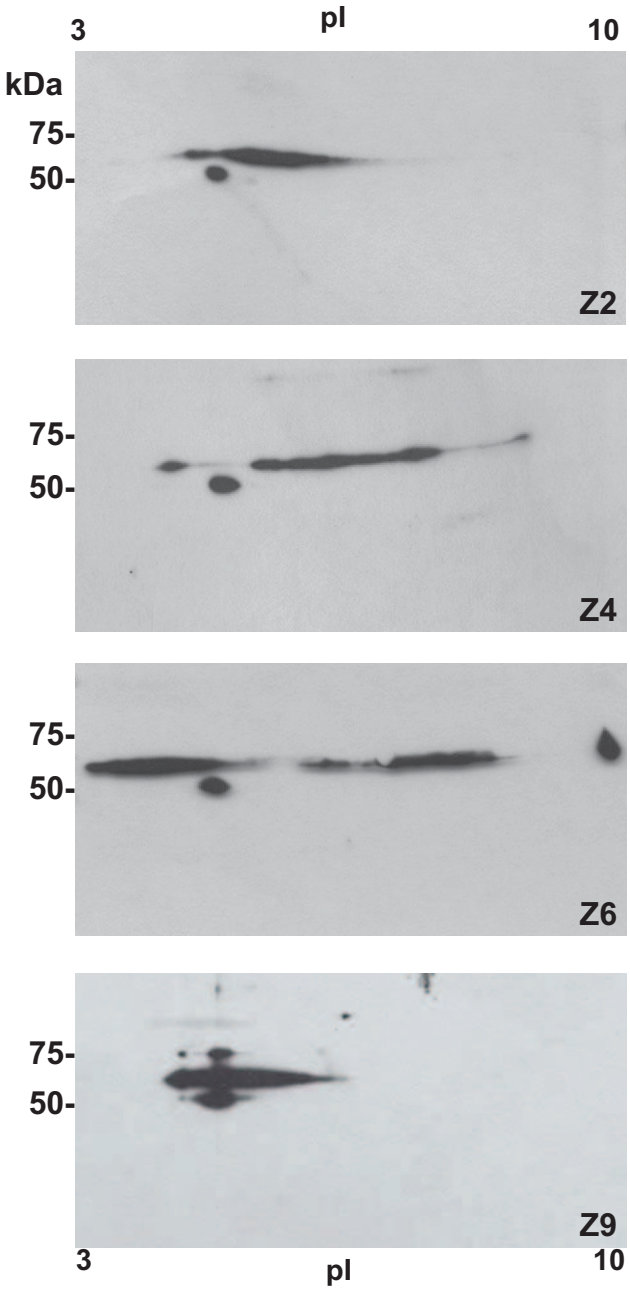

Figure 7 Changes in ADAM-1 properties in bi-dimensional blots. Sperm protein extracted from 4 different epididymal regions, proximal caput (Z2), distal caput (Z4), corpus (Z6) and distal cauda (Z9), were separated by isoelectric focusing on 8-16% SDS-PAGE and then transferred to nitrocellulose. The western blots were probed with the anti-ADAM-1 C-terminal polyclonal antibody. Several different ADAM-1 isoforms can be distinguished along the epididymis.

treatment (NP-40) was used to extract and purified the biotin-labeled membrane surface proteins, the 50 kDa band of ADAM-1 could only be detected in cauda extracts (Figure 9B). When the gel was overloaded, a reactive band was also detected in distal corpus, but not in membrane protein extracts of either the caput or the proximal corpus (Figure 9C). When an anti-ACE was used as a control on the same sperm purified extracts, the presence of ACE was detected on all samples with the best detection for the caput sperm surface, then decreasing in the corpus and cauda, as expected for this germ cell specific enzyme that was previously described to be processed in the epididymis (Figure 9D) [17,20,21].

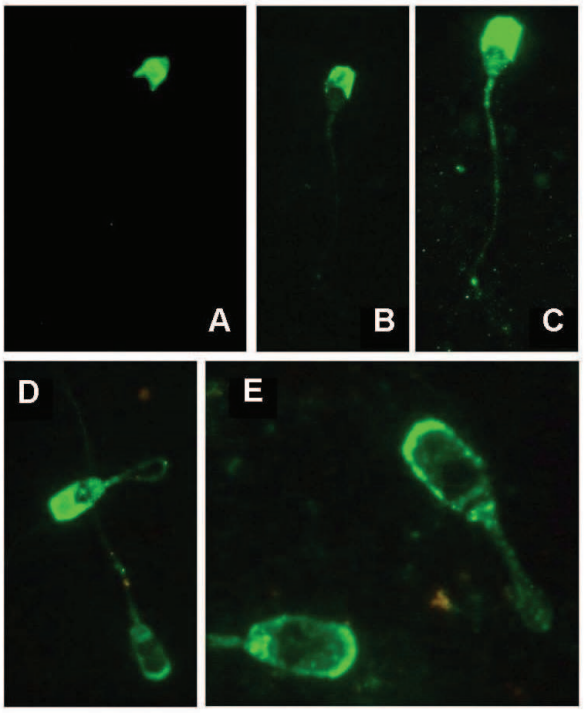

Figure 8 Immunolocalization of fertilin on epididymal spermatozoa. Sperm from caput (**A**, **B** and **C**) and cauda (**D** and **E**) epididymis were incubated with the anti-ADAM-1 C-terminal polyclonal antibody and its localization was revealed with a secondary fluorescent conjugated antibody. Sperm were from the same animal and the observations were done at × 630 (**A** and **B**) and at × 1000 (**C**, **D** and **E**).

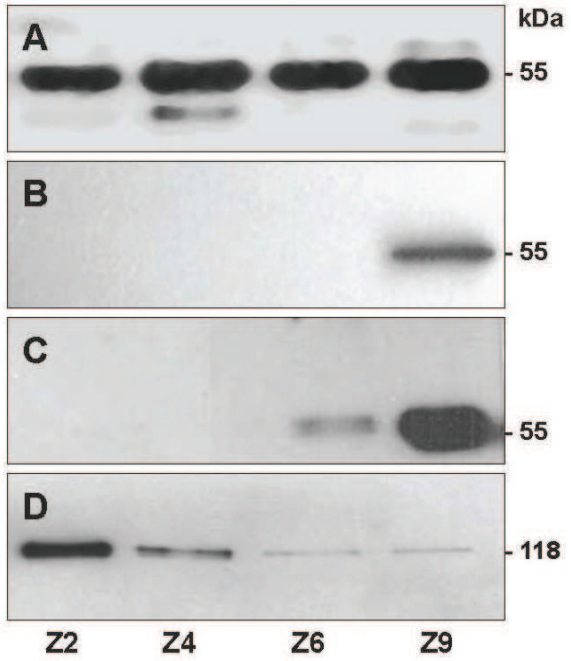

Figure 9 Extractability change of ADAM-1 during sperm maturation. Total SDS sperm protein extracts (**A**) or two different gel loading of purified biotinylated sperm surface proteins (**B** and **C**) from the proximal caput (Z2), distal caput (Z4), corpus (Z6) and distal cauda (Z9) were probed with the anti ADAM-1 C-terminal polyclonal antibody. Purified biotinylated surface proteins (**D**) were probed with the anti-ACE as controls for extraction and purification.

Discussion

ADAM-1 and ADAM-2 are members of the metalloprotease disintegrin protein family (ADAMs) [1,22,23]. To date, at least 34 ADAMs have been identified in a variety of species and in different cells and tissues, and at least 18 of them are expressed in male reproductive organs, including the testis and epididymis [1,23]. Several of these ADAM proteins are also present on the sperm membrane, but their potential roles in fertilization are either not understood at all or need to be clarified [4-8].

In mouse, two different ADAM1 (ADAM1a and ADAM1b sharing 83% homology and 75% identity) have been previously described, that have different roles and sperm localization. ADAM1b forms a complex with ADAM2 on sperm surface, while ADAM1a is restricted to the endoplasmic reticulum of testicular germ cells [7,8]. In databases, we found two sequences for the pig ADAM-1 (that we named ADAM-1a and ADAM-1b) that shared only 76% homology. As the pig genome is still incompletely sequenced, and both sequences are located in a close position on chromosome 14 (Location 14,14 of the *Sus scrofa* reference assembly Sscrofa5), it was not possible to ascertain that these sequences

Table 1 Summary of fertilin pattern distribution after immunocytochemical localization

Region	Pattern 1 Whole acrosome	Pattern2 Acrosomal ridge	Std Dev	SEM	p-value
Proximal Caput	0.9400	0.0600	0.0200	0.0115	$p > 0.05^{a}$
Distal Caput	0.9367	0.0633	0.0058	0.0033	$p > 0.05^{a}$
Proximal Corpus	0.9233	0.0767	0.0208	0.0120	$p > 0.05^{a}$
Distal Corpus	0.8630	0.1370	0.0153	0.0088	$p < 0.001^{b}$
Proximal Cauda	0.2100	0.7900	0.0265	0.0153	$p < 0.001^{c}$
Distal Cauda	0.1200	0.8800	0.0100	0.0058	$p < 0.001^{d}$

Statistical significance of the results tested by the post-hoc multiple comparison procedures (Holm-Sidak method) ($p < 0.05$).

indeed correspond to distinct, functional genes as shown in mouse for ADAM1a and ADAM1b. However, the PCR amplicon obtained from the pig testis (see Additional file 2, Figure S2) showed a much higher sequence identity with the pig ADAM-1a than with ADAM-1b sequence (98% vs 75%, respectively), suggesting that ADAM-1a is likely the major testicular transcript for the pig.

In boar, ADAM-1 and ADAM-2 mRNAs were present in the testis but ADAM-1 mRNA was also observed in all epididymal samples while ADAM-2 was found in the *vas efferens* and caput epididymis. In mouse, ADAM-1a mRNA is also expressed all along the mouse epididymis [24] and expression of ADAM-1 and -2 is not restricted to the male genital tract [25-27].

The process of surface fertilin maturation in epididymal spermatozoa has been mainly described in guinea pig [2,9] and mouse [8] and, recently for ADAM-2 in monkey [28]. In the present work, we show that pig ADAM-1 and ADAM-2 can be found in spermatozoa from the testis and the proximal parts of the epididymal duct under their precursor forms. However, most of ADAM-1 proteins loss the N-terminal part to be reduced to a shorter C-terminal form of about 50-55 kDa, as soon as the sperm get in the *vas efferens*. This molecular weight suggests a removal of the pro-peptide and metalloprotease domains, certainly by cleavage at the conserved pro-protein convertase site, as previously described for rodents [2]. We also observed that not all the ADAM-1 is converted, since a 70 kDa form (with the N-terminal part) is found until the distal corpus where it might be degraded or released within the sperm cytoplasmic droplet.

For ADAM-2, different bands were observed with the anti C-terminal polyclonal antibody, suggesting a sequential proteolytic cleavage pattern of maturation of boar ADAM-2: the testicular form would be processed in the proximal caput into a precursor form of 90 kDa, then to transients forms of 70-75 kDa and 50-55 kDa in the distal caput and corpus, which are further processed in the corpus leading to final form of 40-43 kDa in the cauda. This step by step processing has been reported in rodents [2,5,9], as well as suggested for other ADAM proteins of the membrane of mature spermatozoa [7,22,29-33]. ADAM-2 N-terminal antibody had a faint reactivity, only a 65-70 kDa band being observed in all sperm extracts, which corresponds to one of the transient form of ADAM-2 observed with the anti-C-terminal antibody. Different hypotheses can be proposed to explain this, including a masking of the epitope, its cleavage, or a change in its conformation once the pro-domain is removed. Anyway, these results indicate that the hydrolysis of testicular and precursors forms of ADAM-2 is a specific event restricted to proximal caput and corpus regions, respectively. In rodents, the cleavage of ADAM2 during the transit from distal corpus to proximal cauda is coincident with the relocalization of the fertilin complex on the sperm surface. In guinea pig, fertilin migrates from the whole sperm head to the post-acrosomal region, whereas in mouse it relocalizes in the equatorial region [2,34,35]. We observed a similar phenomenon in boar: in immature spermatozoa from caput and corpus, fertilin is localized over the whole acrosomal region, whereas in mature spermatozoa from cauda it is mainly concentrated on the acrosomal ridge. These results indicate that in boar the migration of fertilin occurs during sperm transit through the distal corpus and is completed during its passage through the proximal cauda. It is interesting to note that it is also in these epididymal regions that most of the membrane surface proteins and glycoproteins labeling changes have been shown to occur, [14-16] and where the number of motile and fertile spermatozoa strongly increases [36,37].

Interestingly, two-dimensional electrophoresis showed that different isoforms of the boar 50-55 kDa ADAM-1 band, differing in Mr and pI, were present in epididymal sperm extracts from the caput and the cauda. Such a variability might result from different glycosylation levels or be related to a possible cleavage of specific residues in C- or N-terminal [5,34] that would render some isoforms more hydrophobic than others and consequently make the focalization on a immobilized gradient strip more difficult. We also observed that fertilin migration or concentration to a new membrane surface domain is coincident with a different detergent extractability of at least a subset of the sperm ADAM-1. This suggests that it moves to a different lipid membrane environment, in agreement with previous data on lipid composition of the sperm membrane domains [38,39]. This change from a lipid environment to another might result from both changes in ADAM2 size and in the properties of the subset of ADAM1, due to post-translational or electrical charge modifications.

The migration of the boar fertilin to the acrosomal ridge is also in agreement with data suggesting that sperm proteins involved in primary binding with the zona pellucida are located in the apical plasma membrane of the head [38] and with a recent report indicating that fertilin beta is one of the sperm proteins retrieved on the porcine ZP [40]. Then, the relocalization in a specific spermatozoa membrane domain resulting in an increase of the local concentration of fertilin, may be crucial for sperm-oocyte interaction. We also observed two main patterns of sperm labeling in the cauda, indicating that at least two populations of spermatozoa with different maturation coexists.

In conclusion, the present work shows that in the boar the fertilin maturation involves a regionalized proteolytic processing, and the modification of both ADAM1 and ADAM-2 as well as the post-translational modifications of a subset of ADAM-1 protein. These changes may have an essential role in the migration of fertilin complexes from the whole acrosomal domain to the acrosomal ridge of the mature spermatozoa. This provides new bases to further decipher the mechanisms involved in the processing and relocalization of the sperm surface proteins during maturation, and thereof provides tools for further studies on epididymal maturation. This kind of study will be more easy in domestic mammals where testicular and epididymal spermatozoa and fluids can be obtained in larger quantity and more easily than in rodents. Moreover, the fine description of mature versus immature patterns of fertilin is an important step toward the use of this molecule as an indicator of the level of mature sperm populations in different males and toward the understanding of a potential relationship between these fertilin patterns and male fertility.

Additional material

Additional file 1: Figure S1. Specificity of the polyclonal antibodies

Additional file 2: Figure S2. PCR amplicons obtained from the pig testis

Additional file 3: Figure S3. Absence of immunoreaction on spermatozoa with preimmune serum

Acknowledgements

The authors would like to thank M. Poirié for careful reading and suggestions on the manuscript and G. Tsikis for technical assistance. The authors gratefully acknowledge the help of the staff at the experimental facilities of the PRC, the different pig insemination organizations for their help in providing the animals used in this study and the staff of Technosperm for their kind technical and scientific support. This work was supported by grants from the French ANR Genanimal (CNRG A 02085 and FNS A 02060) and funding from the INRA PHASE department. Financial support was also provided by grants from the Spanish Ministry of Education for the mobility of pre-doctoral students and from the University of Girona BR07/12.

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Authors' contributions

AF, BG, J-LG performed the experimental work and wrote the manuscript. J-LD, MP, EP, SB participated in the design of the study and its coordination, and correct the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 20 December 2010 Accepted: 30 June 2011
Published: 30 June 2011

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doi:10.1186/1477-7827-9-96
Cite this article as: Fàbrega *et al.*: Expression, immunolocalization and processing of fertilins ADAM-1 and ADAM-2 in the boar (*sus domesticus*) spermatozoa during epididymal maturation. *Reproductive Biology and Endocrinology* 2011 **9**:96.

General discussion

Mammalian spermatozoa are produced in the testis as highly polarized cells but functionally immature. The epididymis has an important role in mammal reproduction conferring the milieu and conditions required for the sperm maturation. Mammalian spermatozoa need to acquire a mature status and the ability to undergo the capacitation and the further acrosomic reaction for the oocyte fertilization. The changes undergone in sperm cells during their passage throughout the epididymal duct included morphological, biochemical, metabolic and movement modifications, which results in the storage of motile and mature spermatozoa in the epididymal cauda. However, several changes are still necessary in mature epididymal spermatozoa to have a fertilizing aptitude. Mature spermatozoa stored in the epididymal cauda in a quiescent state are modified during and after the ejaculation by the accessory gland secretions as well as during their transit across the female reproductive tract and with the contact with the oocyte.

In boars, the changes occurring during the sperm maturation, as well as the role of the organs implicated in these changes have been studied by several authors. The boar epididymal duct structure, components and regions have been accurately described and the specific structures and regions associated with their functionality (Briz *et al.* 1993, Stoffel & Friess 1994, Calvo *et al.* 2000). The morphology of epididymal spermatozoa have been detailed (Jones 1971, Bonet *et al.* 2000) and their motility assessed in several studies (Dacheux *et al.* 1979, Bassols *et al.* 2005, Pruneda *et al.* 2005, Matás *et al.* 2010).

Proteomic secretions of the epididymal epithelial cells (secretome) (Syntin *et al.* 1996, Syntin *et al.* 1999) and their contribution in the sperm maturation (Dacheux *et al.* 2003) were previously described, as well as the protein content of the epididymal fluid (proteome) (Dacheux *et al.* 2009) and, the sperm plasma membrane (Belleannée *et al.* 2011). Epididymal fluid has also been analysed in order to study its composition (Jeulin *et al.* 1987, Cibulková *et al.* 2007, Pruneda *et al.* 2007) and metabolic pathways (Pruneda *et al.* 2006, Han *et al.* 2007). Epididymal cells cultures have been developed (Bassols *et al.* 2006) in order to evaluate the sperm maturation *in vitro* after the co-incubation with epididymal cells (Bassols *et al.* 2005) and proteomic epididymal cell secretions (Bassols *et al.* 2007). Specific fluid and sperm proteins and molecules have been identified in different epididymal regions (Yin-Zhe Jin 1997, Jin *et al.* 1999), and their processing during the epididymal maturation (Gatti *et al.* 1999) and possible implications in the reproductive function have been described (Christopher *et al.* 2007).

In terms of gene expression, the epididymal boar transcriptome was also defined by Guyonnet et al., (Guyonnet *et al.* 2009). The expression and implications of other genes, such as CD9, Retinol-binding protein 4 (RBP4), androgen receptor (AR), relaxin (RLN), acrosin (ACR) or osteopontin have also been reported (Kaewmala *et al.*, Lin *et al.* 2006). Furthermore, specific research in other files such as the study of the effects of the semen-colletion frequency on epididymal sperm maturation (Pruneda *et al.* 2005) have also been done.

This thesis project was focused in the study of boar epididymal spermatozoa and their ability to capacitate under *in vitro* conditions. Several experiments have been developed in order to assess and characterize the sperm quality throughout the epididymal duct and ejaculation in both non-capacitating and capacitating medium conditions. The results have been summarized in Paper I. Moreover, due to the importance of tyrosine phosphorilation during both epididymal maturation and sperm capacitation, the phosphotyrosine patterns of epididymal, ejaculated and *in vitro* capacitated spermatozoa have been analyzed. Complementary techniques have been used in Paper II in order to obtain novel semi-quantitative data about the phosphotyrosine fluorescence intensity, expression and localization throughout epididymal maturation, ejaculation and capacitation.

During the transit throughout the epididymis the sperm plasma membrane is highly modified by both sperm coat interaction with the epididymal fluid and specific sperm cell processes. Taking into account that the plasma membrane maturation is a key process for the sperm-oocyte recognizing and binding, an accurate analysis of carbohydrate content of the plasma membrane proteins have been performed in Paper III in order to further establish possible relations between the glycoside content of the ejaculated spermatozoa with their maturation degree and their implications in boar fertility. Finally the processing of a specific transmembrane protein, fertilin, during the epididymal maturation has been studied in detail in Paper IV.

The trial experiments performed in Paper I mix the classical computerized sperm motility analysis with the novel flow cytometry methods, showing that flow cytometry is an objective and reliable tool which can be used as a complementary procedure for motility assessment. The aim of this study was to obtain an accurate and objective sperm evaluation procedure that lead to a better correlation between the sperm quality parameters of the ejaculate and previous epididymal maturation and further capacitation of spermatozoa. As reported by several authors (reviewed in (Gupta 2005)) mammalian spermatozoa acquire their motility during their transit throughout the epididymal duct from an ineffective swimming motion into a vigorous unidirectional and progressive pattern of motility. The results exposed in Paper I demonstrated that epididymal maturation contributes not only in the acquisition of movement but also in increasing the efficiency of motion. A correct beating frequency (BCF) is the first motility parameter fully acquired since spermatozoa reach the epididymal corpus, whereas total progressive sperm motility is acquired in the epididymal cauda. Nevertheless, straightness, linearity, as well as a correct oscillation movement are not fully acquired until the spermatozoa are ejaculated, as observed in previous studies (Matás *et al.* 2010). *In vitro* capacitation and cryocapacitation has been reported to increase the ALH value over 3.5 (Casas *et al.* 2009) due to the hyperactivation of sperm movement (Yanagimachi 1970) that allows the spermatozoa to pass throughout the viscose oviductal fluid. Low ALH values reported in Paper I indicate that the *in vitro* capacitation protocol used in this trial induces only the earlier stages of the sperm capacitation in ejaculated spermatozoa, on the other hand, non-capacitated corpus and cauda spermatozoa show very high ALH values (›3.5), these results queried the role of ALH as a good capacitation index in epididymal samples. The induction of *in vitro* capacitation not only results in an ALH increase in ejaculated sperm, but it also triggers the fully progressive motility in cauda spermatozoa and the shift from curvilinear to progressive movement, leading to a decrease of VCL. Results obtained in this trial provide novel data showing that even cauda spermatozoa remain stored in a quiescent state,

they are able to undergo a vigorous progressive (increasing its PRG and decreasing its VCL) movement after incubation in a capacitating medium.

Paper I also demonstrates that the mitochondrial sheath integrity is lower in corpus and cauda sperm and it is highly altered after *in vitro* capacitation in ejaculated sperm, and that the increase of mitochondrial sheath membrane potential is necessary for both sperm motility and capacitation acquisition. *In vivo* capacitation is sequentially followed by the acrosome reaction and it is also characterized by a destabilization of sperm plasma membrane and a consequent increase of calcium influx towards the spermatozoon (Yanagimachi 1994). In order to evaluated the epididymal and ejaculated capacity to be in *in vitro*, in our lab we developed an *in vitro* capacitation model for sperm samples (Puigmulé *et al.* 2011); in this trail the plasma membrane fluidity, the intracellular calcium content and the acrosome integrity were assessed in *in vitro* capacitated sperm samples. Results found that neither epididymal maturation nor ejaculation result in an increase of the plasma membrane fluidity or the frequency of acrosome reacted spermatozoa, whereas *in vitro* capacitated spermatozoa from the epididymal cauda and the ejaculate showed an increase of the plasma membrane fluidity. Calcium content was maintained throughout the epididymis and increased in ejaculated sperm and in *in vitro* capacitated ejaculated spermatozoa.

All together, results from Paper I support the hypothesis that only ejaculated spermatozoa are completely mature to undergo a fully capacitation process after *in vitro* capacitation and highlight the importance of epididymal maturation, assessory glands secreations and ejaculation in sperm capacitation. Among epididymal spermatozoa, only those stored in the cauda have a sufficient maturation degree to undergo some of the physiological changes associated with the capacitation process. Cauda spermatozoa are mature cells showing ability to move progressively after *in vitro* capacitation but not capable to undergo the set of changes in intracellular calcium associated with the initial steps of capacitation. On the other hand, the results obtained also indicate that the maturative process made corpus spermatozoa highly sensitive in terms of viability and mitochondrial sheat integrity as compared with both immature caput spermatozoa and quiescent cauda spermatozoa.

The purpose of Paper II was to describe and compare the phosphotyrosine protein patterns in terms of expression and localization among epididymal, ejaculated and *in vitro* capacitated spermatozoa. The pattern of tyrosine phosphorylation is associated with the fertilizing ability through the capacitation process, as studied in different species such as mouse (Visconti *et al.* 1995c, Naz & Rajesh 2004), human (Luconi *et al.* 1996, Naz 1996, Sakkas *et al.* 2003), bovine (Galantino-Homer *et al.* 1997), goat (Chatterjee *et al.* 2010) and pig (Flesch *et al.* 1999, Tardif *et al.* 2001), however little data exist about the phosphotyrosine changes of the sperm during both sperm maturation and capacitation in boars.

Results presented in Paper II provide an integrated study of epididymal, ejaculated and *in vitro* capacitated boar spermatozoa by using complementary techniques of western blot, flow cytometry and immunocytochemistry. The study of the phosphotyrosine expression pattern on sperm proteins during epididymal maturation, ejaculation and *in vitro* capacitation processes demonstrate the presence of four protein groups. The first group was only present in extracts from immature spermatozoa and corresponded to proteins of 93, 66 and 45 kDa. The second group is composed by proteins of 76, 23 and

12 kDa, which showed phosphotyrosine residues in mature spermatozoa from cauda and ejaculate and which mostly increase their phosphotyrosine expression after *in vitro* capacitation.

In the third group several phosphotyrosine proteins of 49, 40, 37, 30, 26 and 25 kDa are observed constitutively, maintaining their phosphotyrosine expression in immature and mature spermatozoa, and mostly increasing their phosphotyrosine expression after maturation and/or *in vitro* capacitation. Previous studies in earlier stages of *in vitro* capacitation on boar sperm (Kalab et al. 1998) also reported several constitutive phosphotyrosine sperm proteins (p44, p40, p38 and p34) in accordance with the constitutive 49, 40, 37 and 30 kDa phosphotyrosine bands described in **Paper II**. In this trial, results highlight that in boars these p44 (49), p40 (40), p38 (37), p34 (30) proteins and also the two new constitutive proteins of 25 and 26 kDa described are tyrosine phosphorylated in early stages of epididymal maturation. This may suggest that most of these mentioned proteins would be involved not only in sperm maturation but also in capacitation process, as their phosphotyrosine expression increases significantly after *in vitro* capacitation. It is also remarkable that one of the constitutive phosphotyrosine proteins described in **Paper II**, the 30 kDa band, could correspond to previously described tyrosine phosphorylated proteins of 32 kDa (p32) by Tardif et al. (Tardif et al. 2001) and the 34 kDa protein described by Kalab et al. (Kalab et al. 1998) and Flesh et al. (Flesch et al. 1999). All this 30-34 kDa bands have a constitutive phosphotyrosine statuts and increased their phosphotyrsosine content after *in vitro* capacitation. The p32 protein has been reported to be located in the cytosolic fraction of boar spermatozoa and only appears in the membrane fraction after capacitation; it may possess tyrosine kinase-like activity (Tardif et al. 2001, Bailey et al. 2005). Further studies currently in progress focused on plasma membrane proteins could help us to elucidate the biological significance of p32 relocalization and expression during capacitation.

Finally, another fourth group is constituted by two proteins of 28 and 20 kDa that only have phosphotyrosine residues after *in vitro* capacitation. Taking into account previous literature on phosphotyrosine proteins, the 20 kDa protein that appears tyrosine phosphorylated in capacitated spermatozoa might correspond to the 21-19 kDa phosphotyrosine protein described previously by Dubé et al., (Dubé et al. 2004) in *Sus scrofa* (p21), by Esworthy et al., (Esworthy et al. 1994) in porcine and by NagDas et al., (NagDas et al. 2005) in hamster as the phospholipid hydroperoxide glutathione peroxidase (PHGPx). PHGPx is a selenoprotein acting as a key enzyme in the protection of biomembranes exposed to oxidative stress (Imai & Nakagawa 2003). In capacitated spermatozoa this enzyme is present as a disulfide cross-linked, membrane-anchored component of the mitochondrial sheath (NagDas et al. 2005). Physiological and molecular implications of PHGPx are poorly known; nevertheless previous data suggest that tyrosine phosphorylation of PHGPx may represent an important event in the signaling cascade associated with capacitation that may regulate the hyperactivation of sperm motility and/or mitochondrial function (NagDas et al. 2005).

Not only the phosphotyrosine protein patterns differed according to the maturative and/or capacitated status of boar spermatozoa, striking differences in the localization and the fluorescent intensity of phosphotyrosine residues between caput and cauda spermatozoa and also between mature non-capacitated and capacitated spermatozoa are also described in **Paper II**. Epididymal maturation of boar

spermatozoa involves dephosphorylation patterns such as in rat spermatozoa (Lewis & Aitken 2001). Results obtained in boars and rats differ from that reported in mouse (Visconti *et al.* 1995a) and goat (Chatterjee et al. 2010), thus suggesting that the phosphorylation pattern throughout the epididymis differs among species. Whereas in distal parts of the murine epididymis the entire sperm tail is tyrosine phosphorylated, and this makes spermatozoa competent for the hyperactivated movement (Aitken et al. 2007), in boar the entire phosphorylation of the sperm tail was not observed. The great differences in labeling patterns between immature and mature spermatozoa together with the significant decrease of the fluorescence intensity are indicative of a dephosphorylation process in the anterior midacrosome and/or a phosphotyrosine concentration in the posterior midacrosome during the epididymal maturation of boars. Results from this trail suggest that these location changes could be associated with the inhibition of premature capacitation according to the quiescence status of mature spermatozoa stored in the epididymal cauda (Jones *et al.* 2007).

After *in vitro* capacitation the phosphotyrosine residues are spreading all over the head, the faint labeling of the tail is recovered, and the phosphotyrosine fluorescence intensity increases according to previous studies performed in boar capacitated sperm (Petrunkina et al. 2001, Tardif et al. 2001). In contrast, in human, monkey, hamster, rat and mouse capacitated spermatozoa (review in (Naz & Rajesh 2004)) an intense tail labeling was described suggesting they may be involved in hyperactivated motility during capacitation. In boar capacitated spermatozoa, the practically absence of tail labeling and the intense labeling over the whole head, specially the acrosome suggest that tyrosine phosphorylation is implicated in capacitation and/or the acrosome reaction (Tardif et al. 2001). An increased tyrosine phosphorylation after *in vitro* capacitation has also been reported in several species such as mouse (Visconti *et al.* 1995b, Visconti *et al.* 1995d), human (Aitken et al. 1996, Aitken et al. 1998, Sakkas et al. 2003), bull (Galantino-Homer et al. 1997, Galantino-Homer et al. 2004), stallion (Pommer et al. 2003), monkey (Mahony & Gwathmey 1999), rat (Lewis & Aitken 2001), hamster (NagDas et al. 2005) and goat (Chatterjee et al. 2010), thus highlighting the importance of phosphorylation in fertilization process.

The glycocalyx represents the primary interface between spermatozoa and the surrounding environment and it is a critical component for sperm maturation, sperm protection (Kirchhoff & Hale 1996), sperm transport and sperm-egg interaction in mammals (Diekman 2003). Therefore, the study of the glycocalyx composition of epididymal spermatozoa performed in **Paper III** contributes to the knowledge of the molecular biology of sperm maturation. Three complementary analyses were performed: 1) global lectin binding over the surface of viable sperm population by flow cytometry; 2) individual analysis of the pattern of lectin binding to the sperm surface by fluorescence microscopy; and 3) electrophoretic characterization of the major sperm surface glycoprotein receptors involved in the lectin binding. Eight lectins were chosen, which fall into five categories according to their sugar affinities: galactose (PNA), glucose/mannose (LCA, PSA), N-acetyl-D-glucosamine (WGA), N-acetyl-D-galactosamine (HPA, PHA-L, SBA), and fucose (UEA-I).

Paper III shows that galactose (Gal), glucose/mannose (Glc/Man), and N-acetyl-D-glucosamine (GlcNAc) residues increased significantly their expression from the caput to the distal cauda. However, the extent of these increases differed among lectins, even if they recognized the same carbohydrate residue (e.g., LCA and PSA). For the other lectin groups (N-acetyl-D-galactosamine (GalNAc) and fucose

(Fuc) carbohydrate residues), the intensity of lectin binding on the sperm surface did not differ statistically among epididymal regions or showed a slight significant decrease either from the proximal or the posterior part of the duct (e.g., for PHA-L and UEA-I). This irregular pattern may be related with the secretion of specific sialoproteins into the corpus epididymal lumen of boars (Harayama et al. 1999) and their further deposition over the sperm surface (Calvo et al. 2000), which could mask the examined carbohydrate residues.

The localization of lectin receptors on the surface of viable spermatozoa varied according with the lectin sugars affinities but also with the stage of sperm maturation. Results show that all lectins labelled almost every part of the sperm surface, illustrating the importance of glycocalyx around the sperm cell. However, only two lectins were found to bind the sperm flagellum significantly: HPA and WGA, being the staining intensity increased with the maturation step of the epididymal spermatozoa. Similar results were observed in hamsters (Calvo et al. 1995).

Two major lectin labeling patterns are observed in the sperm head plasma membrane: the apical ridge and the whole head. It is noticeable that during the epididymal sperm transit these two domains were successively and intensively recognized by most of these lectins, except for WGA (which binds only the apical ridge), HPA and UEA-I (which bind transiently the whole sperm head). The successive changes in the location of lectin binding on the sperm head suggest a redistribution of the same protein over the sperm surface, as described for some sperm surface proteins (Phelps et al., 1990; Petruszak et al., 1991). This was clearly shown here with PSA labelling, whose staining pattern over the whole head of caput spermatozoa moved progressively during epididymal maturation over the apical ridge and the post-acrosomal region. However, PNA is not localized over the head of epididymal spermatozoa. PNA lectin recognizes Gal residues which are known to be localized only on the outer acrosomal membrane of the sperm. Due to Gal localization, spermatozoa with intact acrosomes did not show PNA staining over the head (Fazeli et al. 1997). The acrosomal ridge of mature spermatozoa from the epididymal cauda showed a remarkable abundance of GlcNAc and Glc/Man moieties. This is consistent with the migration of GlcNAc residues toward the sperm head and their involvement in capacitation of ejaculated boar sperm (Jiménez et al. 2003), in sperm-oocyte interaction (Nimtz *et al.* 1999, Töpfer-Petersen 1999), as well as in the prevention of head-to-head agglutination of sperm (Calvo et al. 2000).

The midpiece was labelled intensively by Gal and Glc/Man lectins in all regions of the epididymis; whereas HPA (GalNAc) only stained the midpiece of spermatozoa from the proximal caput. Cytoplasmic droplets showed a different lectin staining from that of the midpiece, despite there being continuity in the membrane domain of both structures: PSA did not stain the droplet surface; SBA and in part HPA labelled only the cytoplasmic droplet but not the midpiece; and PNA, LCA and WGA only stained cytoplasmic droplets in proximal position. These droplets, cytoplasmic remnants of the spermatids, migrate along the midpiece of the flagellum during the epididymal sperm transit by a still unknown intracellular mechanism. In boars the cytoplasmic droplet is released during ejaculation or even in the female tract (Harayama *et al.* 1996, Pruneda *et al.* 2005). In this trial, Gal (PNA) and Glc/man (only LCA) moieties were located over the cytoplasmic droplet of immature spermatozoa but not of mature spermatozoa. In bulls and rams, Gal moieties are also abundant on the cytoplasmic droplet of immature epididymal spermatozoa (Arya & Vanha-Perttula 1985, Magargee *et al.* 1988). Changes in lectin affinity on the surface membrane of cytoplasmic droplets observed in this trail during their migration along the

midpiece indicate that their composition is specifically modified and could be involved in droplet migration. The fluorescence microscopy analysis of the lectin binding on individual spermatozoa gives an accurate localization of the surface domain but it is less informative than flow cytometry, which provides an indication of the global lectin binding on the sperm surface population. So, the individual sperm analysis by fluorescence microscopy and the sperm population analysis by flow cytometry have different sensitivities but they are complementary.

Western blot analysis showed many bands with a wide molecular weight ranging from >250 kDa to 10 KDa. Of these about 13-14 bands were stained more intensively. Several bands of similar molecular mass were stained by different lectins. The 112-102 kDa band exhibited all carbohydrate moieties throughout the epididymis and stained intensely with PNA, LCA and WGA. It also showed a decrease in the degree of glycosylation during the sperm maturation, particularly for GalNac and Fuc residues. In a recent proteomic study of sperm surface in boars, a similar molecular weight protein of 115 kDa was identified as the angiotensin converting enzyme (ACE) (Belleannée et al. 2011). In all mammals ACE localizes over the head and midpiece and is released from the sperm surface into epididymal fluid (Gatti et al. 1999). In rhesus monkeys maturation-dependant 116 kDa glycoproteins with a diversity of oligosaccharides are involved in the fertilizing ability of spermatozoa (Srivastav 2000).

Several major bands, faint bands or bands not stained by all lectins were found only in some sperm maturational steps. Among the most visible, a 151 kDa band containing Glc/Man, GlcNAc and Fuc residues was identified only in spermatozoa from the proximal caput. A 133 kDa band with Fuc residues was present from the distal caput to the cauda. Multiple bands from 91 to 73 kDa with GlcNAc, GalNAc and Fuc residues were only present on the surface of spermatozoa from the proximal and the distal caput. Several bands of low molecular mass with Glc/Man residues were present on the surface of spermatozoa from the proximal caput (16 kDa band) and the distal caput (19 kDa band), and from the corpus to the cauda (23 kDa). Such successive bands could represent several transient forms of a same protein for which the glycan composition is modified during the epididymal transit.

The overall results from the three complementary approaches of **Paper III** showed the increase of galactose, glucose/mannose and N-acetyl-D-glucosamine lectin binding sites and the decrease of N-acetyl-D-galactosamine- and fucose-binding sites in mature spermatozoa stored on the distal cauda. These changes in carbohydrate composition are associated with changes in both the intensity and the localization of binding sites on the sperm surface. This is the first characterization by molecular weight and lectin-binding nature of the major maturation-dependent membrane glycoproteins in boar sperm from a purified surface protein preparation. From all protein bands observed it is possible that the maturational changes include the renewal of proteins, which could appear and disappear from the surface, and the gradual change of a single protein's glycan component upon maturation. Such complex modifications on the sperm surface are the consequence of several interactions between the sperm surface and the epididymal milieu, in particular with several glycosidases known to be in huge concentration in this medium (Syntin et al. 1996).

The knowledge of the protein processing during the sperm maturation in the epididymal duct could adrdress the question of their role in fertility and its potential use as fertility markers. **Paper IV** focuses the attention in a specific transmembrane protein, the fertilin complex (ADAM-1 and ADAM-2). In this

paper fertilin is characterized as an example of a protein processing throughout the epididymal duct of boars. Several ADAMs family proteins are present on the testis and the epididymis, as well as in the sperm membrane, but their potential roles in fertilization are either not completely understood or need to be clarified (Kim et al. 2006, Hunnicutt et al. 2008). Previous studies about fertilin have been performed mostly in guinea pig (Blobel 2000) and mouse (Kim et al. 2006), and although a similar pattern of fertilin modifications was suggested in bulls (Walker et al. 1996) and recently for ADAM-3 in monkeys (Kim et al. 2009), the processing and expression of the fertilin complex during the epididymal maturation in large domestic mammals has never been described in detail until this trail.

The set of experiments performed in **Paper IV** demonstrated that in boar, ADAM-1 and ADAM-2 mRNAs are present in the testis. Nevertheless ADAM-1 mRNA is observed in all epididymal samples, whereas ADAM-2 is only found in the vas efferent and caput epididymis. Previous studies in mouse observed that ADAM-1a mRNA is also expressed along the mouse epididymis (Johnston et al. 2005) and expression of ADAM-1 and -2 is not restricted to the male genital tract (Wolfsberg et al. 1995, Xiang & MacLaren 2002, Murase et al. 2008). Concerning the protein expression of the fertilin complex, precursor forms of boar ADAM-1 and ADAM-2 are found in spermatozoa from the testis and the proximal parts of the epididymal duct. However, most ADAM-1 proteins loss the N-terminal part to be reduced to a shorter C-terminal form of about 50-55 kDa, as soon as the sperm get in the vas efferent. This molecular weight suggests a removal of the pro-peptide and metalloprotease domains, by cleavage at the conserved pro-protein convertase site, as previously described in rodents (Blobel 2000). Our results show that not all the ADAM-1 is converted, since a 70 kDa form (with the N-terminal part) is found until the distal corpus where it might be degraded or released within the sperm cytoplasmic droplet. For ADAM-2, different bands are observed suggesting a sequential proteolytic cleavage pattern of maturation of this boar subunit: the testicular form is processed in the proximal caput into a precursor form of 90 kDa, then to transient forms of 70-75 kDa, 65-70 kDa and 50-55 kDa in the distal caput and corpus, which are further processed in the corpus leading to a final form of 40-43 kDa in the cauda. This step by step processing has been reported in rodents (Blobel et al. 1990, Cho et al. 1998, Blobel 2000), as well as it is suggested for other ADAM proteins of mature spermatozoa (Schlondorff & Blobel 1999, Nishimura et al. 2004, Han et al. 2009). This trial results indicate that the hydrolysis of testicular and precursors forms of ADAM-2 is a specific event restricted to proximal caput and corpus regions, respectively.

Different patterns of localization are observed in boar for ADAM-2, according to previous relocalization dispositions observed in rodents and guinea pigs (Phelps et al. 1990, Yuan et al. 1997, Blobel 2000): in immature spermatozoa from caput and corpus, fertilin is localized over the whole acrosomal region, whereas in mature spermatozoa from cauda it is mainly concentrated on the acrosomal ridge. Results of this trail indicate that the migration of fertilin in boars starts during the sperm transit through the distal corpus, but it is not completed until the sperm reach the proximal cauda. It is interesting to note that the migration process of fertilin is coincident with most of the membrane surface proteins and glycoproteins labeling changes (Dacheux et al. 1989, Belleannee et al. 2010), and with the significant increase in the number of motile and fertile spermatozoa (Dacheux & Paquignon 1980, Yanagimachi 1994). Interestingly, two-dimensional electrophoresis showed that different isoforms of the boar 50-55 kDa ADAM-1 band, differing in Mr and pI, are present in epididymal sperm extracts from caput and cauda. Such a variability might be the result of different glycosylation levels or be related to a possible cleavage

of specific residues in C- or N-terminal (Phelps et al. 1990, Cho et al. 2000) that would render some isoforms more hydrophobic than others and consequently make the focalization on an immobilized gradient strip more difficult. Fertilin migration or concentration to a new membrane surface domain is coincident with a different detergent extractability of at least a subset of the sperm ADAM-1. This suggests that it moves to a different lipid membrane environment, in agreement with previous data on lipid composition of the sperm membrane domains (Christova *et al.* 2004, Jones *et al.* 2007). This change from a lipid environment to another might result from both changes in ADAM-2 size and in the properties of the subset of ADAM-1, due to post-translational or electrical charge modifications. The migration of boar fertilin to the acrosomal ridge is also in agreement with data suggesting that sperm proteins involved in primary binding with the zona pellucida are located in the apical plasma membrane of the sperm head (Jones *et al.* 2007) and with a recent report indicating that fertilin beta or ADAM-2 is one of the sperm proteins retrieved on the porcine ZP (Van Gestel et al. 2007). Then, the relocalization in a specific spermatozoa membrane domain resulting in an increase of the local concentration of fertilin may be crucial for sperm-oocyte interaction.

Moreover, Paper IV also describes that in boars the fertilin maturation involves a regionalized proteolytic processing, and the modification of both ADAM-1 and ADAM-2 as well as the post-translational modifications of a subset of ADAM-1 protein. These changes may have an essential role in the migration of fertilin complexes from the whole acrosomal domain to the acrosomal ridge of the mature spermatozoa. Moreover, the fine description of mature versus immature patterns of fertilin is an important step toward the use of this molecule as an indicator of the level of mature sperm populations in different males. Further studies focused in the description of the potential relationship between fertilin patterns and male fertility could provide new bases to decipher the mechanisms involved in the processing and relocation of the sperm surface proteins during maturation.

In conclusion this thesis project provides new knowledge about the epididymal, ejaculated and *in vitro* capacitated spermatozoa physiology through multiparametric studies (conventional sperm quality and molecular assessment; Paper I), which give a basis for the evaluation of the sperm maturation and give information about the capacitation process. The sperm assessment could become a useful tool to detect abnormal epididymal maturation processes and/or defective capacitation processes of spermatozoa, which decrease the male fertility and prolificacy. Findings about phosphotyrosine expression and localization among maturative and/or capacitation processes (Paper II) highlight novel clue information about the mechanisms by which spermatozoa mature throughout the epididymis and acquire the competence to capacitate and by which they later become programmed for fertilization after capacitation process. Moreover, the knowledge of mechanisms involved in the processing of sperm surface glycocalyx and glycoproteins in the epididymis (Paper III) will provide useful tools to understand the epididymal maturation in both physiological and pathological conditions. Further studies must be oriented towards identifying potential phosphotyrosine proteins, glycoproteins or other markers, such as the fertilin protein complex (Paper IV), and highlighting their specific functions in the fertilization process and male fertility.

Conclusions

From Objective 1

1. Boar spermatozoa acquired their ability to move in the epididymal corpus; however their motility was not linear until the ejaculation.
2. The frequency of viable spermatozoa with intact mitochondrial sheath was higher in caput and ejaculated samples than in corpus and cauda samples, whereas the frequency of spermatozoa with high membrane potential was significantly higher in cauda samples.
3. *In vitro* capacitation resulted in a decrease of the frequency of viable spermatozoa with intact mitochondrial sheath and an increase of the frequency of spermatozoa with high membrane potential in ejaculated samples.
4. Acrosome integrity maintained constant throughout the epididymal duct and after ejaculation and earlier stages of *in vitro* capacitation.
5. Only ejaculated spermatozoa are capable to undergo the set of changes leading to capacitation. Intracellular calcium did not increase until spermatozoa are ejaculated.

From Objective 2

6. Epididymal maturation *in vivo* is associated with a progressive loss of phosphotyrosine residues of the sperm head followed by a subtle increase after *in vitro* capacitation.
7. Tyrosine phosphorylation becomes confined to a triangular band over the posterior part of midacrosome (equatorial) region throughout the epididymal transit and in ejaculated spermatozoa, whereas *in vitro* capacitation causes a spread labeling over the whole head.
8. Different phosphotyrosine patterns were characterized during epididymal maturation, ejaculation and after *in vitro* capacitation: 1) 93, 66 and 45 kDa bands with specific phosphotyrosine expression in immature spermatozoa; 2) 76, 23 and 12 kDa bands with specific phosphotyrosine expression in mature spermatozoa, they being significantly higher after *in vitro* capacitation; 3) 49, 40, 37, 30, 26 and 25 kDa constitutive bands that increased their phosphotyrosine expression after maturation, ejaculation and/or *in vitro* capacitation; and 4) 28 and 20 kDa bands with a specific phosphotyrosine expression in *in vitro* capacitated spermatozoa.

From Objective 3

9. Galactose, glucose/manose and N-acetyl-D-glucosamine binding receptors of the glycocalix increased significantly in the distal regions of the epididymis.
10. The sperm head, cytoplasmic droplet and midpiece were recognized by most of the lectins tested, whereas the principal and terminal pieces of the sperm flagellum were marked only by HPA and WGA lectins.
11. Fourteen sperm surface proteins were observed, with different patterns of lectin expression among epididymal regions in correlation with the degree of ejaculated sperm maturation.

From Objective 4

12. Fertilin alpha (ADAM-1) and beta (ADAM-2) mRNAs were highly produced in the testis, but also in the vas efferens and the epididymis.
13. ADAM-1 subunit appeared as a main reactive band of ~50-55 kDa with different pI isoforms throughout the epididymal duct, especially in the corpus region where isoforms ranged from acidic to basic pI. ADAM-1 proteolytic processing occurs mainly in the testis.
14. ADAM-2 was detected as several bands of ~90 kDa, ~75 kDa, ~50-55 kDa and ~40 kDa, showing a proteolytic progessing of this subunit throughout the epididymal caput. The intensity of high molecular mass bands decreased progressively in the distal corpus where lower bands were also transiently observed. Only the ~40 kDa was observed in mature spermatozoa stored in the epididymal cauda.
15. Fertilin migrates from the acrosomal region to the acrosomal ridge during the sperm transit from the distal corpus to the proximal cauda.
16. The extractability of a part of ADAM-1 changed during the fertilin migration probably due to biochemical changes induced by the epididymal posttranslational processing of both ADAM-1 and ADAM-2.

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A

PROXIMACIÓ MOLECULAR ALS CANVIS QUE ES PRODUEIXEN DURANT LA MADURACIÓ EPIDIDIMÀRIA, EJACULACIÓ I CAPACITACIÓ *IN VITRO* EN ESPERMATOZOIDES PORCINS

Versió en català

Resum

Els espermatozoides de mamífers adquireixen la seva funcionalitat durant la maduració epididimària i l'habilitat de penetrar i fecundar l'oòcit durant la capacitació. En aquest treball s'han avaluat la qualitat espermàtica, el patró d'expressió de proteïnes tirosina fosforilades i dels carbohidrats de la superfície dels espermatozoides, així com l'expressió i el processament d'una proteïna específica de la membrana plasmàtica al llarg del conducte epididimari, per tal de determinar la seva implicació en el procés de maduració i per definir nous marcadors de la maduració espermàtica.

L'anàlisi de la **qualitat espermàtica** en espermatozoides epididimaris, ejaculats i capacitats *in vitro* ha posat de manifest que els espermatozoides epididimaris adquireixen la seva motilitat al corpus, no obstant, la motilitat no esdevé lineal fins que són ejaculats. Els espermatozoides epididimaris no estan capacitats, ja que presenten valors baixos de fluïdesa de membrana i de calci intracel·lular. L'ejaculació en canvi, provoca un augment dels contingut de calci intracel·lular dels espermatozoides, per bé que no modifica la fluïdesa de la seva membrana. La integritat acrosòmica es manté constant al llarg de l'epidídim, així com després de l'ejaculació i de la capacitació *in vitro*. El caput epididimari i l'ejaculat mostren una major freqüència d'espermatozoides amb la beina mitocondrial intacta que al corpus i cauda epididimari. Per contra, els espermatozoides del cauda presenten un major potencial de membrana mitocondrial. La capacitació *in vitro* provoca un increment de la freqüència d'espermatozoides viables amb la membrana mitocondrial alterada, així com un augment del potencial de la membrana mitocondrial dels espermatozoides ejaculats. Els resultats indiquen que tant la **maduració epididimària** com l'**ejaculació** són processos **essencials** per la posterior **capacitació** ja que **només els espermatozoides ejaculats** són **capaços de desencadenar** els processos derivats de la **capacitació**.

L'avaluació dels patrons de tirosines fosforilades desenvolupada en aquest treball demostra que la **maduració epididimària** *in vivo* està associada a una **pèrdua progressiva de residus tirosina fosforilats** a la regió del cap dels espermatozoides, seguit d'un posterior **increment** després de la **capacitació** *in vitro*. Durant el pas dels espermatozoides del caput al **cauda epididimari**, els residus tirosina queden concentrats a una zona triangular de la part **posterior de la regió equatorial**, mentre que durant la **capacitació** *in vitro* el marcatge s'estén a la resta del **cap**. Diferents bandes amb residus tirosina fosforilats han estat detectades durant la maduració epididimària i després de la capacitació *in vitro*: 1) bandes de **93, 66 i 45 kDa** específiques dels **espermatozoides immadurs**; 2) bandes de **76, 23 i 12 kDa** específiques dels **espermatozoides madurs**, les quals augmenten la seva expressió després de la capacitació *in vitro*; 3) bandes de **49, 40, 37, 30, 26 i 25 kDa constitutives** que incrementen la seva expressió de residus tirosina fosforilats després de la maduració i/o capacitació *in vitro*; i 4) bandes de **28 i 20 kDa** específiques dels **espermatozoides capacitats** *in vitro*. L'expressió, localització i els resultats proteòmics obtinguts en aquest treball, demostren la importància dels residus tirosina fosforilats en l'adquisició d'un estatus de competència per a la fecundació de l'oòcit.

La caracterització del glicocàlix dels espermatozoides epididimaris porcins ha permès determinar que durant la maduració epididimària es produeix un **augment** significactiu dels residus **galactosa, glucosa/mannosa i N-acetil-D-glucosamina** a les regions més **distals de l'epidídim**. A més, les regions del cap i la peça intermèdia i la gota citoplasmàtica són reconegudes per la major part de les lectines estudiades, mentre que les peces principal i terminal del flagel només són reconegudes per la HPA i la WGA. En l'estudi s'han observat catorze proteïnes de la superfície espermàtica, amb diferents patrons de marcatge per les lectines estudiades a les diferents regions de l'epidídim. Les modificacions en el glicocàlix observades amplien els coneixement en els canvis moleculars que es produeixen durant la maduració epididimària; l'avaluació dels patrons de glicosilació en els espermatozoides ejaculats poden ser emprats per determinar el grau de maduració epididimària assolit.

Per il·lustrar els canvis que es donen durant la maduració espididmaria en algunes de les proteïnes de la membrana plasmàtica s'ha estudiat l'expressió, la locatització i el patró proteòmic de la fertilina. La fertilina està formada per un complex de dues subunitats, la subunitat alfa (ADAM-1) i la subunitat beta (ADAM-2). La fertilina és una proteïna integral de membrana de la família de les ADAM i està involucarada en processos claus de la interacció entre les membranes de l'espermatozoide i l'oòcit. Els resultats obtinguts demostren que **grans quantitats** de mRNA per la subunitat ADAM-1 i ADAM-2 són **produïts a nivell testicular**, així com també en el **vas eferent** i l'**epidídim**. La subunitat **ADAM-1** apareix en forma d'una banda de **~50-55 kDa** amb diferents isoformes al llarg del conducte epididimari, especialment al corpus on isoformes amb un ampli rang de pI han estat identificades. En canvi, la subunitat **ADAM-2** presenta diversos pesos moleculars, que varien entre ~90 kDa, ~75 kDa, ~50-55 kDa i ~40 kDa. La intensitat de les bandes de major pes molecular disminueix progressivament al corpus distal, i generen bandes transitòries amb un menor pes molecular. Només la banda de **~40 kDa** es pot observa a nivell del **cauda**. Els resultats demostren que mentre la subunitat **ADAM-1 és processada majoritàriament a nivell testicular, la subunitat ADAM-2 és sotmesa a un processament proteolític durant el seu pas del caput al corpus**. La immunolocalització mostra que a més, la **fertilina migra i es concentra a la regió apical del cap durant el seu pas del corpus distal al cauda proximal**. Aquesta migració va acompanyada de canvis important en l'extractabilitat de l'ADAM-1 de la membrana espermàtica. Els resultats suggereixen que la migració a través de la superfície espermàtica de la fertilina pot ser modulada pels canvis bioquímics induïts durant el processament posttransduccional de les subunitats ADAM-1 i ADAM-2. La caracterització dels patrons de maduració del complex fertilina poden suposar un nou pas pel desenvolupament de marcadors de fertilitat basats en canvis en les proteïnes de la superfície espermàtica desenvolupats durant la maduració espermàtica en animals domèstics.

En conjunt, els resultats obtinguts en aquest treball estableixen una base per a l'**avaluació espermàtica** (paràmeters de qualitat, patrons de residus tirosina fosforilats i de residus carbohidrats) per tal d'obtenir informació fiable i robusta del **grau de maduresa** dels espermatozoides ejaculats i obre noves perspectives en la caracterització de **proteïnes** que puguin ser emprades com a **potencials marcados del grau de maduració espermàtica i/o de fertilitat** en els espermatozoides, com ara els patrons de la fertilina, de glicosilació i dels residus tirosina fosforilats.

Introducció

1. Espermatozoide porcí

L'espermatozoide madur porcí és un gàmeta de 45 µm de longitud, amb tres regions morfològicament diferenciades: el **cap**, la **peça de connexió (coll)** i la **cua** (Bonet *et al.* 2000) (Figura 1), i tres regions funcionals: 1) el cap de l'espermatozoide, involucrat en la interacció espermatozoide-oòcit, 2) la peça intermèdia, formada per la beina mitocondrial i involucrada en la producció d'energia i 3) l'estructura flagel·lar de la cua, implicada en la seva motilitat (Flesch & Gadella 2000).

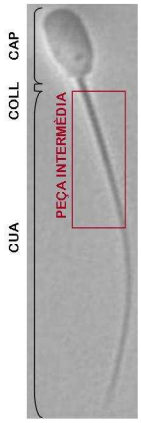

Figura 1. Espermatozoide madur de porcí. Imatge d'un espermatozoide madur de porcí obtinguda amb un microscopi de contrast de fases a x20. Es poden distingir les tres regions morfològiques del cap, coll i cua, així com les regió funcional de la peça intermèdia que conté la beina mitocondrial.

El **cap** és pla i oval, d'uns 7 µm de longitud, 3,7 µm d'ample i 0,4 µm de gruix. Presenta dues cares diferenciades: una de les cara és plana, mentre que l'altra presenta una protuberància apical semblant a una mitja lluna d'uns 0,4 µm d'ample i d'uns 1,2 µm de longitud. A la membrana plasmàtica es poden distingir dos dominis principals, la regió acrosòmica (part anterior) i la regió postacrosòmica (part posterior). El cap conté el nucli, l'espai subacrosòmic, el material fibrós perinuclear, la làmina densa postacrosòmica i l'acrosoma (Eddy & O'Brien 1993, Bonet *et al.* 2000).

Al **nucli** es troba la cromatina molt condensada i formant una estructura molt electrodensa. Els espermatozoides presenten un nucli menys voluminós respecte el nucli de les cèl·lules somàtiques. Té una longitud de 6,6 µm i un gruix de 220 nm a nivell de la regió acrosòmica, mentre que a la regió postacrosòmica augmenta fins als 320 nm. Conté una sola còpia de cada cromosoma, conseqüència de les dues divisions meiòtiques que es produeixen durant l'espermatogènesi (Eddy & O'Brien 1993, Bonet *et al.* 2000).

L'**acrosoma** és un vesícula localitzada a la regió acrosòmica disposada sobre el nucli en forma de caputxa que recobreix el 80% de la longitud nuclear. La matriu acrosòmica està composada per un material citoesquelètic amorf, distribuït homogèniament i ric en hidrolases, que participen a la reacció acrosòmica i en la fusió de les membranes de l'espermatozoide amb l'oòcit (Fawcett 1975, Bonet *et al.* 2000). Tot i que la membrana acrosòmica és contínua, s'hi poden distingir dos dominis: la membrana interna i la membrana externa (Fawcett 1975, Holt 1984, Bonet *et al.* 2000). A la part anterior del cap, la membrana acrosòmica interna es disposa sobre l'embolcall nuclear, mentre que la membrana acrosòmica externa se situa just per sota de la membrana plasmàtica (Eddy & O'Brien 1993). L'acrosoma se subdivideix en tres segments morfològics corresponents als dominis de la membrana plasmàtica: apical, principal i equatorial (Figura 2). El segment apical correspon a la part anterior de l'acrosoma i es troba més dilatat que la resta. En aquest segment la membrana acrosòmica discorre just per sota de la membrana plasmàtica. Pel que fa al segment principal representa la major part de l'acrosoma i juntament amb el segment apical formen el

que també s'anomena cap acrosòmic o acrosoma anterior (Eddy & O'Brien 1993, Bonet *et al.* 2000). El segment equatorial és el que es troba situat a la part posterior de l'acrosoma i és el més electrodens.

El **material fibrós perinuclear** és una estructura citoesquelètica especialitzada que forma una xarxa rígida entre la membrana nuclear i l'acrosoma. A la regió postacrosòmica el material fibrós perinuclear esdevé la **làmina densa postacrosòmica** (Olson 2002), formada per una capa homogènia de material fibrós i electrodens disposat en paral·lel sota la membrana plasmàtica. La làmina densa postacrosòmica recobreix un 20% de la longitud del nucli tot coincidint amb la regió del nucli que no es troba recoberta per la vesícula acrosòmica (Bonet *et al.* 2000). L'estructura rígida de la membrana perinuclear fibrosa ajuda a mantenir la forma del nucli i té una funció mecànica durant el procés de penetració de l'espermatozoide en l'oòcit; d'altra banda, aquesta estructura conté nombrosos factors implicats en l'activació de l'oòcit (Kimura *et al.* 1998, Perry *et al.* 1999). Durant el trànsit al llarg de l'epidídim la membrana perinuclear fibrosa és sotmesa a moltes modificacions: la condensació del material (Bedford & Nicander 1971, Jones 1971), la formació de ponts disulfurs que estabilitzen l'estructura (Calvin & J. 1971, Bedford & Calvin 1974), i l'adopció d'una forma serrada de les vores de la làmina densa postacrosòmica (Juárez-Mosqueda & Mújica 1999).

L'espai format entre el nucli i la membrana acrosòmica interna o amb la cara interna de la làmina densa prostacrosòmica, s'anomena **espai subacrosòmic** o **perinuclear**. Aquest espai és ocupat per material fibrós perinuclear poc electrodens, que esdevé més desenvolupat i electrodens a la regió surpanuclear (Bonet *et al.* 2000).

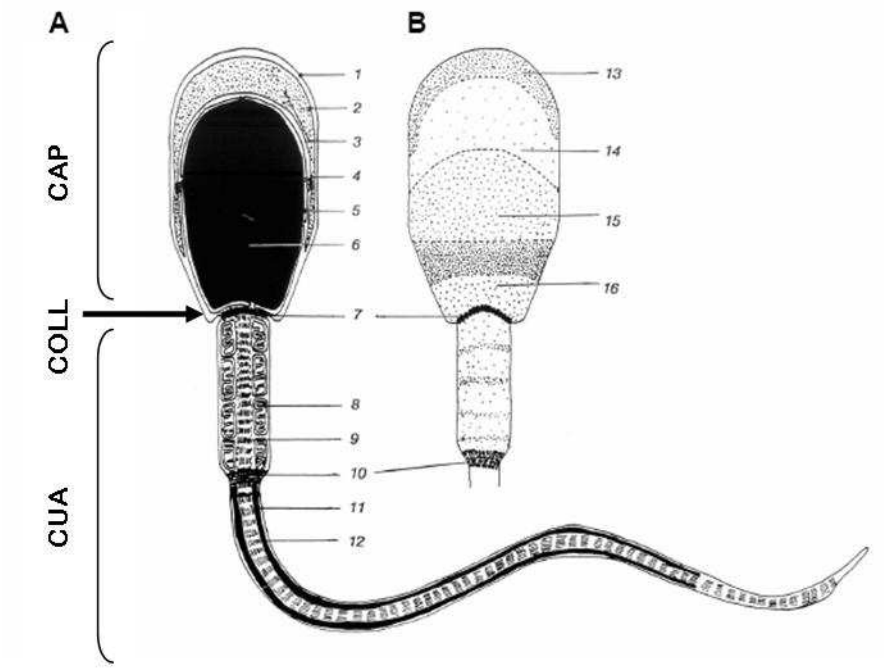

Figura 2. Representació esquemàtica d'un espermatozoide de porcí. (A). Vista en secció de l'espermatozoide porcí. Les línies sòlides representen les membranes de la bicapa 1) membrana plasmàtica, 2) membrana acrosòmica externa, 3) matriu acrosòmica, 4) membrana acrosòmica interna, 5) membrana nuclear, 6) nucli, 7) peça de connexió (coll), 8) peça intermèdia, 9) mitocondris i axonema, 10) *annulus* (anell de Jensen), 11)beina fibrosa i 12) axonema i fibres denses externes. (B). Vista de la superfície del cap i la peça intermèdia de l'espermatozoide amb els subdominis: 13) vora apical, 14) segment principal i 15) segment equatorial; Vista del cap de l'espermatozoide 16) regió postacrosòmica. Modificat de Gadella, 1994 **(Gadella *et al.* 2008)**

La peça de **connexió** o **coll** de l'espermatozoide és la regió que uneix la base del nucli amb el primer mitocondri de la peça intermèdia. A la peça de connexió es poden distingir les següents estructures: la placa basal, els cossos laminars, el *capitulum*, les columnes segmentades, el cos basal i l'axonema. La placa basal està formada per un material electrodens (similar a la làmina densa postacrosòmica) adherit a la membrana externa de l'embolcall nuclear. Els cossos laminars procedeixen d'evaginacions molt pronunciades de l'embolcall nuclear lliures de cromatina. El *capitulum* és una estructura arciforme disposada per sota la placa basal. Les columnes segmentades parteixen dels extrems del *capitulum*; inicialment es troben unides formant una extensió del *capitulum*, i posteriorment s'individualitzen en 9 columnes unides a les fibres denses externes al llarg de la peça intermèdia i principal. El cos basal es troba a la base de la convexitat que forma el *capitulum*. A partir del cos basal s'organitza l'axonema, que presenta l'estructura microtubular típica de 9+2, i en el que cadascun dels doblets de microtúbuls perifèrics es troba associat a una fibra densa externa (Bonet *et al.* 2000).

La **cua** és filamentosa i cilíndrica, i s'hi distingeixen tres regions o peces (Fawcett 1975): la peça intermèdia, la peça principal i la peça terminal.

La peça **intermèdia** s'estén de la part final de la peça de connexió fins a l' *annulus* o l'anell de Jensen, una estructura en forma d'anell electrodens que marca el límit entre la peça intermèdia i la peça principal. A la peça intermèdia i principal, l'axonema ocupa l'eix central entorn al qual es disposen les fibres denses externes que mantenen l'estructura. La beina mitocondrial discorre just per sota del plasmalemma i forma una estructura helicoïdal estabilitzada per ponts disulfur i per un conjunt de grànuls perifèrics localitzats entre les fibres denses de la regió proximal.

A la peça **principal** la beina mitocondrial és substituïda per dos eixos longitudinal continus o columnes, coplanàries amb el parell de microtúbuls centrals, unides a través d'unes costelles circumformes, distribuïdes regularment al voltant de la peça principal formant una beina fibrosa. En aquesta regió els eixos fibrosos es comencen a unir (Bonet *et al.* 2000).

La peça **terminal** és el segment més curt i se situa a la part final de la cua. Aquesta peça no conté estructures citoesquelètiques, sinó que està constituïda per un axomena que es va desorganitzant progressivament a mesura que s'allunya de la peça principal, i que es troba envoltat pel plasmalemma (Bonet *et al.* 2000).

2. Els testicles i els conductes eferents

2.1. Anatomia

En mamífers, els **testicles** estan formats per un parell d'òrgans ovoïdals i encapsulats constituïts per **túbuls seminífers** separats per teixit intersticial. La seva mida varia notablement entre espècies; d'uns 20-22 cm^3 en humans fins a uns 0,062-0,077 cm^3 en ratolins (Kerr *et al.* 2006).

Els espermatozoides es formen als túbuls seminífers i són abocats als conductes de la rete testis. En mamífers, les **cèl·lules de Sertoli** representen el principal tipus cel·lular de l'epiteli seminífer; s'encarreguen de conferir suport físic a les cèl·lules germinals i proveir-les de nutrients i de factors de creixement. L'epiteli seminífer presenta unions especialitzades (unions estretes) entre cèl·lules de Sertoli adjacents, creant una barrera **(barrera hemototesticular)** que el divideix en dos compartiments, el basal i l'adluminal. El compartiment basal conté les formes espermàtiques primerenques, les espermatogònies. Per contra, el compartiment adluminal conté els estadis meiòtics i postmeiòtics del desenvolupament de l'espermatozoide. D'aquesta manera, les cèl·lules de Sertoli mitjançant les unions estretes confereixen una base anatòmica per a la permeabilitat de la barrera hematotesticular, aïllant les cèl·lules germinals del fluid extracel·lular i permetent el pas selectiu de substàncies del torrent sanguini fins a elles (Amann 1989, Pinart *et al.* 2000, Pinart *et al.* 2001).

La **rete testis** forma una xarxa complexa on convergeixen els túbuls seminífers i que els comunica amb els **conductes eferents** (Figura 3), on els espermatozoides són envoltats per un medi fluid i transportats del pol dorsal del testicle fins a l'entrada de l'epidídim. (Setchell *et al.* 1994).

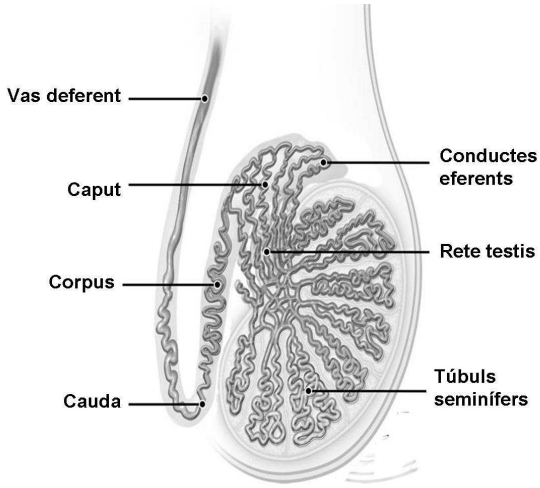

Figura 3. Representació esquemàtica de l'estructura testicular. Vista dels túbuls seminifers connectats amb els conductes eferents mitjançant la rete testis; de l'epidídim format per un sol conducte (caput, corpus i cauda) que desemboca en el vas deferent.

En animals domèstics, el nombre de conductes eferent varia entre 13 i 20 (Saitoh *et al.* 1990). En porcs, es possible distingir-hi dos segments: el segment proximal o intratesticular i el segment distal o epididimari. El conducte presenta una estructura gruixuda que progressivament esdevé més llarga i més flexible. L'epiteli dels conductes eferents està format bàsicament per cèl·lules ciliades i cèl·lules no ciliades, i presenta limfòcits intraepitelials (Arrighi *et al.* 1993, Setchell *et al.* 1994). Les cèl·lules dels conductes

eferents tenen poca capacitat de síntesi i secreció proteica, no obstant, les cèl·lules no ciliades semblen estar activament involucrades en la reabsorció del fluid, mitjançant l'endocitosi per fase-fluid (no modulada per lligants extracel·lulars o per altres inductors naturals coneguts) i absortiva (modulada per la unió dels lligants a receptors de la membrana plasmàtica) (revisat a (Setchell *et al.* 1994)).

2.2. Espermatogènesis

La principal funció del testicle és la **producció** dels **gàmetes masculins** i d'hormones esteroides. L'**espermatogènesi** constitueix el conjunt de processos a través dels quals una espermatogònia primerenca esdevé un espermatozoide als túbuls seminífers dels mascles adults (Figura 4). En el porc domèstic (*Sus domesticus*), a diferència del seu antecessor, el porc senglar (*Sus scrofa*), aquest procés té lloc durant tot l'any, disminuïnt la seva eficàcia reproductiva a l'estiu a causa de l'augment de la temperatura i del fotoperíode (Sancho *et al.* 2004, Sancho *et al.* 2006).

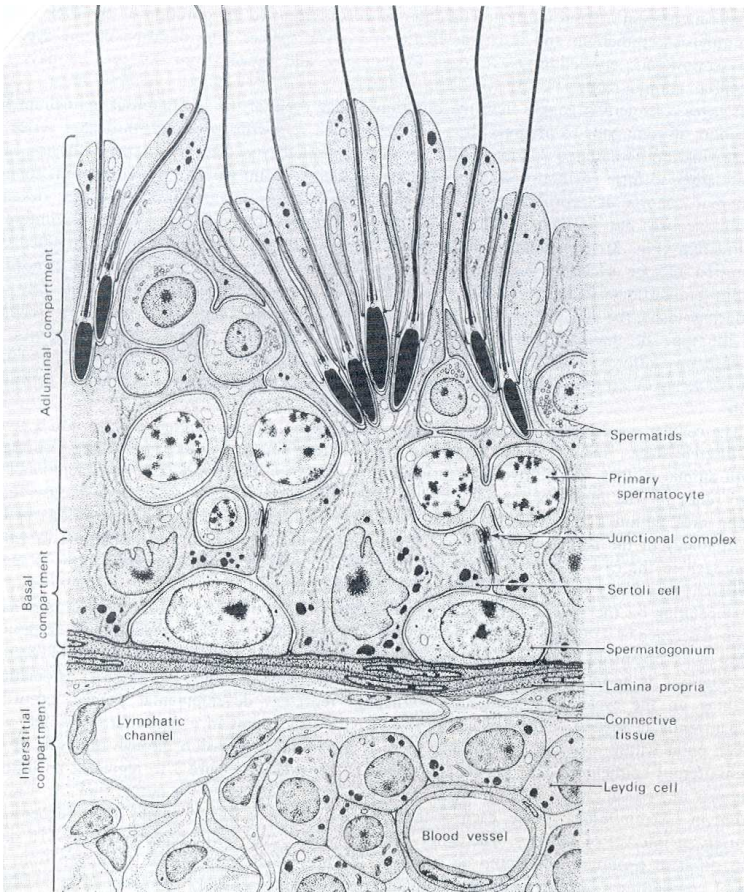

Figura 4. Representació esquemàtica de la formació dels espermatozoides en el túbuls seminífers. Representació de les tipologies espermàtiques presents en els compartiments basal i adluminal de l'epitelí seminífer separats per la barrera hematotesticular. Figura de (Amann 1989).

L'epiteli germinal dels túbuls seminífers presenta una doble funció: produir espermatozoides immadurs de forma contínua i substituir la població d'espermatogònies que els van produint. El procés d'espermatogènesi complet té lloc en tres fases seqüencials de proliferació i diferenciació cel·lular: 1) fase mitòtica (espermatocitogènesi), 2) fase meiòtica, i 3) fase post-meiòtica (espermiogènesi). Durant aquest procés les cèl·lules morfològicament indiferenciades (espermatogònies) es desenvolupen fins a formar els espermatozoides altament diferenciats. El procés d'espermatogènesi té una durada d'uns 73 dies en humà, uns 40 dies en ratolí i uns 34 dies en porc (Amann 1989).

Durant la **fase mitòtica** les espermatogònies primàries proliferen per mitosi donant lloc a successives generacions d'espermatogònies en diferents graus de diferenciació. Es poden distingir dos tipus d'espermatogònies: les espermatogònies A, les quals es caracteritzen per l'absència d'heterocromatina i, les espermatogònies B les quals es caracteritzen per un elevat contingut d'heterocromatina i per donar lloc als espermatòcits primaris, després de la última divisió mitòtica. Les espermatogònies A i B es sotmeten a una sèrie de divisions que resulten en la formació de moltes generacions de cèl·lules intermèdies. No obstant, cada àrea dels túbuls seminífers produeix de forma sincronitzada un mateix grau de diferenciació d'espermatogònies (De Rooij & Grootegoed 1998).

La **fase meiòtica** s'inicia quan els espermatòcits primaris entren en profase I i els cromosomes es condensen. Durant la meiosi els cromosomes són alineats en parelles i tenen lloc processos d'intercanvi genètics entre regions homòlogues de les cromàtides, *crossing-over*. Aquest procés és seguit per dues divisions meiòtiques que no inclouen replicació del DNA i que donen lloc a la formació de les espermàtides, les quals són remodelades fins assolir l'estadi final d'espermatozoide immadur.

La fase final de diferenciació de les cèl·lules germinals masculines abans de ser alliberades de l'epiteli seminífer és la **fase postmeiòtica (espermiogènesi)**. Durant aquesta fase les espermàtides indiferenciades, haploides i arrodonides experimenten complexes canvis morfològics, bioquímics i fisiològics que resulten a la formació de l'estructura flagelar asimètrica de l'espermatozoide. La formació de l'acrosoma, així com de la cua o flagel, són dos dels principals processos de transformació que tenen lloc durant l'espermiogènesi. L'espermatogènesi és un procés relativament llarg en mamífers (entorn a uns 14 dies en ratolí, uns 22 dies en humà i uns 9 dies en porc). Tot i tractar-se d'un procés continu, l'espermatogènesi és pot dividir en diferents fases morfològiques: la fase de formació de l'aparell de Golgi, la fase cap, la fase acrosòmica i la fase de maduració. Durant els primers passos de diferenciació de l'espermatozoide (Fase del Golgi), es formen nombrosos grànuls dins l'aparell de Golgi que acabaran fusionant-se per formar la vesícula acrosòmica, la qual s'adhereix a l'embolcall nuclear. D'altra banda, els centríols es transloquen al pol oposat al que es forma l'acrosoma, establint una polaritat longitudinal dins la cèl·lula. Un dels centríols està implicat a la formació del flagel, mentre que l'altre intervé a la formació de la peça de connexió. Un altre canvi morfològic de la fase de Golgi és l'agrupació i la redistribució dels mitocondris que es troben al voltant del nucli. A la fase cap l'acrosoma s'aplana per sobre l'acroplaxoma (placa citoesquelètica formada per F-actina) i s'uneix a l'envolcall nuclear formant l'estructura del cap. Aquesta estructura inicia el seu descens pels extrems del nucli i l'espermatida es gira situant l'acrosoma en direcció a la làmina basal i l'axomena estenen-se dins el lumen del túbul seminal. Paral·lelament a aquests canvis, s'inicia la fase acrosòmica, durant la qual es desenvolupen els microtúbuls citoplasmàtics formant una estructura cilíndrica transitòria anomenada *manchette*, implicada en l'elongació de l'espermàtida. Durant la fase de maduració, l'acrosoma completa la seva diferenciació i passa a recobrir la meitat de la superfície nuclear, es forma la beina mitocondrial i el nucli es condensa; el *manchette* microtubular desapareix en aquesta fase. Finalment durant la fase de maduració també es completa la formació de la cua, s'allibera l'excés de citoplasma, i continua la condensació i l'estabilització de la cromatina mitjançant la substitució de les histones per protamines (Pinart *et al.* 2000, Pinart *et al.* 2001, Gupta 2005b).

3. Maduració posttesticular

3.1. Epidídim

El conducte epididimari és un sol conducte molt tortuós, originat per la convergència de 5 o 6 conductes eferents (Saitoh *et al.* 1990). L'epidídim es troba íntimament unit a la superfície del testicle, estenent-se del pol anterior fins al posterior de l'òrgan, i unit a la túnica albugínia a través del teixit connectiu. El conducte pot ser dividit en diferents segments delimitats per septes de teixit connectiu i es troba envoltat per una càpsula de teixit fibrós. Ambdos, testicle i epidídim es troben suspesos a l'escrot. Aquesta localització permet el manteniment de la temperatura varis graus per sota de la temperatura corporal en aquests òrgans (Setchell *et al.* 1994, Pruneda 2006). En porcí, el conducte epididimari pesa entorn a 85 g i té una longitud d'uns 27 cm quan es troba cargolat i d'uns 54 m quan es troba completament descargolat i estirat (Pruneda *et al.* 2006, Pruneda *et al.* 2007). Al caput i cauda el conducte epididimari és més ampli que a la zona del corpus on esdevé més prim. L'epidídim està format per tres compartiments diferents: 1) el conducte luminal, que conté els espermatozoides i el plasma epididimari; 2) l'epiteli que limita amb el lumen, i sota el qual si troba una capa de teixit conectiu i musuclatura llisa, i 3) el teixit extern del conducte.

L'**epiteli epididimari** forma una estructura complexa i dinàmica depenent d'andrògens (Bedford 1972). L'epiteli conté diferents tipologies cel·lulars, la proporció de les quals varia al llarg del conducte. En els mascles adults moltes d'aquestes cèl·lules varien en el seu contingut citològic i histoquímic en funció de la regió del conducte a la qual es troben situades (Bedford 1972). La tipologia cel·lular predominant en l'epiteli epididimari dels mamífers són les **cèl·lules principals**, les quals són cèl·lules llargues, estretes i columnars amb estereocilis apicals que van disminuint la seva abundància i longitud al llarg de l'epidídim. Aquestes cèl·lules són presents al llarg de tot el conducte i estan implicades en processos d'absorció i secreció. S'han descrit altres tipus cel·lulars a l'epidídim: les **cèl·lules apicals** presents a la regió del caput, les **cèl·lules basal** presents al corpus i al cauda, les **cèl·lules clares,** descrites a l'epidídim d'altres mamífers però no de porcí, i les **cèl·lules de l'halo** més abundants a les zones proximals que a la resta del conducte (Goyal & Williams 1991, Briz *et al.* 1993, Setchell *et al.* 1994). Les cèl·lules basals són cèl·lules petites i esfèriques que presenten perllongaments a la part basal i que poden estar implicades en l'acidificació i alcalinització del fluid luminal.

El conducte epididimari es troba envoltat pel **teixit connectiu**, el qual està format per fibroblasts, fibres de col·làgena i elàstiques, vasos sanguinis i limfàtics, fibres nervioses, macròfags, leucòcits de vigilància, i per 7 -10 capes concèntriques de **fibres de musculatura llisa** subjacent a l'epiteli (Briz *et al.* 1993, Setchell *et al.* 1994, Stoffel & Friess 1994).

En molts mamífers l'epidídim és divideix en 5 regions funcionals i estructurals: el segment inicial, la zona intermèdia, el caput, el corpus i el cauda (Reid & Cleland 1957, Hamilton 1972, Hamilton 1975, Robaire & Hermo 1988, Hermo 1995). Tot i això, en mamífers l'epidídim es pot dividir també en tres segments en funció de la seva morfologia: **caput**, **corpus** i **cauda**. Les variacions entre espècies en l'estructura i extensió dels diferents segments suggereix variacions a la maduració i emmagatzematge posttesticular dels espermatozoides (Jones 2002). En porcí, els tres segment morfològics principals es

poden subdividir en onze regions: segment inicial (regió **0**), caput (regions **1-4**), corpus (regions **5-7**) i cauda (regions **8-9**) el qual acaba en el conducte deferent (**10**) (Dacheux *et al.* 1984) (Figura 5).

D'altra banda, un altre paràmetre que es veu modificat al llarg del conducte és la concentració espermàtica que augmenta de 10^8 espermatozoides/ml a la rete testis fins a uns 10^9 espermatozoides/ml al conducte deferent, amb un màxim 10^{10} espermatozoides/ml al caput proximal (Dacheux *et al.* 2003), com a conseqüència de la reabsorció del fluid testicular en aquesta regió de l'epidídim (Pruneda *et al.* 2006). D'altra banda, és en aquesta regió on es concentra la major part de la secreció proteica del conducte epididimari (Dacheux *et al.* 2009). Al corpus aquest procés es reverteix i la concentració espermàtica decreix de nou. L'elevada concentració cel·lular observada a les regions proximals coincideix amb una elevada concentració proteica i una baixa concentració en salts, que donen lloc a una pressió osmòtica inferior al 50%, a baixos nivells d'oxigen i a una manca de substrats energètic tals com la glucosa. L'elevada concentració cel·lular facilita les interaccions entre les cèl·lules germinals, els components del lumen i la superfície de les cèl·lules de l'epidídim (Dacheux *et al.* 2005).

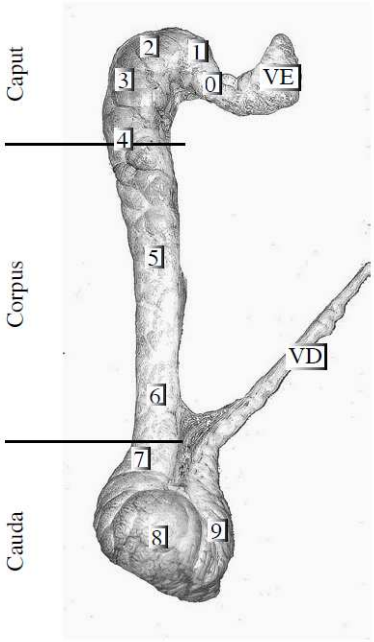

Figura 5. Regions de l'epidídim d'un porc adult. Imatge de l'epidídim d'un porc on es poden diferenciar les diferents regions: VE, vasos eferents; 0 to 8/9, epidídims; VD, vas deferent, i les tres regions morfològiques: caput, corpus, cauda. Figura de (Dacheux *et al.* 1984).

Les **funcions** bàsiques de l'epidídim són: 1) la **maduració espermàtica**, la qual dota l'espermatozoide de motilitat i d'aptitud per reconèixer i unir-se a la zona pel·lúcida (ZP), així com per fusionar-se amb l'oòcit. Aquest procés té una durada d'entre una i dues setmanes en mamífers; 2) el **transport** dels espermatozoides a través de moviments peristàltics generats per la contracció de les fibres de la musculatura llisa i, 3) proveïr d'unes condicions òptimes per l'**emmagatzematge** dels espermatozoides en un estat quiescent abans no siguin ejaculats.

3.2. Conducte deferent

El conducte o vas deferent precedeix el conducte epididimari i es redireciona en direcció al canal inguinal. Està format per un epiteli complex de revestiment i una túnica muscular gruixuda organitzada en estrats concèntrics que afavoreixen el trànsit dels espermatozoides cap a l'uretra en el moment de l'ejaculació (Setchell *et al.* 1994).

3.3. Maduració epididimària

Durant el trànsit per l'epidídim els espermatozoides adquireixen la capacitat de moure's i de fecundar l'oòcit. Els espermatozoides madurats al llarg del conducte epididimari són emmagatzemats en elevades concentracions al cauda, que els confereix un ambient idoni per mantenir-los en un estat quiescent fins el moment de l'ejaculació (revisat a (Jones *et al.* 2007)). Després de l'ejaculació, i ja dins del tracte genital femení, l'espermatozoide és capacitat i té lloc la reacció acrosòmica. Aquests processos confereixen a l'espermatozoide les característiques necessàries perquè l'oòcit pugui ser fertilitzat i van acompanyats de nombrosos processos de relocalització proteica. L'habilitat de l'espermatozoide per desencadenar els mecanismes implicats en la fertilització es desenvolupen al llarg del procés de maduració dels espermatozoides a l'epidídim i inclou: interaccions amb certes proteïnes derivades de les secrecions epididimàries, processos de remodelatge de la superfície espermàtica, la inducció de la condensació de la cromatina, l'adquisició del moviment i el desenvolupament del potencial per desencadenar la capacitació (revisat a (Olson 2002, Gupta 2005a)).

La maduració espermàtica *in vivo* a l'epidídim ha estat descrita com un procés dependent d'andrògens, no obstant, la seva base bioquímica encara és força desconeguda (Gupta 2005a). Mitjançant estudis de microscòpia s'ha demostrat que durant el trànsit pel conducte epididimari els espermatozoides són sotmesos a remodelacions que inclouen canvis en la dimensió i aparença de l'acrosoma i del nucli, canvis estructurals en els orgànuls intracel·lulars i en algunes espècies la migració de la gota citoplasmàtica al llarg de la cua (Olson 2002). Aquests canvis morfològics van acompanyats de modificacions bioquímiques de diverses proteïnes intra-acrosòmiques i de la superfície de la cèl·lula (Cooper 1986, Jones 1998). S'ha demostrat que les secrecions de l'epiteli epididimari són importants per la maduració i la supervivència espermàtica, no obstant el seu paper no està clarament definit. Les modificacions de la membrana espermàtica inclouen la incorporació de certes proteïnes, sucres i lípids. La superfície dels espermatozoides és una estructura dinàmica que no només es modifica al tracte genital masculí, sinó també durant el seu trànsit pel tracte reproductor femení. La modificació de la superfície de l'espermatozoide inclou canvis a la fluïdesa de la membrana plasmàtica, canvis en el perfil de lípids reduint significativament la quantitat de fosfolípids i el contingut glicolípids dels lípids totals. Per contra, la membrana dels espermatozoides madurs del cauda epididimari a diferència dels immadurs del caput presenten un elevat contingut en lípids neutres. La maduresa espermàtica es troba associada a un marcat increment dels nivells d'esterol i d'ester esteris, i a una disminució d'altres lípids neutres units a la membrana. La relació colesterol/fosfolípids i d'àcids grassos saturats/insaturats incrementa significativament a la membrana dels espermatozoides madurs (revisat a (Gupta 2005a)).

La maduració espermàtica a l'epidídim inclou també processos d'estabilització del DNA i un increment dels nivells de monofosfat dadenosina cíclic (cAMP) en els espermatozoides d'ovella, de boví, de porcí i de boc (Gupta 2005a). Els nivells de cAMP es veuen regulats per la relació entre el cDNA sintetitzat i el degradat (Gupta 2005a). En boví la iniciació del moviment espermàtic inclou com a mínim dos processos coneguts, l'augment del contingut intra-espermàtic de cAMP durant el trànsit per l'epidídim i la producció de proteïnes epididimàries específiques per a la motilitat progressiva (Brandt *et al.* 1978). A l'epidídim els espermatozoides passen d'un moviment ineficaç a un patró de moviment vigorós

unidireccional i progressiu. D'aquesta manera, els espermatozoides del caput epididimari de diverses espècies presenten un moviment circular característic. Els espermatozoides del caput de rata, conill i conill d'Índies presenten un patró de moviment circular mentre que els espermatozoides obtinguts del caput de boví, humà i de primats no humans mostren un patró de moviment que varia de la pràctica immobilitat a flagel·lacions febles de la cua (revisat a (Schirren 1980)). S'ha suggerit que el pH intra-espermàtic pot tenir un paper clau en la regulació del inici de la motilitat espermàtica a l'epidídim (Gupta 2005a).

3.4. Fluid Epididimari

En porcí, les proteïnes presents en el fluid epididimari procedeixen de 1) components de la rete testis que entren a través dels conductes eferents fins a les parts proximals de l'epidídim, 2) processos de secreció i absorció epididimària, 3) la proteòlisi de proteïnes preexistents al fluid i 4) de l'activitat metabòlica dels espermatozoides (Dacheux *et al.* 2005). El fluid epididimari confereix als gàmetes un ambient semblant al que ofereix el plasma sanguini als teixits cel·lulars. La presència de la barrera hematotesticular i hematoepididimària fa que la majoria de proteïnes sanguínies no es trobin al plasma epididimari (albúmina, transferrina i altres proteïnes concretes en són una excepció) (Dacheux *et al.* 2009). A diferència de les concentracions constants de proteïnes observades al plasma sanguini, la concentració proteica del fluid epididimari varia significativament al llarg del conducte: entorn a 2-4 mg/ml de proteïna al segment inicial del conducte, 50-60 mg/ml al caput distal i 20-30 mg/ml al corpus i al cauda (Syntin *et al.* 1996, Fouchecourt *et al.* 2000). Centenars de proteïnes epididimàries han estat descrites mitjançant electroforesi, però només un 10% d'aquestes han pogut ser identificades. Al fluid epididimari, s'ha observat que el 60-80% del total de la concentració proteica correspon només a unes 15-20 proteïnes (revisat a (Dacheux *et al.* 2009)). Les proteïnes més comunes trobades al fluid epididimari són la lactoferrina (LF), la procatespsina D (pCD), la proteïna colesterol transferasa (CETP), la glutatió peroxidasa (GPx), la beta-N-acetil-hexosaminidasa, la manosidasa, la galactosidasa, la prostaglandina D2 sintasa (PGDS), la proteïna CRISP (cystein-rich sectory protein) i la proteïna epididimària d'unió a àcid retinoic (E-RAPB). La composició d'aquestes proteïnes canvia contínuament al llarg del conducte, la lactoferrina, la manosida, la PGDS i albúmina són presents en elevades concentracions en cavalls, porcs, bocs i humans, respectivament, mentre que la GPx i la PGDS són absents en humans i porcs, respectivament. La majoria de les proteïnes epididimàries es caracteritzen per tenir nombroses isoformes resultants dels diferents graus de glicosilació als que són sotmeses. És probable que la major part de les proteïnes epididimàries que envolten els gàmetes estiguin més involucrades a la preservació dels espermatozoides que en la inducció de modificacions específiques. L'elevada presència de proteïnes involucrades en la protecció contra l'estrès oxidatiu, com ara la GPx5, la tioredoxina peroxidasa (TPx), la glutatió S-transferasa P (GST-P) i la superòxid dismutasa (SOD) contribueixen significativament a la supervivència de l'espermatozoide durant el seu emmagatzematge al cauda epididimari (revisat a (Dacheux *et al.* 2009)).

3.5. Contribució dels òrgans accessoris

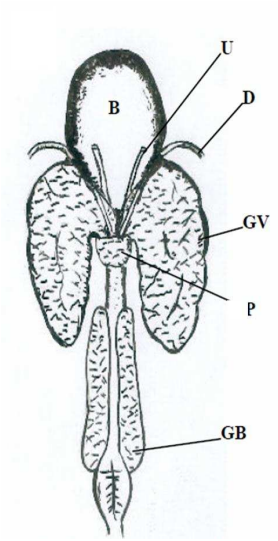

Figura 6. Representació esquemàtica de les glàndules accessòries en porcs. Bufeta urinària (B); vas deferent (D), glàndula bulbouretral (GB); glàndula vesicular (GV), pròstata (P) i Uretra (U).

Durant l'ejaculació, el semen es forma amb les secrecions alliberades per les vesícules seminals, la pròstata i les glàndules de Cowper's (o bulbouretrals) (Figura 6). Les **vesícules seminals** són un parell d'estructures localitzades dorsalment a l'àrea trigonal de la bufeta urinària. Descarreguen un producte ric en fructosa a la uretra mitjançant els conductes ejaculadors en humans i directament a la uretra en porcí. La **pròstata** envolta la base de la uretra i allibera el seu contingut a la uretra durant l'ejaculació a través de nombrosos conductes petits. La pròstata secreta un fluid clar, lleugerament àcid i ric amb àcids fosfatats, àcid cítric, zinc i nombrosos enzims proteolítics. Les glàndules **bulbouretrals** o de **Cowper** aboquen les seves secrecions al bulb uretral. Confereixen una funció lubricant al fluid que protegeix la mucosa de la uretra i neutralitza els residus de l'orina. La principal funció de les secrecions de les vesícules seminals i la pròstata són el transport dels espermatozoides en el moment de l'ejaculació, però també contribueixen breument en el seu metabolisme (Mastroianni & Coutifaris 1990).

4. Fecundació en mamífers

4.1. Transport dels espermatozoides en el tracte genital femení

En porcí, l'ejaculat està composat per tres fraccions diferents i consecutives (Sancho 2002, Yeste 2008, Casas 2010): 1) la fracció **pre-espermàtica**, de 25 cc, té una aparença clara o transparent i una olor forta. Està formada per secrecions de la pròstata, i serveix per eliminar restes d'orina i bacteris de la uretra. Aquesta fracció es caracteritza per no contenir espermatozoides; 2) la fracció **espermàtica** o fracció **rica** presenta una aparença blanca i conté una elevada concentració d'espermatozoides entorn a $0.5\text{-}1\cdot10^9$ espermatozoides per ml (80-90% del total d'espermatozoides). Aquesta fracció té entorn a 40-100 cc i conté secrecions procedents de la pròstata i de les vesícules seminals; 3) la fracció **post-espermàtica** o fracció **pobre**, amb un volum entre 10-400 cc, conté secrecions de la pròstata i vesícules seminals. Té una aparença blanca pàl·lida i presenta una baixa concentració espermàtica, per sota de 10^6 espermatozoides per ml. La fracció post-espermàtica presenta components essencials per a l'activació dels espermatozoides que es troben en estat quiescent al cauda epididimari, tot i que sovint aquesta fracció és descartada per tal de prevenir la dilució de la fracció rica a les dosis seminals. Finalment, a la munta natural es pot distingir una altra fracció anomenada fracció **gelatinosa**, de 20-40 cc, la qual té una consistència gelatinosa i conté secrecions mucoses de les glàndules bulbouretrals, implicada en la formació del "tap mucós" que actua en el segellat del volum ejaculat dins del tracte genital femení.

A moltes espècies, incloses el cavall, el gos, el porc i el ratolí, l'eficiència del transport dels espermatozoides fins el tracte reproductor femení és assegurat per la diposició del semen ejaculat directament dins l'úter. En altres espècies com en humans, conills, vaques, ovelles i cabres el semen és dipositat a la part posterior de la vagina, proper a la part més externa del cèrvix, de manera que els espermatozoides han de trobar el camí des de la vagina fins a les parts superiors de tracte reproductor femení per arribar fins als oòcits (revisat a (Suarez & Pacey 2006) (Suarez 2006)).

4.2. Capacitació

Els espermatozoides epididimaris i ejaculats no són capaços de fertilitzar un oòcit. Per tal que es puguin unir a la ZP de l'oòcit i desencadenar la fecundació és necessari que es produeixin una sèrie de processos que desencadenen canvis en la composició i l'estructura de la membrana plasmàtica (revisat a (Yanagimachi 1994) i (Eddy & O'Brien 1994)). Aquest procés té lloc al tracte femení, i va ser descrit per primera vegada simultàniament i de manera independent per Austin i Chang el 1951 (Austin 1951, Chang 1951). Poc després aquest procés va ser anomenat com a "**capacitació**" per Austin (Austin 1952).

Els principals esdeveniments que es donen durant la capacitació inclouen: 1) la desestabilització de la membrana plasmàtica mitjançant l'eliminació o absorció d'elements a la membrana plasmàtica; 2) un increment de l'influx de calci dins de l'espermatozoide conseqüència de la desestabilització de la membrana (Yanagimachi 1994); 3) l'activació del moviment hiperactiu (Yanagimachi 1970) que permet el pas dels espermatozoides a través del fluid oviductal i, 4) l'activació de vies de transducció del senyal

mitjançant segons missatgers (de Lamirande *et al.* 1997). Tots aquests esdeveniments preparen els espermatozoides per a la posterior reacció acrosòmica i la penetració a la ZP de l'oòcit.

In vivo, la capacitació s'inicia després de l'entrada dels espermatozoides al tracte genital femení. No obstant, el lloc d'unió exacte que desencadena el procés varia en funció del lloc on és dipositat el semen. En algunes espècies, com la bovina, les quals el semen és dipositat a la part anterior de la vagina, la capacitació comença durant la migració dels espermatozoides a través del cèrvix. En d'altres espècies que presenten ejaculació a l'úter, la capacitació espermàtica s'inicia a l'oviducte (Revisat a Bosch i Wright, 2005). En porcí, la capacitació *in vivo* i l'emmagatzematge dels espermatozoides en els reservoris té lloc a nivell de l'itsmus (Hunter 1981). Els espermatozoides són capacitats al tracte genital femení, i són necessàries unes 5-6 hores per tal que adquireixin l'habilitat de penetrar els oòcits (Polge 1978). Els espermatozoides ejaculats experimenten canvis en la composició lipídica de la seva membrana plasmàtica a traves unions amb components d'unió a lípids presents al tracte genital femení, com ara la pèrdua de residus de colesterol. La pèrdua de contingut en colesterol provoca un increment de la desorganització dels fosfolípids de la membrana, iniciant processos en cadena com ara l'increment del pH intracel·lular, l'entrada d'ions calci i bicarbonat, l'activació dels nivells de mono-adenilciclases, augment dels nivells de cAMP, i la fosforil·lació de proteïnes específiques (Harrison 2004) (Figura 7). Tots aquests processos tenen lloc de forma sincronitzada amb l'ovulació (Hunter & Rodriguez-Martinez 2004). En porcí, només uns quants milers d'espermatozoides són capaços d'arribar a l'itsmus i només uns quants d'ells arriben al punt de fecundació, evitant així problemes de polispèrmia (Hunter 1991).

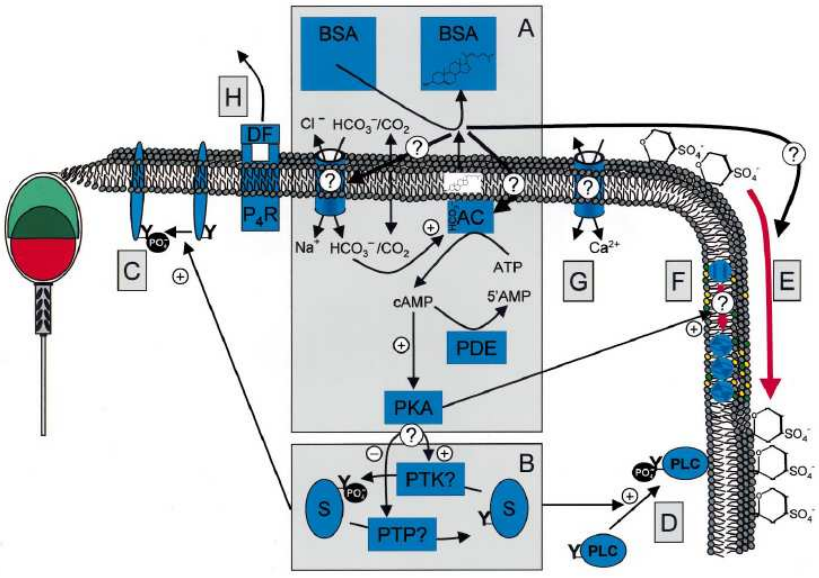

Figura 7. Seqüència d'esdeveniment proposada per la capacitació dels espermatozoides en mamífers. (A) El bicarbonat pot entrar a la cèl·lula espermàtica mitjançant els canals iònics o per difusió en forma de diòxid de carboni. El bicarbonat intracel·lular activa les adenilat ciclasa (AC) i desencadenen la producció de monofosfat d'adenosina cíclic (cAMP) que activa les proteïnes quinases A (PKA). El paper del colesterol en l'activació de les PKA és encara incert. El canvis a la composició de colesterol de la membrana podrien està relacionats en l'entrada d'ions bicarbonat o intervenir directament en l'activació de les AC. (B) Les PKA indueixen la fosforilació de residus tirosina (Y) en molts substrats (S) majoritàriament mitjançant l'activació de PTK o per la inhibició de les tirosina fosfatases presents a la cèl·lula, les proteïnes tirosina fosfatasa (PTP). (C) Les proteïnes d'unió espermatozoide-ZP i altres proteïnes de la membrana plasmàtica esdevenen tirosina fosforilades via bicarbonat-PKA. Aquestes PLC tirosina fosforilades són seguidament translocades a la membrana plasmàtica. (E) L'activació de les PKA indueix canvis a la membrana plasmàtica com ara el redistribució lateral de seminolípids i la translocació de aminofosfolípids. (F) Els aminofosfolípids són translocats de forma PKA depenent. A més, els canvis a la composició de colesterol estan involucrats en les transicions de la membrana plasmàtica. (G) L'entrada de petites quantitats de calci dins la cèl·lula espermàtica tenen un paper clau a la capacitació. (H) Els factors descapacitants (DF) són eliminats de la superfície de la cèl·lula espermàtica, deixant al descobert receptors com els receptors de progesterona (P_4R). Figura obtinguda de (Flesch & Gadella 2000).

4.3. Capacitació *in vitro*

Actualment, és molt difícil avaluar la influència de les cèl·lules oviductals sobre els espermatozoides *in situ* i en el període del cicle reproductiu adequat (Smith 1998, Flesch & Gadella 2000). No obstant, la capacitació potser **induïda *in vitro*** en els espermatozoides epididimaris i ejaculats en medis i condicions físiques específiques (Saravia *et al.* 2007, Ded *et al.* 2010, Puigmulé *et al.* 2011) que mimetitzen la composició d'electròlits del fluid oviductal. Aquests medis de capacitació inclouen normalment una **font energètica** (piruvat, lactat i glucosa), un component proteic (normalment **albúmina sèrica bovina**, BSA), **calci** i **bicarbonat** sòdic. La BSA contribueix a la capacitació *in vitro* actuant com a acceptor del colesterol de la membrana, mentre que el bicarbonat regula diferents funcions espermàtiques que actuen directament sobre la producció de cAMP (Harrison & Miller 2000) i la fosforilació dels residus tirosina de moltes proteïnes (Harrison 2004) (Visconti *et al.* 1995a). De fet, el bicarbonat sembla ser el factor determinat perquè es desencadeni la fosforilació dels residus tirosina de les proteïnes durant la capacitació dels espermatozoides de mamífers (Flesch & Gadella 2000). El calci s'ha descrit que pot actuar de forma sinèrgica amb el bicarbonat a la producció de cAMP (Litvin *et al.* 2003), tot i que el seu paper en la fosforilació dels residus tirosina, en els canvis a la membrana plasmàtica i en l'increment de la motilitat espermàtica encara és desconegut. El bicarbonat té efectes immediats alterant l'arquitectura de la membrana plasmàtica dels espermatozoides de porcí (Harrison *et al.* 1996), i provoca un increment progressiu de la motilitat espermàtica (Holt & Harrison 2002). No obstant, tot i que disposar de protocols definit per desencadenar la capacitació *in vitro* i estudiar els canvis que tenen lloc a la cèl·lula espermàtica durant el procés *in vitro*, no es pot assegurar aquests no presentin lleugeres diferencies en condicions *in vivo*.

4.4. Reacció acrosòmica

La **reacció acrosòmica** (AR) és un procés irreversible d'exocitosi que s'inicia immediatament després de la unió primària de l'espermatozoide capacitat amb l'oòcit. La membrana plasmàtica del cap es fusiona amb la membrana acrosòmica externa, la qual es troba just per sota, en múltiples punts donant lloc a la formació de fenestracions en la membrana plasmàtica per les quals el contingut acrosòmic, que inclou nombrosos enzims hidrolítics i proteolítics, és alliberat (Flesch & Gadella 2000). Els enzims hidrolítics s'encarreguen de disgregar la matriu de la ZP i permetre la penetració de l'espermatozoide a l'espai perivitel·lí (Gadella *et al.* 2001). El contingut acrosomic és alliberat per exocitosi mitjançant un procés mediat per calci (requereix d'un increment massiu dels nivells de calci intracel·lular) com a resposta a senyals específics (Yanagimachi 1994). La membrana plasmàtica del domini equatorial del cap no és fusiona amb la membrana acrosòmica externa i sembla que actua com un lloc específic involucrat en la interacció amb l'oolemma (Flesch & Gadella 2000). No obstant, si la reacció acrosòmica és iniciada de forma primerenca (per exemple abans de la unió de l'espermatozoide a la ZP) els enzims que conté l'acrosoma es perden i l'espermatozoide és incapaç de penetrar la ZP quan es troba amb l'oòcit ni de fertilitzar-lo (Gadella *et al.* 2001) (Figura 8).

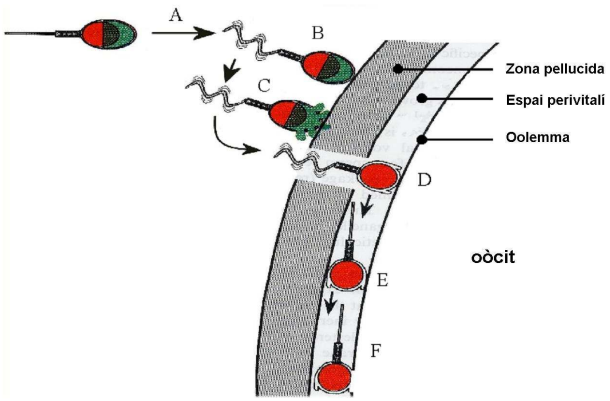

Figura 8. Processos de fertilització en mamífers. Dins el tracte genital femení l'espermatozoide és sotmès a la capacitació (A) que li confereix un moviment hiperactivat (B). L'hiperactivació de la motilitat permet que l'espermatozoide es pugui moure a través de l'epiteli oviductal i li confereix la motilitat necessària per poder penetrar a través de la ZP. L'espermatozoide s'uneix a la ZP (B) i el seu acrosoma reacciona (C) alliberant gran quantitat d'enzims hidrolítics que lisen la ZP, permeten el pas de l'espermatozoide per l'espai perivitel·lí i la seva unió amb l'oolemma, primerament la zona apical (D) i posteriorment a través d'unions laterals (E). Finalment l'espermatozoide es fusiona amb l'oòcit (F). Figura obtinguda de (Flesch & Gadella 2000).

La reacció acrosòmica ***in vitro*** pot ser induïda mitjançant una combinació de calci i ionòfor de calci, els quals indueixen la fusió de la membrana plasmàtica amb les membranes acrosòmiques; les cèl·lules espermàtiques no capacitades presenten una membrana plasmàtica rígida que no permet la fusió d'aquestes membranes sota les mateixes condicions (Cheng *et al.* 1996). S'ha observat que existeixen diferències importants entre la reacció acrosòmica espontània, la induïda amb ionòfor de calci i la que es produeix de forma fisiològica.

4.5. Fecundació

La fecundació es defineix com el procés d'unió de dues cèl·lules germinals, l'oòcit i l'espermatozoide, que dóna lloc a la recuperació del nombre de cromosomes típic de les cèl·lules somàtiques i a la iniciació del desenvolupament d'un nou individu. Els passos finals de l'oogènesi i l'espermatogènesi en mamífers preparen l'oòcit i l'espermatozoide, respectivament, per a la fecundació.

La membrana plasmàtica que recobreix el cap de l'espermatozoide és el loci primari d'interacció amb l'oòcit, i per tant, conté moltes proteïnes funcionalment importants per desencadenar el procés. Cadascuna de les regions de la superfície del cap de l'espermatozoide té una funció específica que permet que aquest pugui fecundar l'oòcit (Gadella *et al.* 2008) (Figura 9). L'espermatozoide dels mamífers interacciona amb l'oòcit en quatre nivells diferents durant la fecundació: 1) a nivell del *cumulus*; 2) a nivell de la ZP induïnt l'exocitosi del contingut acrosòmic; 3) a nivell de la membrana plasmàtica de l'oòcit, iniciada per l'adhesió a la membrana plasmàtica de l'oòcit i que conclou amb la fusió de les membranes de l'espermatozoide i l'oòcit; i 4) a nivell del citoplasma on es produeix la descondensació del nucli espermàtic (revisat a (Evans 2002)). En mamífers, la interacció espermatozoide-oòcit és específica per a cada espècie i inclou nombrosos receptors implicats en la reorganització de les glicoproteïnes de la ZP. La ZP està composada per tres glicoproteïnes altament específiques amb una elevada quantitat de glicans (Revisat a (Detlef *et al.* 2005)). Segons els gens codificants s'han descrit tres tipus de glicoproteïnes, que poden ser processades i modificades posttranscripionalment: ZP1 (~ 200 kDa; dímer), ZP2 (~ 120 kDa) i ZP3 (~ 83 kDa) (Wassarman 1999, Wassarman 2008).

Figura 9. Representació esquemàtica de la seqüència d'interaccions que tenen lloc entre l'espermatozoide i l'oòcit durant la fecundació. (1) Unió de l'espermatozoide a la ZP (zona apical); (2) reacció acrosòmica (fusió de la zona apical i la preequatorial); (3) la penetració de la ZP (la membrana equatorial roman intacte i s'alliberen les vesícules mixtes resultants de la reacció acrosòmica); (4) unió i fusió amb la membrana plasmàtica de l'oòcit (zona equatorial); (5) activació de l'oòcit fecundat mitjançant factors solubles de l'espermatozoide; (6) bloqueig de la poliespèrmia mitjançant la reacció cortical. Figura modificada de Flesch et al., 2000 (Gadella *et al.* 2008)

El reconeixement del gàmeta, així com la seva unió i fusió es donen mitjançant processos regulats molt específicament, que inclouen nombrosos canvis bioquímics. La unió primària entre l'espermatozoide capacitat i intacte amb la ZP inclou moltes molècules especialitzades de la membrana plasmàtica apical del cap de l'espermatozoide: la proteïna lactadherina P47 (P47), les proteïnes d'adhesió a la ZP, galactosiltransferases, proteïna espermàtica de superfície de 56 kDa (sp56) i proteïna de 95 kDa (p95) (Revisat a (Wassarman 1999, Mori *et al.* 2000, Detlef *et al.* 2005)). Aquestes proteïnes reconeixen i s'uneixen a receptors primaris associats a residus carbohidrats *O-linked* de la ZP (ZP3 glicoproteïnes). Aquesta unió inicial entre l'espermatozoide i la ZP dóna lloc a la reacció acrosòmica, i conseqüentment a la unió dels lligants secundaris i els receptors presents a la matriu acrosòmica i/o la membrana acrosòmica interna, que queden exposats a la superfície de l'espermatozoide després de la reacció acrosòmica (Wassarman 1999, Howes & Jones 2001, Detlef *et al.* 2005). Aquesta unió secundària també desencadena la motilitat del flagel, la seva hiperactivació i l'acció lítica de la membrana acrosòmica, mentre l'espermatozoide comença a penetrar per la ZP i migra per l'espai perivitel·lí (Bedford 1998). La proacrosina, les proteïnes espermàtiques de 38 kDa (sp38) i 17 kDa (sp17) i la hialuronidasa PH-20 (SPAM-1 o PH-20) han estat descrites com a mediadors de la unió secundària amb les glicoproteïnes ZP2 (Wassarman 1999, Mori *et al.* 2000, Detlef *et al.* 2005). L'acrosina i la PH-20 a més de tenir afinitat per la ZP, presenten activitats proteïnasa i hialuronidasa (Urch & Patel 1991, Gmachl *et al.* 1993).

Finalment, la unió terciària i fusió inclouen la participació d'un tercer tipus de receptors espermàtics, com els receptors per les integrines alfa-6-beta-1 de l'oòcit i la fertilina (PH-30 alfa/beta o ADAM-1/2), així com integrines o receptors C9 de l'oolemma (Howes & Jones 2001). També s'han descrit proteïnes de la membrana acrosòmica interna implicades en el procés, com ara la ciristestina 1 (ADAM-3) de la matriu acrosòmica (Forsbach & Heinlein 1998).

La ZP de l'oòcit porcí està formada també per un grup específic de tres glicoproteïnes anomenades ZPA (~ 90-70 kDa), ZPB (~55-59 kDa) i ZPC (~55-46kDa) (Harris *et al.* 1994, Mori *et al.* 2000, Sinowatz *et al.* 2001). En base a la seqüència de la ZPA, la ZPB i la ZPC aquestes són homologues a les glicoproteïnes ZP2, ZP1 i ZP3 descrites en ratolí, respectivament (Harris *et al.* 1994). A més, a l'oòcit porcí s'han descrit associacions d'heterocomplexes formats per l'associació de ZPB-ZPC (Yurewicz *et al.* 1998). La ZPC i la ZPB tenen activitats d'unió independents, però els heteròmers ZPB-ZPC esdevenen funcionals i estructuralment units a la matriu extracel·lular amb una gran afinitat per les molècules presents a la membrana de l'espermatozoide porcí (Yurewicz *et al.* 1998, Sinowatz *et al.* 2001). Diversos assajos d'unió a la ZP han mostrat que les proteïnes d'unió es troben concentrades a la zona apical de l'acrosoma de l'espermatozoide. Aquests estudis també mostren que els espermatozoides procedents del corpus epididimari ja són capaços d'unir-se i fecundar l'oòcit (Burkin & Miller 2000).

5. Composició de l'espermatozoide

5.1. Composició proteica

L'espermatozoide és una cèl·lula molt polaritzada i especialitzada amb un baix contingut citosòlic i pocs orgànuls (Eddy & O'Brien 1994, Yanagimachi 1994), però amb abundants proteïnes. L'espermatozoide no conté reticle endoplasmàtic, aparell de Golgi, lisosomes, peroxisomes o ribosomes, i per tant perd el seu potencial d'expressió gènica i tant la transcripció com la traducció es troben silenciades (Boerke *et al.* 2007).

Milers de proteïnes constitueixen el contingut proteic de les cèl·lules espermàtiques, tal i com demostra la identificació proteòmica de les proteïnes de l'espermatozoide humà realitzat per Martínez-Hereida (Martínez-Heredia *et al.* 2006). Entre aquestes proteïnes s'inclouen una gran quantitat de proteïnes del flagel (revisat a (Oliva *et al.* 2008, Oliva *et al.* 2009)), com ara la tubulina (Mohri 1968), proteïnes acrosòmiques (Tanii *et al.* 2001, Yoshinaga *et al.* 2001) com l'acrosina (Brown & Harrison 1978, Puigmulé *et al.* 2011), proteïnes mitocondrials (NagDas *et al.* 2005, Aitken *et al.* 2007), proteïnes del citoesquelet (Shaman *et al.* 2007), proteïnes nuclears, entre les quals les protamines són les més abundants (Felix 1960, Oliva 2006) i proteïnes de membrana (Peterson & Russell 1985, Hunnicutt *et al.* 1996).

5.2. Composició de la membrana plasmàtica

La membrana plasmàtica és una estructura molt dinàmica (revisat a (Flesch & Gadella 2000)). Durant el trànsit dels espermatozoides per l'epidídim, la composició de la membrana plasmàtica canvia amb processos de redistribució, modificacions i absorcions de proteïnes i lípids. La distribució lateral polaritzada descrita a la membrana plasmàtica dels espermatozoides, així com els estudis de fraccionament per congelació indiquen que cada domini conté una concentració i distribució de proteïnes transmembrana diferents (Flesch & Gadella 2000). Aquesta reorganització també té lloc durant el pas dels espermatozoides pel tracte genital femení per tal que acabin d'adquirir la capacitat per poder fecundar l'oòcit.

Composició lipídica i implicacions a la superfície espermàtica

La superfície de membrana dels espermatozoides madurs no està en contacte amb les membranes intracel·lulars ja que el transport mitjançant vesícules es troba bloquejat. L'única excepció es dóna quan la membrana plasmàtica apical es fusiona amb la membrana acrosòmica externa, durant la reacció acrosòmica. La composició i organització inusual dels lípids a la membrana plasmàtica és un reflex de les propietats específiques de l'espermatozoide (revisat a (Flesch & Gadella 2000)). Tot i que hi ha variacions considerables entre els diferents mamífers, en general la membrana plasmàtica dels espermatozoides conté aproximadament un 70% de fosfolípids, un 25% de lípids neutres i un 5%

glicolípids (en base molar); el factor més variable entre els mamífers és la quantitat de colesterol que conté la membrana plasmàtica. En humans, els espermatozoides contenen altes quantitats de colesterol (40 % del total de lípids en base molar), mentre que en porcí els espermatozoides contenen menys colesterol (només un 22%) (Mann & Lutwak-Mann 1982). Nivells baixos en la relació colesterol/fosfolípids, així com un alt contingut en fosfolípids insaturats, fan que la membrana plasmàtica del espermatozoides porcins sigui particularment propensa a formar fases gelatinoses (Parks & Lynch 1992). Aquesta composició particular de la membrana plasmàtica és possiblement una conseqüència del procés de maduració epididimària dels espermatozoides, durant el qual disminueix el contingut de colesterol de les membranes espermàtiques (Nikolopoulou *et al.* 1985). S'ha demostrat que aquesta composició fa a l'espermatozoide porcí especialment sensible al xoc fred durant la maduració epididimària i després de l'ejaculació (Simpson *et al.* 1987).

Composició proteica i implicacions a la superfície espermàtica

Les proteïnes de la membrana plasmàtica tenen una funció essencial per les cèl·lules, interaccionant amb components cel·lulars i extracel·lulars, estructures i molècules de senyalització (Josic & Clifton 2007).

El procés de maduració de les proteïnes de la superfície espermàtica és molt actiu i específic per a cada funció: 1) les proteïnes es localitzen en dominis específics, com ara l'ADAM2 o la PH-20 que són processades i relocalitzades (Primakoff *et al.* 1985, Overstreet *et al.* 1995, Blobel 2000) perquè puguin participar activament en la interacció oòcit-espermatozoide i la fecundació (Jury *et al.* 1997, McLaughlin *et al.* 1997, Waters & White 1997); 2) algunes proteïnes secretades per les cèl·lules epitelials de l'epidídim s'adhereixen a la superfície espermàtica durant el pas per l'epidídim (revisat a (Gupta 2005a)), com ara les proteïnes epididimàries de ratolí (MEPs) o l'antigen de maduració espermàtica 4 (SMA-4) en ratolí, la proteïna secretora específica d'epidídim 3 (E-3) en rates, la proteïna epididimària de hàmster 64 (HEP64) o la proteïna espermàtica de hàmster de 26 kDa (P26h) en altres rosegadors, i la proteïna 135 kDa en grans animals domèstics (Okamura *et al.* 1992); 3) finalment, altres proteïnes són alliberades de proteïnes d'ancoratge al medi epididimari, com ara l'enzim conversiu de l'angiotensina I (ACE) (Gatti *et al.* 1999).

A més, durant la capacitació espermàtica l'organització proteica i lipídica de la membrana plasmàtica també canvia severament com a resultat de la capacitació i la reacció acrosòmica, a fi de poder unir-se posteriorment amb la ZP (Gadella *et al.* 2008).

Glicocàlix

La superfície de l'espermatozoide dels mamífers es troba recobert d'una capa densa de molècules riques en carbohidrats d'uns 20-60 nm de gruix anomenada **glicocàlix**. El glicocàlix comprèn els residus carbohidrats que s'uneixen als lípids i a d'altres estructures moleculars. Aquestes estructures poden ser intercalades o ancorades a la doble membrana lipídica, o associats superficialment amb la membrana mitjançant grups polars o interaccions hidrofòbiques (Schroter *et al.* 1999). A més, la major part dels residus glicosídics que es troben units a les proteïnes estan també integrats en la membrana espermàtica o estan més o menys associats a ella. S'estima que el glicocàlix està format per centenars de glicoproteïnes

diferents, sintetitzades als testicles o a nivell post-testicular per l'epiteli dels conductes eferents, de l'epidídim i per part de les glàndules accessòries.

L'adquisició d'un glicocàlix madur és important per a l'adquisició de la capacitat fecundant en els espermatozoides. El glicocàlix té un paper destacat en la comunicació intracel·lular del gàmeta ja que forma la superfície que embolcalla l'espermatozoide (Flesch & Gadella 2000). Fins que s'estableixi de forma completa l'estructura del glicocàlix, la seva funció romandrà incerta, tot i que es creu que està relacionada amb la maduració de la membrana i la immunoprotecció dels espermatozoides al tracte genital femení, també se li atribueix un paper destacat en la unió de l'espermatozoide a la ZP i en la fecundació (Schroter *et al.* 1999).

La majoria (~92 %) de les proteïnes de la membrana plasmàtica de les cèl·lules són glicosilades. L'oòcit també es troba recobert per una matriu glicosilada, la ZP, que difereix del glicocàlix de l'espermatozoide pel seu gruix (16 µm en porcs) i per la seva complexitat. El glicocàlix de l'oòcit està format només per tres glicoproteïnes principals (ZP1, ZP2 i ZP3), mentre que el glicocàlix de l'espermatozoide conté entre 50 i 150 glicoconjugats diferents (Schroter *et al.* 1999).

Les lectines són proteïnes normalment d'origen vegetal capaces de reconèixer estructures oligosacàrides específiques (Taula 1). La conjugació de les lectines amb fluorocroms o biotina permet l'estudi dels sucres de la superfície de les cèl·lules i l'observació de canvis en la composició glicosídica durant el creixement cel·lular, la diferenciació o en casos de metàstasi (Lis & Sharon 1998) i, també són una eina útil per a la caracterització de les estructures dels residus carbohidrats presents a les glicoproteïnes, ja que poden discriminar diferents glicoconjugats a la superfície de l'espermatozoide.

Els estudis amb lectines han permès la descripció dels motius glicoproteics presents a la membrana plasmàtica i les modificacions que es donen durant el trànsit epididimari per part de les fucosiltransferases i les beta-D-galactosidases presents al fluid epididimari (Gupta 2005a).

Taula 1. Afinitat especifica de les lectines pels grups carbohidrats.

Lectina	Especificitat	Sucre inhibidor	Grup carbohidrat
Arachis hypogaea **(Peanut) agglutinin (PNA)**	β-Gal	Lactosa (200 mM)	**Gal**
Lens Culinaris **Agglutinin (LCA)**	α-Man; α-Glc	Metil α-D-manopiranosida (200 mM)	**Glc/man**
Pisum Sativum **Agglutinin (PSA)**	α-Man; α-Glc	Methyl α-D-mannopyranoside (200 mM)	
Wheat germ agglutinin (WGA)	β-GlcNAc ; Neu5Ac	N-Acetyl-D-glucosamine (500 mM)	**GlcNAc**
Helix pomatia **Agglutinin (HPA)**	α-GalNAc	N-Acetil-D-galactosamina (200 mM)	**GalNAc**
Phytohemagglutinin-L (PHA-L)	Gal ; GalNAc	N-Acetil-D-glucosamina (500 mM)	
Glycine max **(Soybean) agglutinin (SBA)**	α- or β-GalNAc	N-Acetyl-D-galactosamine (200 mM)	
Ulex europaeus Agglutinin I (UEA-I)	α-Fuc	L-fucosa (500 mM)	**Fuc**

Abreviacions: Galactosa (Gal); Glucosa (Glc); Manosa (Man); N-acetilglucosa (GlcNAc); N-acetilgalactosa (GalNAc); Fucosa (Fuc); B-galactosa (β-Gal) ; α-D-manosa (α-Man); α-D-glucosa (α-Glc); β-N-acetil-D-glucosa (β-GlcNAc); àcid N-acetilneuraminic (Neu5Ac) ;α or β N-acetil-galactosamina (α- or β-GalNAc); α-fucosa (α-Fuc).

5.3. Enzim conversor de l'angiotensina I (ACE)

L'enzim conversor de l'angiotensina I (ACE; EC 3.4.15.1) és una proteïna zinc-metalopeptidasa ancorada a la membrana plasmàtica que converteix els dipèptids C-terminals de molts substrats oligopeptídics,

com l'angiotensina circulatòria I, la bradiquinina i el pèptid hemoregulatori N-acetil-SDKP. Aquest enzim normalment es relaciona amb la regulació de la pressió sanguínia, tot i que alguns estudis han demostrat que té un paper important en la reproducció (revisat a (Sabeur *et al.* 2001)). S'ha observat la presència d'aquest enzim als espermatozoides (gàmeta ACE, gACE) i s'ha establert que té un paper important en la fecundació. En mascles de rates *knockout* per l'enzim gACE s'ha observat que l'absència d'aquest enzim provoca subfertilitat. Tot i que no s'ha pogut establir amb precisió com actua aquest enzim en la fertilitat masculina s'ha postulat un possible paper en la formació de l'estructura i del remodelatge de la membrana espermàtica (Esther *et al.* 1997, Gatti *et al.* 1999, Metayer *et al.* 2002, Thimon *et al.* 2005) .

Els mascles de mamífers presenten dues isoformes de l'enzim convertidor de l'angiotensina, la isoforma somàtica i la germinal. Ambdues isoformes estan codificades pel mateix gen (Metayer *et al.* 2001). La isoforma **somàtica (sACE)** té un pes molecular aproximat de 140-180 kDa, s'expressa en nombrosos teixits i té un paper destacat en el control de la pressió sanguínia actuant sobre els pèptids de bradiquinina i l'angiotensina. La seva seqüència esta formada per dos dominis redundants (dominis N- i C-terminals) que contenen un lloc d'unió a zinc i un centre funcional catalític (revisat a (Metayer *et al.* 2001)). La isoforma **germinal (gACE)** s'expressa a les cèl·lules germinals masculines haploides per l'activitat específica d'un promotor testicular situat al dotzè intró del gen somàtic (Gatti *et al.* 1999). Aquesta isoforma germinal de 90-110 kDa es troba restringida al domini C-terminal del sACE, i inclou el punt hidrofòbic d'ancoratge a la membrana (que permet la inserció a la membrana cèl·lular) i una curta cua citoplasmàtica, però que té una seqüència específica de 60 aminoàcids en el domini N-terminal i només un del dos centres catalítics i llocs d'unió a zinc (Gatti *et al.* 1999, Metayer *et al.* 2001, Thimon *et al.* 2005) (Figura 10).

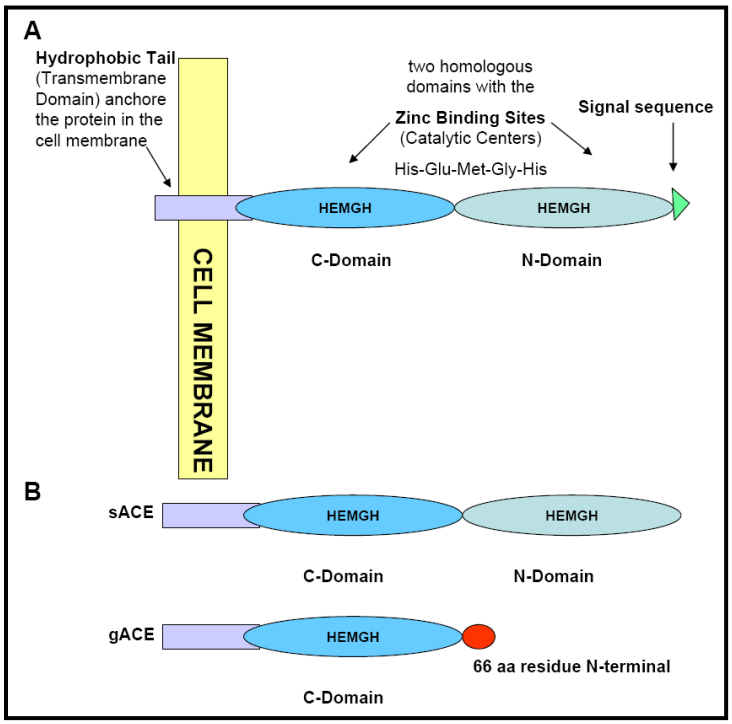

Figura 10. Estructura de l'enzim convertidor de l'angiotensina (ACE). A. Forma somàtica de l'ACE formada per dos dominis homòlegs flanquejats per una seqüència senyal i un domini hidrofòbic transmembrana, el qual ancora la proteïna a la membrana cel·lular. Cadascun dels dominis homòlegs conté un lloc d'unió a zinc consens His-Glu-Met-Gly-His (HEMGH), el qual es pot unir a una molècula de zinc i que actua com a centre catalític. La seqüència senyal és alliberada durant el processament i no es troba present a la forma madura de l'ACE. **B.** Estructura de la forma somàtica (sACE) i de la forma testicular (gACE) . La forma gACE conté només el domini C-terminal de la sACE i una seqüència addicional de 66 aminoàcids específica. Figura adaptada de (Esther *et al.* 1997).

Tant la sACE com la gACE són formes solubles produïdes per una associació específica a la membrana i un processament que provoca l'alliberament del domini extracel·lular d'aquest enzim (Metayer *et al.* 2001, Metayer *et al.* 2002). La forma soluble present a

l'epidídim i al plasma seminal gACE deriva de la forma present a la membrana plasmàtica alliberada en aquests fluids mitjançant un procés proteolític del domini extracel·lular (Gatti *et al.* 1999, Metayer *et al.* 2002, Thimon *et al.* 2005). La proteïna de 94-105 kDa és alliberada de l'espermatozoide al caput proximal en animals domèstics (Gatti *et al.* 1999) i en rosegadors (Metayer *et al.* 2002). Aquesta forma alliberada és la responsable de l'activitat ACE detectada al fluid epididimari (Gatti *et al.* 1999). L'alliberació localitzada d'ACE als espermatozoides epididimaris suggereix la necessitat d'un ambient específic per tal que tingui lloc aquest processament (Gatti *et al.* 1999). El canvi de pes molecular de 105 KDa a 94 kDa pot atribuir-se a modificacions posteriors de l'enzim secretat com ara en el seu estat de glicosilació o a la pèrdua del punt d'ancoratge a la membrana plasmàtica (Gatti *et al.* 1999, Metayer *et al.* 2002). Una altra forma de 10 kDa pot ser observada en el fluid i correspon als dominis transmembrana i intracel·lulars dels gACE derivats del processament proteolític de les serina proteases durant el trànsit per l'epidídim (Gatti *et al.* 1999, Thimon *et al.* 2005).

La immunolocalització de l'enzim ACE mostra un marcatge intens a la peça intermèdia i difús a la regió acrosòmica dels espermatozoides testiculars i al principi del caput epididimari. En espermatozoides de zones més distals del caput, corpus i cauda, així com en espermatozoides ejaculats, l'enzim ACE es localitza a la zona apical de l'acrosoma (Gatti *et al.* 1999).

No totes les formes del gACE testicular són alliberades dels espermatozoides durant la maduració epididimària. Alguns estudis mostren restes d'activitat i de marcatge a la regió acrosòmica dels espermatozoides de totes les regions epididimàries i dels espermatozoides ejaculats (Kohn *et al.* 1998), indicant que la localització intracrosòmica podria protegir l'ACE de l'activitat proteolítica i del seu alliberament al fluid epididimari (Hagaman *et al.* 1998, Gatti *et al.* 1999)

5.4. Fertilina (PH-30 o ADAM-1 i ADAM-2)

La fertilina és una glicoproteïna heterodimèrica transmembrana (~75 kDa), membre de la família de les proteïnes ADAMs que es localitza a la part posterior del cap dels espermatozoides madurs de conill d'Índies (Blobel *et al.* 1990, Blobel 2000) (Figura 11).

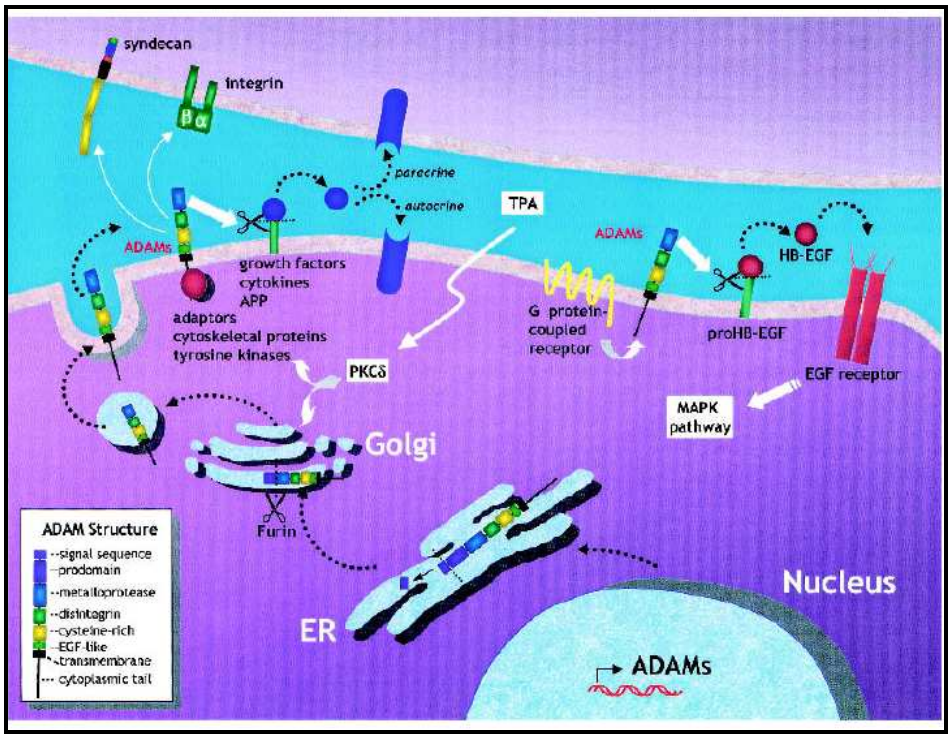

Figura 11. Esquema de la síntesi, processament i funcions de la família de proteïnes ADAMs. Nombroses activitats de les proteïnes ADAMs es troben esquematitzades en aquesta figura, però no totes les descrites per aquesta família. Figura obtinguda de (Seals & Courtneidge 2003).

La família de proteïnes ADAMs pertany a la superfamília de les zinc proteases, que tenen un paper essencial en la fecundació i el desenvolupament cel·lular, però que també participen en nombrosos processos biològics com ara el processos cancerosos, les imflamacions i la neurogènesi (Primakoff & Myles 2000, Seals & Courtneidge 2003). Les proteïnes de la família ADAM presenten dominis multifuncionals prodominis, dominis metalloproteasa, dominis desintegrina, dominis rics en cisteïnes, dominis semblants a factors de creixement epidèrmic (*EGF-like*) i dominis citoplasmàtics els quals es creu que donen suport a diferents funcions (Seals & Courtneidge 2003) (Figura 12).

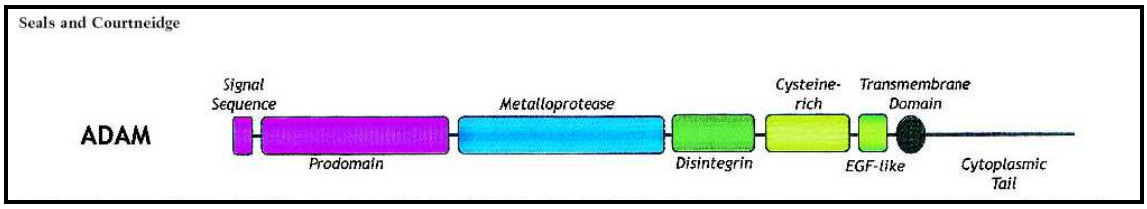

Figura 12. Representació gràfica de les ADAMs. Estructura dels dominis generals que conformen la família de les proteïnes ADAMs: prodomini, domini metaloproteasa, domini desintegrina, domini ric en cisteina, domini *EGF-like*, domini transmembrana i domini de la cua citoplasmàtica. Figura obtinguda de (Seals & Courtneidge 2003).

La fertilina és una proteïna essencial per a la migració de l'espermatozoide a través de l'oviducte, per a la unió a la ZP, i per a la iniciació de la fecundació de l'espermatozoide durant la interacció espermatozoide-oòcit (Primakoff & Myles 2000, Seals & Courtneidge 2003). Està formada per dues subunitats estretament associades i immunològicament diferenciades, la subunitat **alfa** (subunitat-α o ADAM1) de ~45 kDa i la subunitat **beta** (subunitat-β o ADAM2) de ~ 27 kDa (Blobel *et al.* 1990, Janice 2001) (Figura 13).

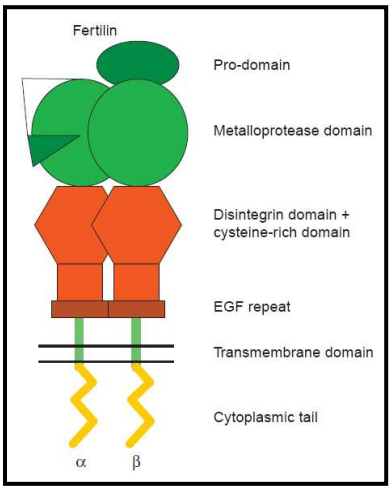

Figura 13. Model de l'estructura heterodimèrica de la fertilina proposada per Bobel, 1990. Figura obtinguda de (Blobel 2000).

Les subunitats de la fertilina es formen a través de precursors que són processats en diferents estadis del desenvolupament. La subunitat-α és processada primer a nivell testicular (Blobel *et al.* 1990), mentre que la subunitat-β és processada durant el pas dels espermatozoides pel conducte epididimari, entre el corpus distal i el cauda proximal, moment en què els espermatozoides adquireixen la motilitat i la competència per fecundar (Van Gestel *et al.* 2007).

En conills d'índies, el pes molecular aparent és d'uns 45 kDa per la subunitat-α i d'uns 27 kDa per la subunitat-β (Blobel *et al.* 1990). Pel que fa als espermatozoides de *Macaca fascicularis* s'ha descrit un pes molecular de 47 kDa per la subunitat-β madura i d'uns 100 KDa pel seu precursor (Kim *et al.* 2009) (Figura 14). La subunitat-β de la fertilina té un paper essencial en la fecundació, ja que s'ha associat al procés de fusió dels gàmetes mitjançant les regions riques en cisteïna, semblant a la fusió viral de pèptids (Janice 2001). En ratolins *knockout* per a l'expressió de β-fertilina s'ha demostrat que els passos d'adhesió (a través del domini desintegrina) i de fusió són defectius, per bé que aquests ratolins encara són fèrtils tot i la poca capacitat d'unió a la ZP (Seals & Courtneidge 2003, Van Gestel *et al.* 2007). La β-fertilina és creu que és proteolíticament inactiva fins que el prodomini i el domini metalloproteasa són alliberats. Aquest alliberament té lloc un cop l'espermatozoide ha adquirit la competència per fecundar i l'activitat proteasa és necessària (revisat a (Seals & Courtneidge 2003)).

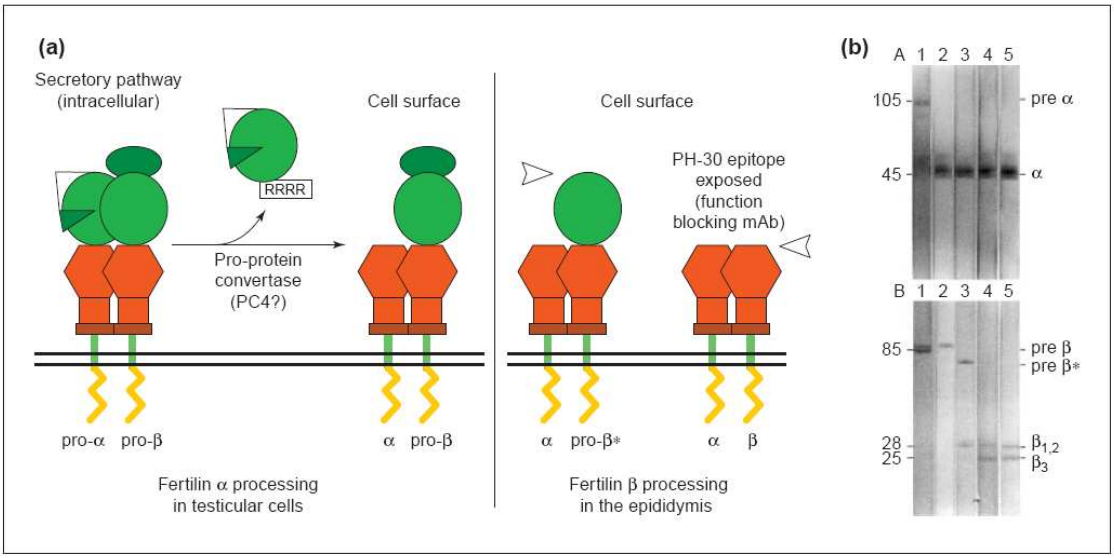

Figura 14. Processament proteolític de la fertilina durant la maduració espermàtica al testicle i l'epidídim. Figura obtinguda de (Blobel 2000)

Les dues subunitats de la fertilina es troben directament associades a través de la unió d'un dels seus dominis però es desconeix a través de quin. Tot i que la subnitat-α no és funcional per si sola en alguns primats, l'existència del complex fertilina encara genera resultats controvertits ja que l'eliminació de la subunitat-α evita la presentació de la subunitat-β a la superfície espermàtica, i per tant es creu que pot tenir un paper important en la formació del complex fertilina a la superfície espermàtica (Kim *et al.* 2009).

En conill d'índies, la immunolocalitzacio de la fertilina també difereix al llarg de l'epidídim. El processament proteolític de la fertilina es troba associat a la seva redistribució a nivell de la superfície espermàtica; en els espermatozoides testiculars, la fertilina es troba distribuïda en tota la superfície espermàtica, però durant el trànsit per l'epidídim migra cap a la part posterior del cap de l'espermatozoide (Blobel 2000).

5.5. Proteïnes tirosina fosforilades

La fosforilació de residus tirosina és una de les modificacions post-transcripcionals de les proteïnes que permeten a la cèl·lula controlar varis processos. L'estat de fosforilació de les proteïnes és controlat per l'activitat de les proteïnes quinases i fosfatases (Urner & Sakkas 2003). A l'espermatozoide de mamífers, l'estat de fosforilació és regulat per l'activació de les vies de transducció de la senyal intracel·lulars.

Els processos de fosforilació de residus tirosina estan associats amb la capacitació en moltes espècies, com en ratolí (Visconti *et al.* 1995b), humà (Luconi *et al.* 1996), boví (Galantino-Homer *et al.* 1997), oví (Chatterjee *et al.* 2010) i porcí (Flesch *et al.* 1999). Les proteïnes tirosina fosforilades regulen les funcions espermàtiques en mamífers, com ara l'hiperactivació de la motilitat (Vijayaraghavan *et al.* 1997) (Nassar *et al.* 1999), la unió a la ZP (Pukazhenthi *et al.* 1998), la reacció acrosòmica (Pukazhenthi *et al.* 1998) i la fusió espermatozoide-oòcit (Urner & Sakkas 2003, Naz & Rajesh 2004).

Els espermatozoides capacitats en humans, primats, hàmsters, rates i ratolins (revisat a (Naz & Rajesh) presenten un marcatge intens de residus tirosina fosforilats al flagel. En aquestes espècies, la fosforilació de proteïnes de la cua és dóna a la beina fibrosa, suggerint la seva implicació en la hiperactivació del moviment, associat a la capacitació i/o a la reacció acrosòmica (Tardif *et al.* 2001). Pel que fa als espermatozoides de porcí la capacitació també sembla estar associada a la fosforilació de residus tirosina de certes proteïnes, però no s'ha pogut relacionar amb canvis en la motilitat espermàtica (Bailey *et al.* 2005).

La maduració epididimària i els esdeveniments primerencs per adquirir la funcionalitat espermàtica estan també implicats amb patrons de fosforilació de residus tirosina (Tardif *et al.* 2001). En rates la maduració epididimària comporta patrons de desfosforilació proteica (Lewis & Aitken 2001), mentre que en ratolins l'espermatozoide adquireix la capacitat de fosforilar tota la cua a mesura que es prepara per generar el moviment hiperactivat (Aitken *et al.* 2007). A més, la maduració epididimària comporta canvis en els patrons de fosforilació dels residus tirosina de l'acrosoma, s'observa com aquests passen d'ocupar la totalitat de la regió acrosòmica en els espermatozoides del caput, a concentrar-se a la regió posterior de la vesícula acrosòmica en els espermatozoides del cauda (Lewis & Aitken 2001).

S'han descrit nombroses proteïnes que presenten canvis en la fosforilació dels seus residus tirosina durant la maduració espermàtica i/o capacitació. Estudis de capacitació *in vitro* d'espermatozoides porcins (Kalab *et al.* 1998) han identificat certes proteïnes amb residus tirosina fosforilats de forma constitutiva (p44, p40, p38 i p34), mentre que en d'altres s'ha observat que incrementa el seu grau de fosforilació després de la capacitació *in vitro*, com en el cas de la p32 (Tardif *et al.* 2001). Aquesta proteïna probablement correspon a la proteïna de 34 kDa amb residus tirosina fosforilats identificada

posteriorment per Kalab (Kalab *et al.* 1998) i Flesh (Flesch *et al.* 1999). La p32 possiblement presenta una activitat semblant a la tirosina quinasa; es troba a la fracció citosòlica de l'espermatozoide porcí, i només apareix a la membrana un cop desencadenada la capacitació (Tardif *et al.* 2001, Bailey *et al.* 2005).

Una altra proteïna amb residus tirosina fosforilats és la p21, prèviament descrita per Dubé et al., (Dubé *et al.* 2004) com l'enzim glutatió peroxidasa (PHGPx) porcí (Esworthy *et al.* 1994) i en espermatozoides capacitats de hàmster com una proteïna de 19 kDa (NagDas *et al.* 2005). La PHGPx és una selenopoteina i un enzim clau en la protecció de les biomembranes exposades a l'estrès oxidatiu (Imai & Nakagawa 2003); en els espermatozoides capacitats és present en forma de component ancorat a la membrana de la beina mitocondrial (NagDas *et al.* 2005). Les implicacions fisiològiques i moleculars de la PHGPx són poc conegudes, no obstant dades prèvies suggereixen que la fosforilació de residus tirosina de la PHGPx poden representar un procés important en la cascada de senyalització associada a la capacitació i que regula la hiperactivacio de la motilitat i/o la funció mitocondrial (NagDas *et al.* 2005).

6. Citometria de flux

Fonaments de la citometria de flux

La citometria de flux té com a finalitat la mesura de determinades característiques físiques i químiques de cèl·lules o partícules biològiques en suspensió. Entre les característiques físiques, els citòmetres proporcionen una estimació de la mida i la complexitat, a través de la dispersió de la llum que provoquen les partícules en impactar contra el làser. Les característiques químiques s'analitzen majoritàriament a través del marcatge amb fluorocroms, els quals s'han de seleccionar en funció de l'equip que es disposa i de les aplicacions que se'ls vulgui donar.

Els citòmetres de flux consten bàsicament d'una **font de llum** (làser o làmpada d'arc), un **sistema de flux cel·lular**, uns **components òptics** per diferenciar i seleccionar les modificacions de la llum, i uns **elements electrònics** capaços d'amplificar i processar el senyal resultant en un ordinador (Figura 15)

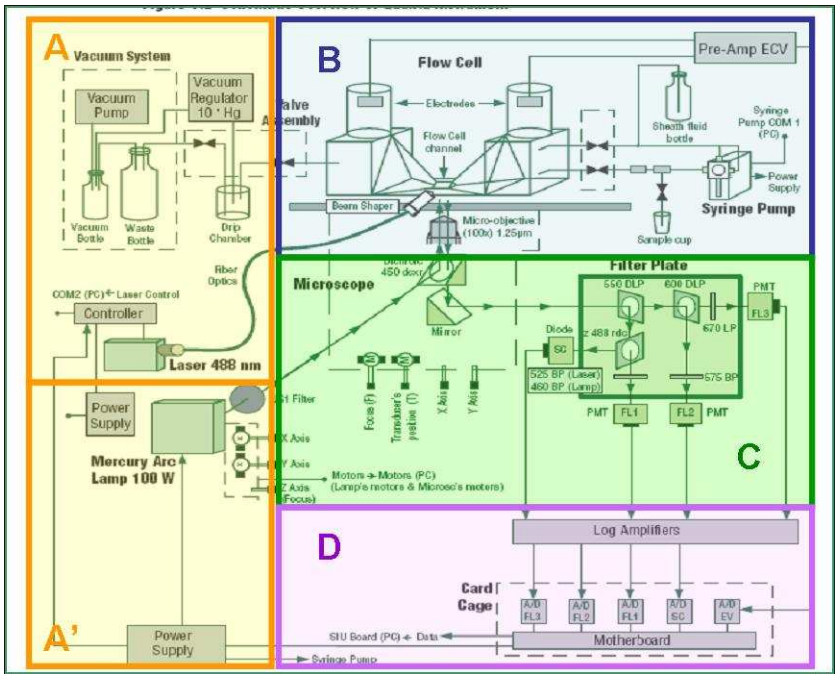

Figura 15. Components d'un citòmetre de flux. Components d'un citometre Beckman coulter Cell Lab Quanta SC: A) font de llum amb un làser i A') una làmpada d'arc, B) sistema de fluid cel·lular, C) components òptics i D) elements electrònics.

Les **fonts de llum** més comunes en citometria són els làsers (light amplification by stimulated emission of radiaton) i les làmpades d'arc. Els làsers generen feixos de llum monocromàtics molt intensos i focalitzats en un petit diàmetre. Els equips més avançats estan dotats de fins a tres o quatre làsers, tot i que els equips més comuns són els que disposen d'un únic làser de gas d'argó que emet a 488nm (espectre del blau). Les **làmpades d'arc**, a diferència dels làsers, no produeixen llum monocromàtica i requereixen d'un complex sistema òptic per focalitzar la llum en un punt del flux.

El **flux cel·lular** es basa en un sistema hidràulic que permet que les cèl·lules o partícules de la suspensió passin individualment davant la font de llum i es focalitzin al centre del fluid per evitar obturacions. Per tal que això sigui possible, és indispensable que el flux sigui laminar tal com descriu el principi de Reynolds de 1883. La generació del flux laminar s'aconsegueix gràcies al diàmetre del capil·lar i a la velocitat del líquid envolvent o d'arrossegament. A la cambra de flux definida per Crosland-Taylor el 1953 es produeix aquest enfocament hidrodinàmic a través de la disminució de la secció del tub, basada en el principi de Bernoulli. Segons aquest principi, en disminuir la secció del tub es produeix un augment de la velocitat de fluid i una disminució de la pressió. D'altra banda, l'augment de la velocitat és major al centre del flux de manera que enfoca les cèl·lules cap el capil·lar i permet el pas individual davant la font de llum (Figura 16).

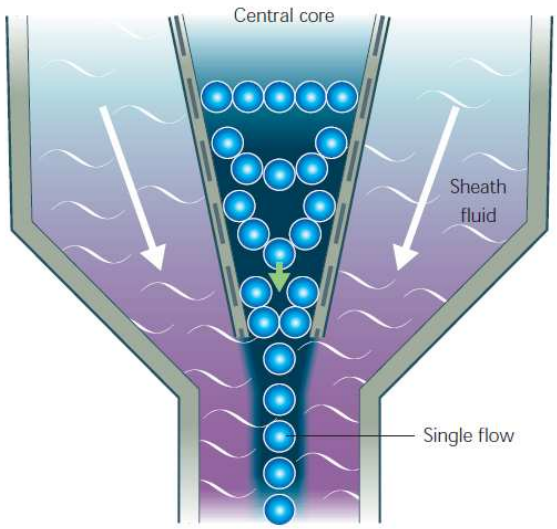

Figura 16. Sistema fluídic d'un citometre de flux. L'enfoc hidrodinàmic genera un flux de partícules individualitzades que permet que puguin ser avaluades una a una davant la font de llum. Figura obtinguda de http://www.spacesrl.com/wp-content/uploads/2011/02/FlowCytometry_V3_20101.pdf.

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En citometria la dispersió de la llum produïda per les cèl·lules dóna informació sobre les seves característiques físiques. La mida cel·lular, el nucli, la membrana cel·lular i el material granular de l'interior de cada cèl·lula provoquen una dispersió concreta de la llum emesa pel làser en xocar contra la seva superfície, donant lloc a un canvi de direcció però no de longitud d'ona de la llum, que permet diferenciar diferents poblacions i subpoblacions cel·lulars. Es poden diferenciar dos tipus de dispersió de la llum: la llum dispersada cap endavant ("forward scatter", FSC), la qual proporciona una estimació de la mida cel·lular, i la llum dispersada en angle de 90º ("side scatter", SSC) que depèn de la densitat i granulositat cel·lular, i per tant, és un indicador de la complexitat cel·lular (Peña i Rodríguez, 2006).

A més de l'anàlisi de les característiques físiques, la citometria de flux també permet valorar les propietats bioquímiques de les cèl·lules a través de la llum emesa per fluorocroms o compostos fluorescents units específicament a les cèl·lules. Els fluorocroms són compostos que absorbeixen llum d'una determinada longitud d'ona i emeten fotons a un nivell energètic inferior, i per tant de major longitud d'ona, que pot ser detectada i identificada a través dels jocs de filtres i detectors acoblats al citòmetre.

Per tal de discriminar i detectar la dispersió de la llum i les diferents longituds d'ona produïdes, els citòmetres disposen de diferents tipus de **filtres òptics** que permeten classificar els senyals produïts per les cèl·lules en impactar amb la font de llum. Una vegada classificats els senyals, els **fotodetectors** acoblats a cada conjunt de filtres són capaços de detectar i transformar la llum en impulsos elèctrics. Aquests polsos elèctrics constitueixen el senyal analògic que posteriorment podrà ser convertit en valors digitals processables per un sistema informàtic. Els citòmetres més comuns són aquells que presenten cinc sistemes òptics de mesura: dos de dispersió (FSC per a la dispersió frontal i SSC per a la dispersió lateral) i tres de fluorescència (normalment, FL1 per a la longitud d'ona de la banda del verd, FL2 per a la longitud d'ona de la banda del taronja i FL3 per a la longitud d'ona de la banda del vermell) (Figura 17).

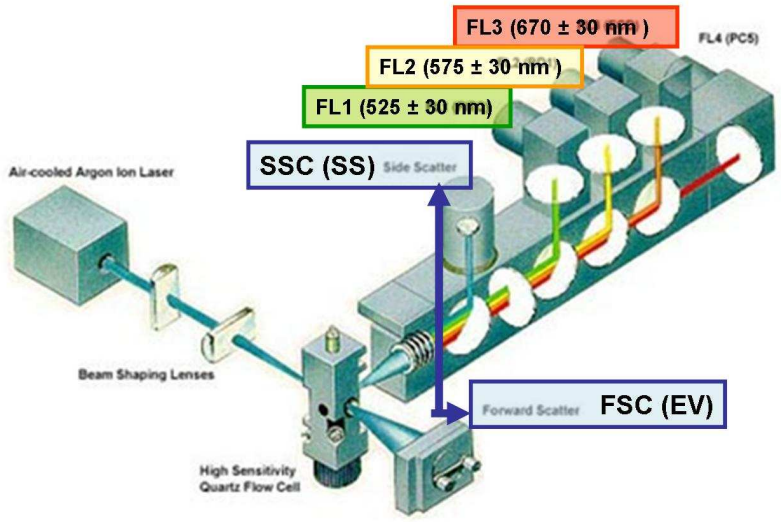

Figura 17. Diagrama dels components òptic d'un citòmetre de flux. Components òptics i col·locació de la cambra de flux en un citòmtre Beckman coulter Cell Lab Quanta SC. El sistema òptic està format per el detector forward scatter (EV) el qual proporciona informació sobre la mida cel·lular, i per un detector side scatter (SS) que fa una aproximació de la complexitat cel·lular. A més, s'observen 3 fotodetectors que es troben connectats a cadascun dels filtres (FL1 525 ± 30 nm, FL2 575 ± 30 nm i FL3 670 ± 30 nm).

Discussió General

L'espermatozoide porcí és produït als testicles com una cèl·lula altament polaritzada però funcionalment immadura. L'epidídim té una funció important en la reproducció animal conferint el medi i les condicions necessàries per a la maduració espermàtica. En mamífers, els espermatozoides han d'esdevenir madurs i adquirir l'habilitat de desencadenar la capacitació i la reacció acrosòmica per tal de poder fecundar els oòcits. Els canvis que es donen durant el trànsit dels espermatozoides pel conducte epididimari inclouen modificacions morfològiques, bioquímiques, metabòliques i canvis en el moviment, els quals donen lloc a l'adquisició de la motilitat i a la maduració dels espermatozoides un cop arriben al cauda epididimari. No obstant, després de la maduració epididimària encara sónn necessaris molts canvis en els espermatozoides epididimaris madurs per tal que adquireixin l'aptitud fecundant. Els espermatozoides madurs emmagatzemats al cauda epididimari es troben en un estat quiescent i són modificats durant i després de l'ejaculació per part de les secrecions de les glàndules accessòries, així com durant el seu trànsit pel tacte genital femení i en la interacció amb l'oòcit.

En porcí, els canvis que es donen durant la maduració espermàtica, així com el paper dels òrgans implicats en aquests canvis han estats estudiats en detall per diferents autors. Pel que fa al conducte epididimari porcí, els seus components i regions ha estat descrites detalladament, així com les estructures específiques i les regions associades a la seva funcionalitat (Briz *et al.* 1993, Stoffel & Friess 1994, Calvo *et al.* 2000). La morfologia dels espermatozoides epididimaris ha estat detallada amb anterioritat (Jones 1971, Bonet *et al.* 2000) i la seva motilitat ha estat avaluada en nombrosos estudis previs (Dacheux *et al.* 1979, Bassols *et al.* 2005, Pruneda *et al.* 2005, Matás *et al.* 2010).

Les secrecions proteiques de les cèl·lules epitelials (secretoma) (Syntin *et al.* 1996, Syntin *et al.* 1999) i la seva contribució en la maduració espermàtica (Dacheux *et al.* 2003) han estat descrites amb anterioritat, així com el contingut proteic del fluid epididimari (proteoma) (Dacheux *et al.* 2009) i, més recentment la composició de les proteïnes de la membrana plasmàtica dels espermatozoides (Belleannée *et al.* 2011). El contingut del fluid epididimari ha estat analitzat per tal de determinar-ne la seva composició (Jeulin *et al.* 1987, Cibulková *et al.* 2007, Pruneda *et al.* 2007) i estudiar-ne diferents rutes metabòliques (Pruneda *et al.* 2006, Han *et al.* 2007). *In vitro*, també s'han desenvolupat cultius de cèl·lules epididimàries (Bassols *et al.* 2006) per tal d'emprar-los en l'avaluació *in vitro* de la maduració espermàtica en porcs mitjançant la co-incubació amb espermatozoides immadurs (Bassols *et al.* 2005) i per tal d'observar les secrecions proteiques de les cèl·lules epididimàries *in vitro* (Bassols *et al.* 2007). Algunes proteïnes específiques del fluid i dels espermatozoides ha estat identificades en diferents regions epididimàries (Yin-Zhe Jin 1997, Jin *et al.* 1999), i se n'ha descrit el seu processament al llarg de la maduració epididimària (Gatti *et al.* 1999) i les seves possibles implicacions en la funció reproductiva (Christopher *et al.* 2007).

A nivell d'expressió gènica, el transcriptoma del conducte epididimari porcí ha estat descrit per Guyonnet i col., 2009 (Guyonnet *et al.* 2009). L'expressió d'altres gens, com ara el CD9, la proteïna 4 d'unió a retinol (RBP4), el receptor androgen (AR), la relaxina (RLN), l'acrosina (ACR) o osteopontina, i les seves implicacions en la funció reproductiva també ha estat publicada per diferents autors (Kaewmala

et al., Lin *et al.* 2006). D'altres estudis més específics també s'han desenvolupat prèviament, com ara l'estudi dels efectes de la freqüència de la recol·lecció de mostres seminals en els espermatozoides epididimaris (Pruneda *et al.* 2005),.

El present projecte doctoral s'ha centrat en l'estudi dels espermatozoides epididimaris porcins i la seva habilitat per desencadenar processos de capacitació *in vitro*. S'han realitzat nombrosos experiments per tal d'avaluar i caracteritzar la qualitat espermàtica al llarg del conducte epididimari i durant l'ejaculació en mostres mantingudes en condicions no capacitants i en medis específics que mimetitzen les condicions que es produeixen durant la capacitació. Els resultats obtinguts ha estat resumits i publicats a l'**Article I**. D'altra banda, la fosforilació de residus tirosina ha estat descrita per diversos autors com un dels principals esdeveniments implicats en la maduració epididimària i claus durant el procés de capacitació. Per aquest motiu, es van incloure diferents experiments per determinar els patrons de residus tirosina fosforilats en mostres d'espermatozoides epididimaris, ejaculats i capacitats *in vitro*, els resultats dels quals es mostren a l' **Article II**. En aquest article s'han desenvolupat diferents tècniques complementàries per tal d'obtenir noves dades semi-quantitatives avaluant els valors de intensitat de fluorescència, d'expressió i de localització dels residus tirosina fosforilats al llarg de la maduració epididimària, l'ejaculació i la capacitació *in vitro*.

Durant el trànsit per l'epidídim la membrana plasmàtica dels espermatozoides també es veu altament modificada per la interacció amb el fluid epididimari, així com pels processos específics de maduració epididimària que hi tenen lloc. Tenint en compte que la maduració de la membrana plasmàtica és un dels processos claus pel reconeixement entre l'espermatozoide i l'oòcit i per la seva unió, s'ha realitzat un estudi acurat del contingut de carbohidrats de la membrana plasmàtica a l'**Article III**. En aquest article s'ha caracteritzat la composició del glicocàlix en mostres d'espermatozoides epididimaris i s'han establert possibles relacions entre el contingut en glicoproteïnes de la superfície dels espermatozoides porcins i la maduració espermàtica. Finalment, el processament específic d'una proteïna transmembrana, la fertilina, durant la maduració epididimària ha estat descrit a l'**Article IV**.

Els experiments realitzats a l'**Article I** inclouen l'anàlisi computeritzada clàssica de la motilitat espermàtica, així com mètodes de citometria de flux, demostrant que la citometria de flux és una eina objectiva i fiable que pot ser emprada de forma complementària amb les tècniques clàssiques de motilitat espermàtica. L'objectiu principal d'aquest estudi ha estat la utilització de processos d'avaluació acurats i objectius per a l'avaluació de la qualitat espermàtica que permeten una bona correlació entre els paràmetres de qualitat espermàtica i els processos de maduració i capacitació espermàtica. Tal i com han descrit anteriorment diversos autors (revisat a (Gupta 2005)) en mamífers els espermatozoides adquireixen la seva motilitat durant el seus trànsit pel conducte epididimari, passant d'un moviment inefectiu a un moviment unidireccional vigorós i progressiu. Els resultats observats a l'**Article I** demostren que la maduració epididimària contribueix no només en l'adquisició del moviment sinó també en el increment de l'eficiència del moviment. Els resultats obtinguts mostren que l'adquisició d'una freqüència de batec correcte (BCF) és el primer paràmetre de motilitat que s'adquireix completament quan l'espermatozoide assoleix el corpus epididimari, mentre que la motilitat progressiva total no és

adquirida fins que l'espermatozoide arriba a nivell del cauda epididimari. Pel que fa a la motilitat la rectitud i linealitat del moviment, així com l'adquisició d'un moviment oscil·latori correcte no es produeix de forma completa fins que l'espermatozoide és ejaculat, tal i com s'ha observat en estudis previs (Matás *et al.* 2010). D'altres estudis descriuen que durant la capacitació *in vitro* i la criocapacitació els valors d'ALH augmenten per sobre de 3,5 (Casas *et al.* 2009) a causa de la hiperactivació del moviment que es produeix en els espermatozoides capacitats (Yanagimachi 1970) i que permet el seu pas a través del fluid oviductal de consistència viscosa. Els resultats mostrats a l'**Article I** indiquen que el protocol de capacitació *in vitro* utilitzat en aquests experiments només indueix els estadis inicials de la capacitació espermàtica en els espermatozoides ejaculats, ja que els valors d'ALH obtinguts es troben per sota del 3,5 prèviament observat en mostres capacitades *in vitro* o criocapacitades. D'altra banda, els resultats obtinguts mostren que els espermatozoides no capacitats del corpus i del cauda epididimari, presenten valors d'ALH molt per sobre del 3,5, posant en dubte l'ús d'aquest paràmetre com a un bon indicador de l'índex de capacitació per mostres epididimàries. La inducció de la capacitació *in vitro* no només dóna lloc a un augment d'ALH sinó que també desencadena l'adquisició de la motilitat progressiva total en els espermatozoides del cauda, així com una disminució de la VCL. El cauda epididimari confereix un ambient únic que permet mantenir els espermatozoides emmagatzemats en un estat de quiescència metabòlica abans de l'ejaculació (revisat a(Jones *et al.* 2007b)). La dilució dels espermatozoides en el plasma seminal durant l'ejaculació permet l'adquisició de la plena motilitat dels espermatozoides (Matás *et al.* 2010). Els resultats obtinguts mostren noves dades de l'estat quiescent en que es troben emmagatzemats els espermatozoides al cauda epididimari, i descriuen la seva capacitat de desenvolupar un moviment vigorós i progressiu (augmentant la seva PRG i disminuint la seva VCL) en incubar-los en un medi capacitant.

Pel que fa a la integritat de la beina mitocondrial s'ha observat que es menor en les mostres espermàtiques del corpus i cauda, i que es veu especialment alterada per la capacitació *in vitro* en els espermatozoides ejaculats. Els resultats de l'**Article I** també mostren la necessitat d'un increment del potencial de la membrana mitocondrial per desencadenar la motilitat i la capacitació espermàtica durant el trànsit pel conducte epididimari i després de la capacitació *in vitro*. La capacitació *in vivo* comporta una desestabilització de la membrana plasmàtica i el conseqüent increment de l'influx de calci en els espermatozoides que precedeix a la reacció acrosòmica (Yanagimachi 1994). Per aquesta raó al nostre laboratori s'ha desenvolupat un protocol de capacitació espermàtica *in vitro* (Puigmulé *et al.* 2011); en aquest experiment es va procedir a l'avaluació de la fluïdesa de la membrana plasmàtica, dels nivells de calci intracel·lulars i de la integritat acrosòmica de les mostres espermàtiques. Els resultats obtinguts mostren que ni la maduració epididimària ni l'ejaculació comporten un increment de la fluïdesa de la membrana plasmàtica ni de la freqüència d'acrosomes reacionats, mentre que la capacitació *in vitro* sí que augmenta la fluïdesa de la membrana plasmàtica dels espermatozoides del cauda i ejaculats. El contingut de calci intracel·lular es manté durant el trànsit dels espermatozoides per l'epidídim, augmentant en els espermatozoides ejaculats i quan aquests són capacitats *in vitro*.

En conjunt, els resultats obtinguts a l'**Article I** donen suport a l'hipòtesi que només els espermatozoides ejaculats són completament madurs per desencadenar un procés complet de capacitació en condicions *in vitro* i revelen la importància de la maduració epididimària, de les secrecions de les glàndules accessòries i

l'ejaculació en el procés de capacitació espermàtica. Els espermatozoides epididimaris emmagatzemats al cauda són els únics que es troben prou madurs per desenvolupar alguns dels canvis fisiològics associats al procés de capacitació espermàtica; són cèl·lules madures capaces de moure's progressivament després de la capacitació *in vitro* però incapaces de desencadenar els canvis associats a l'augment del calci intracel·lular dels passos inicials de la capacitació. Els resultats també indiquen que durant el procés maduració espermàtica els espermatozoides del corpus són especialment més sensibles que els espermatozoides immadurs del caput o els espermatozoides quiescents del cauda.

A l'**Article II** s'han descrit i comparat els patrons de proteïnes amb residus tirosina fosforilats, la seva expressió i la seva localització en mostres epididimàries, ejaculades i capacitades *in vitro*. Els canvis en els patrons de fosforilació dels residus tirosina de les proteïnes espermàtiques han estat associats anteriorment amb la capacitació espermàtica i en la aptitud fecundant dels espermatozoides en diferents espècies com ara ratolí (Visconti *et al.* 1995c, Naz & Rajesh 2004), humà (Luconi *et al.* 1996, Naz 1996, Sakkas *et al.* 2003), boví (Galantino-Homer *et al.* 1997), oví (Chatterjee *et al.* 2010) i porcí (Flesch *et al.* 1999, Tardif *et al.* 2001). No obstant, són pocs els estudis que s'han realitzat per determinar el paper dels residus tirosina fosforilats en la maduració epididimària i en la posterior capacitació dels espermatozoides porcins.

L'**Article II** correspon a un estudi integral dels espermatozoides porcins epididimaris, ejaculats i capacitats *in vitro* mitjançant l'ús de les tècniques complementàries de Western blot, citometria de flux i immunocitoquímica. L'estudi de l'expressió dels patrons de tirosines fosforilades en les proteïnes espermàtiques porcines durant la maduració epididimària, l'ejaculació i la capacitació *in vitro* ha demostrat la presència de quatre grups de proteïnes. El primer grup de proteïnes amb residus tirosina fosforilats només es troba als extractes dels espermatozoides immadurs i correspon a les bandes de 93, 66 i 45 kDa. El segon grup composat per proteïnes de 76, 23 i 12 kDa, només presenten residus tirosina fosforilats en els espermatozoides madurs del cauda i ejaculats i majoritàriament incrementen la seva expressió de tirosines fosforilades després de la capacitació *in vitro*.

Un tercer grup amb nombroses proteïnes de 49, 40, 37, 30, 26 i 25 kDa presenta de manera constitutiva residus tirosina fosforilats tant en els espermatozoides immadurs com madurs, i majoritàriament incrementen la seva expressió de residus tirosina fosforilats després de la maduració i/o capacitació *in vitro*. Estudis previs d'estadis inicials de la capacitació *in vitro* en espermatozoides porcins (Kalab et al. 1998) també han descrit proteïnes amb residus tirosina fosforilats constitutives (p44, p40, p38 i p34) amb concordança amb les proteïnes amb residus tirosina fosforilats constitutives de 49, 40, 37 i 30 kDa descrites a l'**Article II**. Els resultats d'aquest estudi demostren la presència de dues proteïnes més amb residus tirosina fosforilats constitutives de 25 i 26 kDa. Aquests resultats suggereixen que la major part de les proteïnes mencionades es troben involucrades no només en la maduració espermàtica sinó també en el procés de capacitació, ja que la seva expressió de residus tirosina fosforilats es veu significativament incrementada després de la capacitació *in vitro*. Cal destacar, que la banda proteica constitutiva de 30 kDa descrita a l'**Article II**, podria correspondre's a la proteïna tirosina fosforilada de 34 kDa descrita inicialment per Kalab (Kalab et al. 1998) i més tard per Flesh (Flesch et al. 1999), així com a la proteïna de 32 kDa (p32) observada per Tardif (Tardif et al. 2001). Totes aquestes proteïnes de 30-34 kDa presenten

un pes molecular similar, una fosforilació constitutiva dels seus residus tirosina i incrementen l'expressió d'aquests residus després de la capacitació *in vitro*. La proteïna p32 és una proteïna citosòlica de l'espermatozoide porcí que només apareix a la membrana plasmàtica després de la capacitació i que posseeix activitat semblant a les tirosina quinases (Tardif et al. 2001, Bailey et al. 2005). Caldran estudis més exhaustius de les proteïnes de la membrana plasmàtica per elucidar la significació biològica de la relocalització i expressió de la proteïna p32 durant la capacitació.

Finalment, s'ha observat un quart grup de proteïnes de 28 i 20 kDa que únicament presenten residus tirosina fosforilats després de la capacitació *in vitro* de l'espermatozoide porcí. Estudis previs han descrit una proteïna de 21-19 kDa que presenta residus tirosina fosforilats en els espermatozoides capacitats de *Sus scrofa* (p21) (Dubé et al. 2004), de *Sus domesticus* (Esworthy et al. 1994) i d'hàmster (NagDas et al. 2005). Aquestes proteïnes s'han identificat com la glutatió peroxidasa (PHGPx). La PHGPx és una selenoproteïna que actua com un enzim clau en la protecció de les biomembranes exposades a estrès oxidatiu (Imai & Nakagawa 2003). En els espermatozoides capacitats aquest enzim es troba ancorat a la membrana de la beina mitocondrial (NagDas et al. 2005). La implicació fisiològica i molecular de la PHGPx en la capacitació és poc coneguda, no obstant les dades prèvies suggereixen que la fosforilació dels residus tirosina de la PHGPx podrien representar un pas important en la cascada de senyalització associada a la capacitació i implicada en la regulació de la hiperactivació de la motilitat espermàtica i/o la funció mitocondrial (NagDas et al. 2005).

A més del canvis en els patrons de proteïnes tirosina fosforilades observats en funció de l'estat maduratiu i/o de capacitació dels espermatozoides porcins, a l'**Article II** també s'han descrit importants canvis a la localització i a la intensitat d'aquests residus entre els espermatozoides del caput i els del cauda, així com entre els espermatozoides madurs no capacitats i els capacitats *in vitro*. La maduració epididimària dels espermatozoides porcins inclou patrons de desfosforilització com en el cas dels espermatozoides de rata (Lewis & Aitken 2001). Per contra, els resultats obtinguts en porcs i rates difereixen dels resultats publicats en ratolí (Visconti *et al.* 1995b) i boc (Chatterjee et al. 2010), suggerint que els patrons de fosforilació al llarg de l'epidídim són variables entre espècies. Mentre a les parts distals de l'epidídim de ratolí la totalitat de la cua dels espermatozoides es troba tirosina fosforilada, contribuïnt a la hiperactivació del seu moviment (Aitken et al. 2007), als espermatozoides porcins no s'ha observat aquest patró de marcatge. Els espermatozoides porcins pateixen una desfosforilització de la part anterior de l'acrosoma concentrant els residus tirosina fosforilats a la part posterior de la regió equatorial durant la maduració epididimària. Els resultats d'aquest estudi suggereixen que els canvis de localització dels residus tirosina fosforilats observats podrien estar associats als processos d'inhibició de la capacitació prematura descrits pels espermatozoides emmagatzemats en estat quiescent al cauda epididimari (Jones et al. 2007a).

Després de la capacitació *in vitro* s'observa com els residus tirosina fosforilats s'estenen per tot el cap de l'espermatozoide, i apareix un lleuger marcatge a la cua; aquests canvis van acompanyats d'un increment de la intensitat de fluorescència que ja havia estat descrita amb anterioritat en espermatozoides porcins capacitats (Petrunkina et al. 2001, Tardif et al. 2001). Per contra, en humans, primats, hàmsters, rates i ratolins els espermatozoides capacitats presenten un marcatge intens de la cua que podria estar relacionat amb la hiperactivació del moviment durant el procés de capacitació (revisat a (Naz & Rajesh

2004)). Els espermatozoides porcins capacitats pràcticament no mostren marcatge a la cua, sinó que concentren gran part dels residus tirosina fosforilats a l'acrosoma, suggerint una implicació d'aquest residus en el procés de capacitació i/o de reacció acrosòmica (Tardif et al. 2001). No obstant, l'increment de tirosines fosforilades després de la capacitació *in vitro* ha estat descrita en moltes espècies com ara en ratolí (Visconti *et al.* 1995a, Visconti *et al.* 1995c), humà (Aitken et al. 1996, Aitken et al. 1998, Sakkas et al. 2003), boví (Galantino-Homer et al. 1997, Galantino-Homer et al. 2004), equí (Pommer et al. 2003), primats (Mahony & Gwathmey 1999), rosegadors (Lewis & Aitken 2001)(NagDas et al. 2005) i boc (Chatterjee et al. 2010), destacant la importància de la fosforilació en el procés de fecundació.

El glicocàlix és la primera barrera entre els espermatozoides i el medi que els envolta. En mamífers s'ha descrit com un element essencial per a la maduració espermàtica, la protecció dels espermatozoides (Kirchhoff & Hale 1996), el seu transport i la interacció amb l'oòcit (Diekman 2003). Es per això que a l'**Article III** s'ha procedit a descriure la composició del glicocàlix dels espermatozoides epididimaris, amb l'objectiu d'aprofundir en el coneixement sobre la seva implicació en el procés de maduració espermàtica. Per tal de dur a terme aquest estudi s'han emprat tres tècniques complementàries: 1) s'ha estudiat la unió de les lectines a la superfície dels espermatozoides viables per citometria de flux; 2) s'han analitzat els patrons d'unió de les lectines a la superfície dels espermatozoides de manera individual mitjançant el microscopi de fluorescència; i 3) s'ha caracteritzat mitjançant electroforesi les glicoproteïnes de la membrana espermàtica a les que s'uneixen les diferents lectines. En l'estudi s'han utilitzat vuit lectines, classificades en cinc grups diferents d'afinitat per carbohidrats: galactosa (PNA), glucosa/manosa (LCA, PSA), N-acetil-D-glucosamina (WGA), N-acetil-D-galactosamina (HPA, PHA-L, SBA) i fucosa (UEA-I).

A l'**Article III** s'ha observat que els residus galactosa (Gal), glucosa/manosa (Glc/Man) i N-acetil-D-glucosamina (GlcNAc) incrementen significativament la seva expressió entre el caput i el cauda distal. Per contra, aquest increment és variable entre les diferents lectines tot i reconèixer el mateix tipus de residus carbohidrats (Exemple, LCA i PSA). Per a la resta de grups de lectines (lectines que reconeixen residus N-acetil-D-galactosamina (GalNAc) i fucosa (Fuc)), la intensitat del marcatge sobre la superfície espermàtica no difereix significativament entre regions epididimàries, o en tot cas disminueix subtilment entre regions (Exemple per PHA-L i UEA-I). Aquest patró irregular podria relacionar-se amb les secrecions de sialoproteïnes específiques al lumen del corpus epididimari (Harayama et al. 1999) i amb la seva posterior deposició a la superfície espermàtica (Calvo et al. 2000). Aquests residus sialics podrien trobar-se a la superfície espermàtica emmascarant d'altres residus carbohidrats presents.

La localització del marcatge de les lectines sobre la superfície dels espermatozoides viables varia en funció de l'estadi de maduració espermàtica. Els resultats mostren que majoritàriament totes les lectines s'uneixen a l'espermatozoide, il·lustrant la importància del glicocàlix a la superfície espermàtica. No obstant, només s'ha descrit marcatge del flagel per dues lectines, la HPA i la WGA, incrementant la seva afinitat a mesura que avança el procés de maduració epididimària. Resultats similars als obtinguts en aquest estudi han estat descrits en hàmsters (Calvo et al. 1995).

Dos patrons majoritaris de marcatge s'han observat a la superfície del cap dels espermatozoides: un marcatge concentrat a la vora apical del cap, o bé un marcantge en el conjunt de la superfície cefàlica. També cal destacar que durant la maduració epididimària aquests dos patrons s'alternen i són reconeguts intensament per la major part de les lectines, excepte la WGA (la qual únicament s'uneix a la vora apical

del cap), i la HPA i l'UEA-I (les quals s'uneixen únicament de forma transitòria al conjunt de la superfície espermàtica). Els canvis successius de localització en el cap de l'espermatozoide suggereixen processos de redistribució d'un conjunt de proteïnes a la superfície espermàtica, tal i com suggereixen estudis previs amb proteïnes espermàtiques de superfície (Phelps et al., 1990; Petruszak et al., 1991). Aquesta redistribució s'observa especialment en el cas de la PSA, el patró de marcatge de la qual difereix passant d'un marcatge de la totalitat de la superfície espermàtica en els espermatozoides del caput, a un progessiu desplaçament durant la maduració epididimària vers la vora apical i la regió post-acrosòmica del cap. Cal destacar però, que la PNA no mostra afinitat pel cap dels espermatozoides epididimaris, sinó que reconeix residus Gal localitzats només a la membrana acrosòmica externa. Es per això que la superfície de l'acrosoma dels espermatozoides epididimaris no presenten marcatge per aquesta lectina (Fazeli et al. 1997). La vora acrosòmica dels espermatozoides madurs del cauda mostren una abundància remarcable de residus GlcNAc i Glc/Man. Aquesta migració dels residus GlcNAc vers al cap dels espermatozoides s'ha descrit prèviament en processos de capacitació en espermatozoides ejaculats porcins (Jiménez et al. 2003), en processos d'interacció espermatozoide-oòcit (Nimtz *et al.* 1999, Töpfer-Petersen 1999), així com en processos implicats en la prevenció de l'aglutinació cap amb cap dels espermatozoides (Calvo et al. 2000).

La peça intermèdia presenta un marcatge intens de residus Gal i Glc/Man en totes les regions epididimàries, mentre que l'HPA (GalNAc) només presenta marcatge a la peça intermèdia en els espermatozoides el caput proximal. La gota citoplasmàtica mostra un marcatge diferent al descrit a la peça intermèdia, tot i tractar-se d'estructures continues de la membrana plasmàtica. La PSA no marca la superfície de la gota, mentre que la SBA i en part l'HPA marquen només la gota citoplasmàtica però no la peça intermèdia; Pel que fa a la PNA, la LCA i la WGA només marquen la gota citoplasmàtica en les posicions més proximals de l'epidídim. Les gotes citoplasmàtiques, migren al llarg de la peça intermèdia del flagel durant la maduració epididimària, tot i que encara se'n desconeix el mecanisme. En porcs la gota citoplasmàtica no és alliberada fins al moment de l'ejaculació o fins i tot fins que es troba al tracte femení (Harayama *et al.* 1996, Pruneda *et al.* 2005). En aquest estudi, s'han observat residus Gal (PNA) i Glc/Man (només LCA) a la superfície de la gota citoplasmàtica dels espermatozoides immadurs però no en espermatozoides madurs. En boví i bocs, els residus Gal també són abundants a la gota citoplasmàtica dels espermatozoides immadurs (Arya & Vanha-Perttula 1985, Magargee *et al.* 1988). Els canvis en l'afinitat de les lectines per la superfície de la membrana de la gota citoplasmàtica observats en l'estudi indiquen que la seva composició de carbohidrats és especialment modificada i podria estar involucrada en la migració de la gota. L'anàlisi per microscòpia de fluorescència aporta informació sobre la localització concreta dels residus en la superfície espermàtica però només permet l'anàlisi d'un nombre limitat d'espermatozoides, mentre que la citometria de flux dóna una informació més global de la unió de les lectines en el conjunt de la població espermàtica. Es tracta doncs, de dues tècniques que aporten informacions amb diferent sensibilitat (individualitzada en el cas de la microscòpia i del conjunt de la població en el cas de la citomètria de flux), però complementàries.

L'anàlisi de les proteïnes de membrana mitjançant la tècnica de Western blot mostra nombroses bandes de proteïnes que abarca un ampli rang de pesos moleculars >250 kDa a 10 KDa, de les quals 13-14 bandes tenien una major intensitat. Moltes bandes de similar pes molecular presenten marcatge per diferents lectines. Un cas concret és la banda de 112-102 kDa, amb marcatge per tots els residus carbohidrats a

totes les regions epididimàries estudiades, i especialment intens per la PNA, la LCA i la WGA. A més, per aquesta banda s'observa una disminució del grau de glicosilació durant la maduració espermàtica, en especial pels residus GalNac i Fuc. Un estudi proteomic recent centrat en la caracterització de les proteïnes de la membrana dels espermatozoides porcins, descriu una proteïna amb un pes molecular similar de 115 kDa, la qual s'ha identificat com l'enzim convertidor d'angiotensina (ACE) (Belleannée et al. 2011). En mamífers l'ACE es localitza al cap i la peça intermèdia dels espermatozoides i és alliberada de la superfície espermàtica al fluid epididimari (Gatti et al. 1999). En els primats *Rhesus* també s'ha caracteritzat una glicoproteïna depenent de la maduració de 116 kDa amb una elevada diversitat d'oligosacàrids i que determina la capacitat dels espermatozoides per poder fecundar els oòcits (Srivastav 2000).

Nombroses bandes amb un marcatge molt lleu o no marcades per totes les lectines s'han observat només en certes etapes de la maduració espermàtica. Entre les més destacades la banda de 151 kDa amb residus Glc/Man, GlcNAc i Fuc, només observada en els espermatozoides del caput proximal, la banda de 133 kDa amb residus Fuc que apareix en espermatozoides del caput distal i es manté fins al cauda i les bandes de 91 a 73 kDa amb residus GlcNAc, GalNAc i Fuc, les quals només marquen la superfície dels espermatozoides del caput proximal i distal. Moltes altres bandes de menor pes molecular amb residus Glc/Man són presents a la superfície dels espermatozoides del caput proximal (16 kDa) i caput distal (19 kDa), i del corpus als cauda (23 kDa). La similitud en els pesos molecular d'aquestes bandes, així com el seu patró d'expressió a l'epidídim, podrien indicar que es tracten de formes transitòries d'una mateixa proteïna, la composició en glicans de la qual es va modificant durant el trànsit dels espermatozoides per l'epidídim.

El conjunt dels resultats presentats a l'**Article III** mostren un increment dels residus galactosa, glucosa/manosa i N-acetil-D-glucosamina i una disminució dels residus N-acetil-D-galacatosamina i fucosa en els espermatozoides madurs emmagatzemats al cauda. Aquests canvis en la composició de carbohidrats està associada a canvis tant d'intensitat com de localització a la superfície espermàtica. Aquests resultats representen la primera caracterització de les principals glicoproteïnes de la membrana dels espermatozoides porcins (per pes molecular i capacitat d'unió a lectines) implicades en la maduració espermàtica. En totes les bandes observades és possible observar processos de remodificació: les glicoproteïnes observades apareixen i desapareixen de la superfície dels espermatozoides i/o varien la seva composició en glicans durant la maduració. Aquestes modificacions complexes de la superfície dels espermatozoides són una conseqüència de les nombroses interaccions entre la superfície espermàtica i el medi epididimari, en particular amb les glicosidases presents en elevades concentracions al fluid epididimari (Syntin et al. 1996).

El coneixement dels canvis als que són sotmeses les proteïnes durant la maduració espermàtica en el conducte epididimari permet establir el seu paper en la fertilitat i el seu potencial com a marcadors de fertilitat. L'**Article IV** focalitza la seva atenció en una proteïna transmembrana, el complex fertilina (ADAM-1 i ADAM-2). En aquest article es caracteritza la fertilina com un exemple del processament proteic a que són sotmesses certes proteïnes al llarg del conducte epididimari en porcs. Nombroses proteïnes de la família ADAMs són present als testicles i epidídims, així com a la membrana espermàtica, però el seu rol potencial en la fecundació no està del tot elucidat (Kim et al. 2006, Hunnicutt et al. 2008). Estudis previs sobre la fertilina s'han desenvolupat principalment amb conills d'Índies (Blobel 2000) i

ratolins (Kim et al. 2006), i tot i que s'ha suggerit un patró de modificació semblant per boví (Walker et al. 1996) i recentment per l'ADAM-3 en primats (Kim et al. 2009), el processament i l'expressió del complex fertilina durant la maduració epididimària en porcí no havia estat encara descrit.

El conjunt d'experiments realitzats a l'Article IV demostra la presència d'mRNA per l'ADAM-1 i l'ADAM-2 en els testicles dels porcs, però mentre l'expressió de l'ADAM-1 és continua, observant-se en totes les mostres epididimàries, l'expressió d'ADAM-2 només s'observa en el vas eferent i el caput epididimari. Estudis previs amb ratolins han descrit que l'mRNA d'ADAM-1 és expressat també al llarg del conducte epididimari dels ratolins (Johnston et al. 2005) i l'expressió d'ADAM-1 i 2 no es troba restringida al tracte genital masculí (Wolfsberg et al. 1995, Xiang & MacLaren 2002, Murase et al. 2008). Pel que fa a l'expressió proteica de la fertilina, s'observen formes precursores de les subunitats ADAM-1 i ADAM-2 en els espermatozoides porcins del testicle i de les regions més proximals dels conducte epididimari. No obstant, la major part de l'ADAM-1 perd la part N-terminal, quedant una forma més curta C-terminal d'uns 50-55 kDa tan bon punt l'espermatozoide arriba al vas eferent. El pes molecular d'aquesta proteïna suggereix una pèrdua del pro-peptid i del domini metalloproteasa, trencant el lloc convertasa conservat de la pro-proteïna, com prèviament s'havia descrit en rosegadors (Blobel 2000). També s'observa que no tota l'ADAM-1 és convertida, ja que la forma de 70 kDa (amb la part N-terminal) es pot observar fins a la regió del corpus distal on potser és degradada i alliberada amb la gota citoplasmàtica. Per l'ADAM-2, les diferents bandes observades suggereixen un patró del processament proteolític seqüencial per a la maduració d'aquesta subunitat en els espermatozoides porcins: la forma testicular és processada al caput proximal cap a una forma precursora de 90 kDa, i seguidament a les formes transitòries de 70-75 kDa, 65-70 kDa i 50-55 kDa al caput distal i corpus, les quals són posteriorment processades al corpus donant lloc a una forma final de 40-43 kDa al cauda. Aquest processament pas a pas també s'ha descrit en rosegadors (Blobel et al. 1990, Cho et al. 1998, Blobel 2000), així com també ha estat suggerit com a model per altres proteïnes ADAMs de la membrana dels espermatozoides madurs (Schlondorff & Blobel 1999, Nishimura et al. 2004, Han et al. 2009). En aquest estudi, els resultats indiquen que la hidròlisi de les formes testiculars i de les precursores de l'ADAM-2 és dóna de forma específica i restringida al caput proximal i el corpus, respectivament.

Diferents patrons de localització es poden observar en porcí per l'ADAM-2, en concordança amb la relocalització descrita prèviament en rosegadors i conills d'Índies (Phelps et al. 1990, Yuan et al. 1997, Blobel 2000): en els espermatozoides immadurs del caput i corpus, la fertilina es localitza sobre la totalitat de la regió acrosòmica, mentre que en els espermatozoides madurs del cauda és manté conservada a la vora acrosòmica. Els resultats de l'estudi indiquen que la migració de la fertilina en porcs s'inicia durant el trànsit dels espermatozoides pel corpus distal, però que no s'acaba de completar fins que els espermatozoides assoleixen el cauda proximal. És interessant destacar que el procés de migració de la fertilina coincideix amb el de moltes proteïnes de la superfície de la membrana , amb els canvis en el marcatge de glicoproteïnes (Dacheux et al. 1989, Belleannee et al. 2010) i amb un increment significatiu del nombre d'espermatozoides mòbils i fèrtils (Dacheux & Paquignon 1980, Yanagimachi 1994). Els gels d'electroforesi bidimensionals mostren diferents isoformes de la subunitat porcina de 50-55 kDa ADAM-1 que difereixen en pes molecular i pI entre regions. Aquest variabilitat pot ser el resultat de diferents nivells de glicosilació o està relacionada amb possibles eliminacions de residus específics en els dominis C- i N-terminal (Phelps et al. 1990, Cho et al. 2000) que podrien inactivar algunes de les isoformes més

hidrofòbiques fent més difícil la focalització d'aquestes isoformes en els gels. La migració de la fertilina i la concentració a un nou domini de la superfície de la membrana coincideix amb les diferències d'extractibilitat observades per la subunitat ADAM-1 als detergents. Aquest fet suggereix que el complex fertilina es mou pels diferents ambients lipídics de la membrana i concorda amb les dades prèvies de la composició lipidica dels dominis de la membrana espermàtica (Christova *et al.* 2004, Jones *et al.* 2007a). El canvi en l'ambient lípidic pot resultar en canvis tant en la mida de l'ADAM-2 com en les propietats de la subunitat ADAM-1 a causa de modificacions post-traduccionals o canvis en la càrrega elèctrica. La migració de la fertilina porcina cap a la vora acrosòmica concorda amb altres estudis que suggereixen la concentració de les proteïnes espermàtiques involucrades a la unió primària amb la ZP a la zona apical de la membrana plasmàtica del cap de l'espermatozoide (Jones *et al.* 2007a) i amb un estudi recent que indica que la beta fertilina o ADAM-2 és una de les proteïnes espermàtiques implicades en la unió de l'espermatozoide a la ZP de l'oòcit porcí (Van Gestel et al. 2007). Un cop relocalitzada en aquest domini específic de la membrana de l'espermatozoide s'observa un increment de la concentració local de la fertilina que podria ser crucial per la interacció espermatozoide-oòcit.

A més, l'**Article IV** també descriu que la maduració de la fertilina de l'espermatozoide de porcí inclou el processament proteolític de l' ADAM-1 i de l'ADAM-2 en regions epididimàries molt concretes, així com nombroses modificacions posttraduccionals de la subunitat ADAM-1. Aquests canvis poden tenir un rol essencial en la migració del complex fertilina de la totalitat del domini acrosòmic cap a la vora acrosòmica en l'espermatozoide madur. Una acurada descripció del patrons que presenta la fertilina madura versus la fertilina immadura permetrà avançar en l'ús d'aquesta molècula com a indicador del nivell de maduració de la població espermàtica en diferents mascles. Futurs estudis enfocats en la descripció de la relació potencial entre els patrons de fertilina i la fertilitat masculina podrien establir noves bases per elucidar els mecanismes involucrats en el processament i la relocalització de les proteïnes de l'espermatozoide durant la maduració.

En conclusió aquest projecte de tesi ofereix nous coneixements sobre la fisiològica dels espermatozoides epididimaris, ejaculats i capacitats *in vitro* mitjançant estudis multiparametrics (anàlisis convencionals de qualitat espermàtica i moleculars; **Article I**), els quals donen una base per a l'avaluació de la maduració espermàtica i nova informació sobre el procés de capacitació. L'anàlisi espermàtica pot esdevenir una eina molt útil per a la detecció de processos anormals de maduració epididimària i/o processos defectius de capacitació espermàtica, els quals disminueixen la fertilitat masculina i la prolificitat. Els resultats sobre l'expressió de residus tirosina fosforilats i la seva localització a llarg dels processos maduratius i/o de capacitació (**Article II**) aporten nova informació sobre els mecanismes mitjançant els quals l'espermatozoide maduran al llarg de l'epidídim i adquireix la competència necessària per ser capacitat i programat per la fecundació. A més, el coneixement dels mecanismes involucrats en el processament del glicocàlix i les glicoproteïnes de la superfície espermàtica al llarg de l'epidídim (**Article III**) confereixen futures eines per entendre la maduració epididimària en condicions fisiològiques i patològiques. Futurs estudis orientats en la identificació de les proteïnes tirosina fosforilades, glicoproteïnes o d'altres proteïnes, com ara el complex proteic fertilina (**Article IV**), implicadades en la maduració i la capacitació permetran establir el seu paper potencial com a marcadors de fertiliat, així com estudiar les seves funcions específiques en el procés de fecundació i en la fertilitat masculina.

Girona, gener 2012