Electron microscopy of cryopreserved boar spermatozoa

With special reference to cryo-scanning electron microscopy and immunocytochemistry

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Abstract

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Cryopreservation of boar spermatozoa is as yet sub-optimal, presumably owing to variation in dehydration and membrane intactness among boars. This thesis work investigated, using cryo-scanning electron microscopy (Cryo-SEM), how cooling rates below –5°C/min affect the frozen structure of boar semen in four different packages/volumes (maxi-straws and medium straws, FlatPacks [FPs] and MiniFlatPacks [MFPs]). The hypothesis tested was that the degree of dehydration differs between containers, as well as by cooling rate, which would explain differences in post-thaw viability among boars. Cryo-SEM allows the study of the fully hydrated ultrastructure of frozen sperm samples without introducing artefacts caused by fixation or processing. It further allows visualisation of the topography of the sample, including the distribution and amount/area of the frozen water which, when sublimated, leaves the partly or fully dehydrated extender and the spermatozoa intact. Furthermore, the thesis work investigated the effect of cooling/re-warming on the localisation of the hexose substrate carriers GLUT-3 and GLUT-5 and water/cryoprotectant channels (Aquaporin 7 [AQP7]) in the plasma membrane. Use of different cooling speeds and containers clearly affected the degree of (de)hydration of the extender/spermatozoa, thus explaining recorded differences in post-thaw sperm survival, but not inter-boar variation. The degree of dehydration was most favourable for the MFPs owing to their cryogenic properties. Ultrastructural analyses by SEM and transmission electron microscopy (TEM) of immunolabelling clearly showed that boar spermatozoa express the hexose/glucose transporters GLUT-3 and GLUT-5 in both the outer and the inner plasmalemma. Their distribution in the plasma membrane changed during the process of cryopreservation, particularly for GLUT-3, whose labelling decreased dramatically post-thaw. Likewise, the channel protein AQP7, involved in the transport of water and glycerol through the plasma membrane, is present in boar spermatozoa collected from the cauda epididymides and from different fractions of the ejaculate. The domain localisation changed in ejaculated spermatozoa during cryopreservation, with a clear redistribution and increasing intensity following freezing-thawing. Both hexose (GLUT-) transporters and AQP7 are markers of interest for non-empirical studies of boar semen cryopreservation.

Key words: spermatozoa, cryopreservation, cooling rates, cryo-packages, electron microscopy (EM), Cryo-SEM, TEM, immunocytochemistry, hexose transporters (GLUT), aquaporins (AQPs), pig.

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Appendix

Papers I–V

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Ekwall, H. 2007: Cryo-scanning electron microscopy discloses differences in dehydration of frozen boar semen stored in large containers. *Reproduction in Domestic Animals* (in press) DOI No.: 10.1111/j.1439-0531.2007.00994.

II. Hernandez, M., Ekwall, H., Roca, J., Vazquez, J.M., Martinez, E., & Rodriguez-Martinez, H. 2007: Cryo-scanning electron microscopy (Cryo-SEM) of semen frozen in medium-straws from good and sub-standard freezer AI-boars. *Cryobiology* 54: 63–70.

III. Ekwall, H., Hernandez, M., Saravia, F., & Rodriguez-Martinez, H. 2007: Cryo-scanning electron microscopy (Cryo-SEM) of boar semen frozen in medium-straws and MiniFlatPacks. *Theriogenology* 67: 1463–1472.

IV. Sancho, S., Casas, I., Ekwall, H., Saravia, F., Rodriguez-Martinez, H., Rodriguez-Gil, J.E., Flores, E., Pinart, E., Briz, M., Garcia-Gil, N., Bassols, J., Pruneda, A., Bussalleu, E., Yeste, M., & Bonet, S. 2007: Effects of cryopreservation on semen quality and the expression of sperm membrane hexose transporters in the spermatozoa of Iberian pigs. *Reproduction* 134: 111–121.

V. Ekwall, H., & Rodriguez-Martinez, H. 2007: Immunocytochemical localization of aquaporin 7 (AQP7) in boar spermatozoa. (Submitted)

Abbreviations

AI	artificial insemination
ANOVA	analysis of variance
AQP	aquaporin
ART	assisted reproductive technique
BSA	bovine serum albumin
BSD	back scatter detector
BTS	Beltsville thawing solution
CASA	computer-assisted sperm analysis
CPA	cryoprotectant agent
cryo-SEM	cryo-scanning electron microscopy
DNA	deoxyribonucleic acid
EM	electron microscopy
ET	embryo transfer
FITC-PNA	fluorescein isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin
FP	FlatPack
GLM	General Linear Model
GLUT	hexose transporter proteins
GLUT-3	hexose transporter protein-3
GLUT-5	hexose transporter protein-5
IgG	immunoglobulin
KO	knockout
LEY	lactose-egg yolk
LEYGO	lactose-egg yolk-glycerol-OrvusEsPaste
LIN	linearly motile
LN_2	liquid nitrogen
MANOVA	multi-analysis of variance
MFP	MiniFlatPack
NGS	normal goat serum
P1, P2	portion 1, portion 2
PATN	pattern analysis technique
PBB	phosphate-BSA buffer
PBS	phosphate-buffered saline
PI	propidium iodide
PMI	plasma membrane integrity
R123	rhodamine-123
RT	room temperature
S1–4	stages 1–4
SD	standard deviation
SDS	sodium dodecyl sulphate
SED	secondary electron detector
SEM	scanning electron microscopy
SLU	Swedish University of Agricultural Sciences
TEM	transmission electron microscopy
TSM	total sperm motility
VAP	average path velocity
VCL	curvilinear velocity
VIA	sperm viability
VSL	straight linear velocity
ZP	zona pellucida

Introduction

Cryobiology is the multidisciplinary science that studies the physical and biological behaviour of cells, tissues and organs at temperatures below the freezing point of water (0°C), with reference to their long-term storage for scientific research or medical application (Walcerz & Karow 1996; Karlsson 2002). Its impact follows the successful short-term preservation at above-zero temperatures of, among others, blood and bone marrow cells for transplantation (King 2004). Mammalian cells exist in an ionic environment and are kept viable by maintaining a similar concentration of ionic salts in the intra-cellular space to balance the osmotic pressure across the plasma membrane. Therefore, cryobiology is primarily concerned with the changes of the phase of water (including changes from the liquid to/from the solid phase, and even sublimation/condensation), with the amount of free and bound water, and also, with the occurrence of changes in ionic concentration, caused by directional movement of water across the membrane, following phase changes (e.g. formation/dissolution of ice and de/rehydration phenomena) (Mazur 1963; 1984).

When a mammalian cell is subjected to slow cooling to temperatures below zero (which is the most commonly used procedure in biomedicine), water freezes to form ice, primarily extra-cellularly. This ice becomes, over time, surrounded by an increasing number of solutes which disturb the homeostatic osmotic pressure of the cell. The cell responds by allowing water to leave the intra-cellular compartment, thus compensating for the fact that the extra-cellular milieu is becoming increasingly hyper-osmotic. Such a phenomenon leads not only to dehydration, but also, to a hyper-concentrated solute environment within the cell, which ultimately affects its viability (toxic effect) (Watson & Fuller 2001). These changes during cooling and freezing reverse when thawing or re-warming occurs, which is also an essential component of cryobiology. Slow re-warming allows water to reflux to the places where solutes are concentrated, but since these concentrations *per se* are toxic, cells are unable to survive the process. A more rapid re-warming is therefore desirable, since these toxic solute concentrations are then present only momentarily. However, both customary cooling and re-warming rates affect the fluidity of the plasma and organelle membranes by rearrangement of structural proteins and dislocation of the constituent lipids. They can even disrupt the cytoskeletal proteins or the chromatin structure to such an extent that the deoxyribonucleic acid (DNA) may be damaged (Watson 1981; Watson & Fuller 2001). In cells in suspension, such as spermatozoa, both the form and volume of the sample to be cooled/re-warmed, and the concentration of the contained cells play major roles during the most damaging interval in the process, i.e. during the changes in phase of the extra-cellular water, when heat is either dissipated (during cooling) or incorporated (during re-warming) (Mazur *et al* 1972; Mazur 1984; Mazur & Cole 1985; 1989; Morris *et al* 1999).

Obviously, the challenge has been, and still is, to take specimens through the temperature region in which damage can occur (i.e. cooling) and back again (re-

warming) without causing irreparable cell damage. Thus far, this challenge has been successfully met only for some cell suspensions and several relatively simple tissue systems (Mazur & Cole 1985; 1989; Morris *et al* 1999), despite the use of cooling and re-warming protocols that involve addition of cryoprotectant agents (CPAs) (often toxic substances), use of specific cooling rates run by programmable freezers, and use of suitable cryogenic containers and handling protocols (Watson & Fuller 2001). Interestingly, such combinations of procedures have resulted in findings that question the previously mentioned classic theory of freezing and thawing events (Mazur 1984), and the reasons for cell damage, particularly when specific cells such as spermatozoa are considered (Morris 2006; Morris *et al* 2007).

The spermatozoon, a highly differentiated, terminal cell with several sub-cellular compartments, has been used as a model for investigating the effects of cryopreservation procedures on cell survival and function (Mazur & Cole 1985; 1989; Yudin *et al* 1988). To be fully functional, spermatozoa must reach the site of fertilisation and the oocyte; they must capacitate, undergo the acrosome reaction when in contact with the zona pellucida (ZP), penetrate it, and fuse with the oolemma. Freezing could interfere with, or ablate, the capacity of the spermatozoon to undergo one or more of these steps (Critser, Ameson *et al* 1987; Critser, Huse-Bender *et al* 1987). Consequently, multiple levels of biological function must be considered in assessing the role of the biophysical parameters that are regarded as critical for cell survival during freezing and thawing (Hammerstedt *et al* 1990; Rodriguez-Martinez 2007b). For gene banking or breeding through assisted reproductive techniques (ARTs), such as artificial insemination (AI) or embryo transfer (ET), both gametes (but particularly spermatozoa) and embryos are cryopreserved (Watson & Holt 2001). Semen from both domestic and wild animals, as well as humans, has been frozen following extrapolation of protocols used for other species, with varying results post-thaw (Gao *et al* 1997; Curry 2000; Holt 2000a). Spermatozoa from some species (including avian and human) are easy to cryopreserve, as indicated by the good survival rates achieved, while others are more difficult (such as the equine or porcine species), or nearly impossible (such as marsupials) (Watson & Holt 2001).

One of the species in which AI has increased exponentially during the past years has been the porcine, using liquid-stored semen. Boar ejaculated spermatozoa (often from the sperm-rich fraction of the ejaculate) are re-suspended at low concentrations in chemically defined extenders and stored at 16–20°C for several days before use. Artificial insemination with these spermatozoa usually results in fertility rates similar to those obtained after natural mating (for a review, see Rodriguez-Martinez 2007a). However, boar spermatozoa are one of the most demanding cell types with respect to sustaining viability during freezing and thawing, with a large proportion of the spermatozoa not surviving these procedures

(Penfold & Watson 2001). Those spermatozoa that do survive are, moreover, usually a mixture of cells, some of which survive well while others show modified motility and a shortened lifespan, factors which compromise their fertilising ability. Insemination with such spermatozoa leads, ultimately, to lowered pregnancy rates and fewer piglets born, compared with AI using liquid-stored semen (Larsson & Einarsson 1976; Larsson & Ersmar 1980; Johnson 1985; Johnson *et al* 2000).

Because of low post-thaw survival, boar semen has been traditionally frozen in large containers, such as 5 mL maxi-straws, holding about 5 billion spermatozoa (Westendorf *et al* 1975). Such large cylinders are cryogenically unsuitable, since because of their large section-to-surface ratio the dissipation of heat during cooling and the consequent incorporation of energy during re-warming take very long time (Weitze *et al* 1987; Yoshida 2000; Watson & Fuller 2001). Efforts have been made to use other, cryogenically more suitable packaging, such as the 5 mL FlatPack (FP) (Eriksson & Rodriguez-Martinez 2000), or smaller containers such as 0.5 mL medium-straws (Roca, Rodriguez-Martinez *et al* 2006) and the novel, 0.5 mL, MiniFlatPack (MFP) (Saravia *et al* 2005). More efficient freezing and re-warming of the spermatozoa would be expected in these latter types of packaging, leading to higher survival rates among the processed cells (Eriksson *et al* 2002; Roca, Rodriguez-Martinez *et al* 2006; Saravia *et al* 2005; 2007; Wongtawan *et al* 2006). However, whether these survival rates are a consequence of improved, more homogenous cooling and freezing remains to be seen, particularly considering the interactions that occur during cryopreservation between cooling rates, and the volume and shape of the samples to be frozen, among other factors.

In studies of cryopreservation, the emphasis has been on how cell survival relates to the physical responses of the cell to the physico-chemical events involved in the process, primarily the intactness of the plasmalemma. However, the other membrane-bound organelles present within the cell may also suffer (Armitage & Mazur 1984). As yet, little has been done to determine how these intra-cellular structures and organelles respond to freezing, partly because it is difficult to assay their state and function *in situ* in an instantly recognisable fashion. Cryo-substitution (Bwanga, Ekwall *et al* 1991) and the related ice granulometry determinations at transmission electron microscopy (TEM) level (Courtens & Paquignon 1985; Courtens *et al* 1989; 1992; Ekwall, Eriksson *et al* 1997; Rodriguez-Martinez & Ekwall 1998) have been the methods used to determine, indirectly, the presence of ice crystals in frozen spermatozoa, including those of pigs. Boar semen slowly frozen in extender containing glycerol as CPA, and packaged in maxi-straws or plastic bags (FPs) showed larger globular masses of extra-cellular ice (lakes of free water) and freeze-concentrated extender/CPA-containing spermatozoa (veins). Differences in the size of both lakes and veins were evident between containers, and also, between regions within the maxi-straws. These differences reflect variation in the formation of ice and the ability of water to leave the CPA-sperm-bearing extender, which showed varying degrees of compaction/dehydration. In those areas with less dehydration, such as the centre of the maxi-straws, intra-cellular ice crystal shadows were present in the acrosome, between the acrosome and nucleus, in the mitochondria and the sub-plasmalemma, and along the entire spermatozoon, especially when ultra-rapid cooling was used

(Bwanga, Ekwall *et al* 1991), compromising ~20% of these spermatozoa (Ekwall, Eriksson *et al* 1997). However, studies of other species, such as the equine or human species, whose spermatozoa are more hydrated, particularly at the sperm head region, than those of the pig, have failed to identify the presence of intra-cellular ice crystals during slow (10°C/min) or rapid (3,000°C/min) cooling (Morris *et al* 1999; 2007; Morris 2006). Further detailed studies are needed to elucidate inter-species differences.

Although valuable evidence is provided by the use of granulometry, in combination with freeze substitution, to determine the presence and size of ice, the sequence of practical steps required before the samples can be analysed makes the procedure fairly demanding and time-consuming, as well as potentially introducing artefacts (Rodriguez-Martinez & Ekwall 1998). Other methods are now available, such as cryo-scanning electron microscopy (cryo-SEM), which make it possible to directly study cells in their frozen state, including visualisation of inner structures (Ekwall, Eriksson & Rodriguez-Martinez 1997; Rodriguez-Martinez & Ekwall 1998; Nissje & Van Aelst 1999; Wyss *et al* 2002). In brief, frozen semen samples are fractured at liquid nitrogen (LN_2) temperature and the fracture surface is etched by controlled sublimation of the frozen aqueous phase. This is subsequently examined by cryo-SEM after platinum/palladium coating. The assessment of these non-chemically fixed, fully hydrated samples provides instant knowledge of the different components (such as extender and free water) and the microstructure of included cells. Moreover, cryo-SEM enables quantitative image analysis, thus allowing the study of differences between cooling rates, containers and location within frozen samples. Such data would be beneficial for developing new handling protocols for both freezing and thawing (Morris *et al* 1999; 2007), particularly in the porcine species.

Selection for sperm freezability has been pursued for decades among AI bull sires and has resulted in minor inter-animal variation, despite a common freezing method utilized for all sires. Such selection has not been applied in the pig. Despite efforts made to accommodate freezing methodologies (Hernandez *et al* 2007), the large variation seen in post-thaw sperm survival is still being attributed to individual boars, consequently forcing their pre-selection (for a review, see Roca, Hernandez *et al* 2006). This pre-selection for semen processing is usually based on post-thaw sperm characteristics, such as motility, membrane integrity and chromatin structure, and results in classifying the boars as “good-” or “bad freezers” (Hernandez *et al* 2006; 2007). Owing to the low degree of intra-boar variability present, this classification is useful, but tedious. Moreover, it is not known why these differences exist between boars. Variations in the plasma membrane (Ortman & Rodriguez-Martinez 1994; Medrano *et al* 2002), motility (Thurston *et al* 1999), chromatin stability (Hernandez *et al* 2006) and genetics (Thurston, Siggins *et al* 2002; Thurston, Watson *et al* 2002) have been listed as possible causes. Cryo-SEM studies of semen in a frozen state may disclose whether differences in freezing patterns can be seen among boars as a cause of variation.

Cold shock injury during temperature reduction in spermatozoa is a general phenomenon whose intensity varies among species, the pig being highly sensitive

(Wales & White 1959; Watson & Plummer 1985; Watson & Morris 1987). The possible mechanisms of cold shock injury include thermotropic instability of the plasma membrane, which may cause differences in solute concentration around the spermatozoa. There may also be protein dislocation and/or denaturation, causing loss in sperm membrane permeability and the membrane's eventual disruption during thawing (Waterhouse *et al* 2006, Saravia *et al* 2007). Examples of vital proteins are those belonging to the hexose transporter (GLUT) family, which play a role in the regulation of the glucose and fructose metabolism of unfrozen cells. These proteins are mainly responsible for the passage of hexoses across mammalian sperm membranes (Burant *et al* 1992; Angulo *et al* 1998), including those of the dog (Rigau *et al* 2002), rat (Farooqui *et al* 1997), human (Kokk *et al* 2005) and boar (Medrano *et al* 2006). Since the major substrate provided in most boar semen extenders used for cooling and freezing is glucose, studying the presence and temporal localisation of these GLUT proteins during the different steps of cryo-processing is of utmost importance in determining whether their function is impaired and therefore related to the decreased cell survival post-thaw.

Water transport across cell membranes is, as already pointed out, of vital significance, particularly with regard to fluid flow among cells and tissues. It is also of importance when cells such as spermatozoa are confronted to different media during their transport in the female genitalia or during their manipulation and processing for AI or storage (including cryopreservation). Water moves across the plasma membrane by two main mechanisms: by passive osmotic pressure, and by active transport through molecular water channels. These channels are built by specific membrane proteins, the so-called "Aquaporins (AQPs)" (Verkman *et al* 1996; Borgnia *et al* 1999; Izumi *et al* 2006). Aquaporins have been demonstrated in cell membranes with a tenfold to 100-fold higher capacity for water permeation than diffusion (Agre *et al* 2002), and have been detected in both rat and human testicular spermatozoa (Calamita *et al* 2001; Saito *et al* 2004). Functionally, AQPs are divided into those that selectively transport water, and those that transport glycerol (Rodriguez *et al* 2006) and possibly other small solutes (Wang *et al* 2006), in addition to water, being also called "aquaglyceroporins". One of the latter group, AQP7, has been found in the rat testis, and its gene is particularly expressed by spermatids, where it has been attributed a role for glycerol transport as a carbon source for mature sperm metabolism (Borgnia *et al* 1999). As mentioned previously, in the cryopreservation of cells, the movement of water and CPAs such as glycerol across plasma membranes plays a central role in their survival post-thaw. The replacement of water by glycerol, which occurs during cell cryopreservation (Mazur 1984), may therefore be modulated by some types of AQPs, such as AQP7, which are involved in transporting neutral solutes with a small molecular weight, such as glycerol or urea (Ishibashi *et al* 1994; 1997; Tsukaguchi *et al* 1998), as well as water. It is noteworthy that glycerol is by far the most widely used CPA for boar semen preservation. Therefore, studies aiming at disclosing whether AQP7 is present in boar spermatozoa and whether its location is affected by handling and cryopreservation, would provide cues for the non-empirical design of better cryopreservation methods.

The presence and localisation of both GLUT and AQPs in spermatozoa can be studied using different methods depending on the objective of the study. For

instance, molecular determination by blotting methods provides evidence of the presence of GLUT and AQPs, but does not disclose their precise cell location. Fluorescent light microscopy after immunolabelling gives a general, but imprecise, view of the binding pattern, although it studies only a few cells. Flow cytometry, a very fast technique able to analyse thousands of spermatozoa per minute, can be used instead, but the method does not indicate where the binding sites are, for instance whether the labelling is in the inner or outer membrane face. On the other hand, immunocytochemistry at the electron microscopy (EM) level exposes details of the binding sites thanks to its higher resolution. Immunocytochemistry for GLUT or AQP7 proteins has not been previously applied to boar spermatozoa when studied at the various stages of the cryopreservation procedure.

Aims of the study

The general aim of this thesis work was to investigate, using cryo-SEM, how extracellular ice formation occurs during freezing of boar semen in containers of various cryo-biological properties, at different cooling velocities, and using the semen of different males, in the hope of helping to facilitate the development of this sub-optimal biotechnology. Moreover, the presence, localisation and temporal modifications of membrane molecules involved in the transport of hexoses (GLUTs) or water/cryoprotectants (AQPs) in relation to sperm handling and cryo-processing were disclosed using immunocytochemistry, combined with SEM and TEM.

The specific aims were to determine, using cryo-SEM –

* how freezing rates affect the degree of dehydration of frozen boar semen in two different, large packages (5 mL) for single AI doses;

* whether the degree of dehydration and ice crystal distribution differs among boars whose spermatozoa display different post-thaw viability, when processed in medium-straws (0.5 mL); and

* how small, 0.5 mL containers, such as medium-straws or MFPs, affect the degree of *in situ* dehydration and levels of homogeneity of boar semen.

Also, the thesis work aimed to determine –

* where the hexose-specific transporters GLUT-3 and GLUT-5 are located in boar spermatozoa, and whether their location varies during cooling and thawing; and

* whether AQP7 is present in the membrane of boar spermatozoa, both epididymal and ejaculated, in relation to their extension, and to cooling and freezing-thawing.

Materials and Methods

Animals

Ejaculated spermatozoa were collected from 29 sexually mature boars (aged 8 months to 5 years) of various breeds (Swedish Yorkshire, Swedish Landrace, Iberian) or commercial hybrid lines, while epididymal spermatozoa were removed from the caudae epididymides of six Swedish Yorkshire boars slaughtered at a local abbatoir. All boars were fed commercial pig feed, according to national (Swedish or Spanish) standards, and provided with water *ad libitum* while being housed in individual pens. The boars were selected according to either normal semen quality and proven fertility (**Papers I–V**) or normal development (for slaughtered boars, **Paper V**). Boars were either not pre-selected for freezability (**Papers I**, **III–V**) or classified as having different freezability (**Paper II**). The experimental protocol was designed in accordance with the relevant regulations on animal welfare. These were the guidelines established by the Animal Welfare Directive of the Government of Catalonia (Spain, **Paper IV**), the Region of Murcia (Spain, **Paper II**), or the Ethics Committee for Experimentation with Animals, Uppsala, Sweden, which reviewed and approved the experimental design in advance (**Papers I**, **III** and **V**).

Collection of semen

Semen was collected once or twice per week using the gloved-hand technique, either selecting the sperm-rich fraction of the ejaculate, collected in an insulated thermos flask (**Papers I–IV**), or as selected portions of the ejaculate (**Paper V**). In the latter case, two portions were collected, the first 10 mL from the sperm-rich fraction (portion 1 [P1]), which was collected in a 15 mL plastic tube, and the rest of the ejaculate (portion 2 [P2]), which was collected in a plastic bag, both inside an insulated thermos flask. In **Papers I**, **III** and **V** the spermatozoa were processed immediately, while in **Papers II** and **IV** the spermatozoa were suspended (1:2, v/v) in Beltsville thawing solution (BTS) (IMV, Beltsville, MD, USA), cooled and maintained at 17ºC for shipment, within 24 h of collection, to the laboratory where they were cryopreserved and stored (University of Murcia, **Paper II**; and the Swedish University of Agricultural Sciences (SLU), **Paper IV**).

Sperm evaluation pre-freezing

Only ejaculates (n= 64) with at least 70% of motile spermatozoa and 75% of morphologically normal spermatozoa were used (**Papers I**, **III–V**). Ejaculates included in **Paper II** had ≥200 x 10^6 spermatozoa/mL, ≥85% spermatozoa with normal morphology, ≥75% of spermatozoa that were progressively motile and ≥80% displaying intact membranes before cooling and freezing.

Sperm freezing

Semen was processed using documented protocols, in **Paper I** following the method described by Eriksson & Rodriguez-Martinez (2000), in **Paper II** using the straw freezing procedure described by Westendorf *et al* (1975) and modified by Thurston *et al* (1999) and Carvajal *et al* (2004), and in **Papers III–V** following Saravia *et al*'s protocol (2005). In brief, the BTS-extended semen was spun in a programmable refrigerated centrifuge, whereupon the supernatant was discarded and the resulting pellets were re-extended in lactose-egg yolk (LEY, extender II) extender to a concentration of 1.5 x 10^9 cells/mL. After further cooling to 5ºC within 90–120 min, the extended spermatozoa were re-suspended with LEY-glycerol-OrvusEsPaste (LEYGO) extender (extender III) to yield a final concentration of 1 x 10^9 cells/mL and a final amount of glycerol of 3% (v/v). The cooled spermatozoa were then packed into 5 mL maxi-straws (**Paper I**), 5 mL FPs (**Paper I**), or 0.5 mL medium- plastic straws (**Papers II–V**) or MFPs (**Paper III**) before being frozen using controlled-rate freezers (**Papers I**, **III**, **V**: Mini Digitcool 1400; IMV, L'Aigle, France; **Paper II**: IceCube 1810; Minitüb, Tiefenbach, Germany). The cooling started from +5°C to –5°C at rates of 3 or 6°C/min, followed by different freezing rates being used from –5°C (**Paper I**: 2°C, 50°C or 1,200°C/min, the last by direct plunging into LN_2; **Paper II**: from –5°C to –80°C at 40°C/min, held for 30 sec at –80°C, then cooled at 70°C/min to –150ºC; **Papers III–V**: 1 min for crystallisation at –5°C and thereafter cooling at 50°C/min from –5 to –140°C). In all cases, the straws/bags were thereafter plunged into LN_2 (–196°C) for storage.

In addition, in order to determine eventual changes in viability and immunolocalisation during semen processing (**Papers IV** and **V**), spermatozoa, after being extended in BTS and kept chilled at 17°C for approximately 24 h (A), or after being re-extended in extender-II and cooled to 5°C for 2 h (B), or (C) post-thaw (**Paper IV**) were also studied during different stages of cryopreservation. In particular, they were studied for sperm viability (VIA) (motility and membrane integrity) as well as for GLUT-3 and GLUT-5 immunolocalisation. For AQP7 immunocytochemistry (**Paper V**), spermatozoa were studied at +20–21°C (stage 1 [S1]), at +16°C (stage 2 [S2]), at +5°C (stage 3 [S3]) and post-thaw (stage 4 [S4]).

Semen evaluation during processing and post-thaw

As mentioned, spermatozoa were studied for VIA (motility and membrane integrity) during different stages of cryopreservation and post-thaw. Frozen samples were thawed in circulating water at 50°C (**Papers I** and **II**) for 40 sec (maxi-straws), 13 sec (FPs) and 12 sec (medium-straws), or at 35°C for 20 sec (medium-straws and MFPs, **Papers III–V**).

Sperm motility

Sperm motility was assessed with computer-assisted sperm analysis (CASA) instruments (**Papers I**, **III** and **V**: SM-CMA; MTM Medical Technologies, Montreaux, Switzerland; **Paper II**: Sperm Class Analyzer®; Microptic, Barcelona, Spain) following incubation at 37–38°C for at least 30 min, using microscopes

equipped with a warm stage and phase contrast optics (200 x). One hundred to 200 spermatozoa were counted per sample, using pre-warmed Makler counting chambers (Sefi Medical Instruments, Haifa, Israel) to determine the percentages of motile spermatozoa and, among these, of those linearly motile (LIN) spermatozoa, after counting between three and eight predetermined optic fields (**Papers I** and **II**). As well, the following motility variables were recorded: percentage of LIN spermatozoa (%), straight linear velocity (VSL) (μm/s), average path velocity (VAP) (μm/s), and curvilinear velocity (VCL) (μm/s) (**Papers III** and **IV**).

Sperm viability

Sperm viability was evaluated in terms of plasma membrane integrity (PMI) (**Papers I–IV**) using the DNA-specific fluorochrome propidium iodide (PI) (**Papers I** and **II**) or the LIVE/DEAD Sperm Viability Kit L-7011 (Molecular Probes Inc., Eugene, OR, USA) (**Papers III** and **IV**) and flow cytometry. In addition, mitochondrial membrane potential and acrosomal integrity were microscopically determined in thawed samples (**Paper II**) using a triple fluorescent staining method which included PI, rhodamine-123 (R123) and fluorescein isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA).

Classification of semen freezability

Following evaluations of post-thaw sperm quality, cryopreserved ejaculates included in **Paper II** were classified by multivariate pattern analysis technique (PATN) (Belbin 1993; Abaigar *et al* 1999) into three groups, as described in detail by Hernandez *et al* (2006). Those boars with the best frozen-thawed sperm characteristics (>60% of total sperm motility [TSM] and VIA) were identified as "good" freezers. By contrast, boars showing reduced frozen-thawed sperm characteristics were considered as "moderate" (40–60% TSM and VIA) or "bad" freezers (<40% TSM and VIA). Only straws from boars classified as "good" (n= 5) or "poor" (n= 5) freezers were used.

Cryo-scanning electron microscopy (cryo-SEM, Papers I–III)

Cryo-SEM was performed on frozen-fractured, etched samples to examine their fine structure, in particular the size (area) of the solid-state water in the extra-cellular, outer-extender areas ("ice lakes"). Inside LN_2, the containers were manually broken, the plastic was removed, and a cylinder (maxi- or medium straw) or rectangle (FP or MFP) of the frozen semen was mounted in a mechanical grip holder. The grip holder was transferred to a cryostage (Oxford HT1500F Cryo System; Oxford systems, Oxon, UK) and then to a chamber attached to the microscope (JEOL Scanning Microscope 6320F; JEOL Ltd, Tokyo, Japan). Once the sample was inside the chamber, it was fractured in order to obtain a fresh, clean surface for examination. The temperature of the sample was raised by heating the holder to –92°C for 30 sec in order to etch the surface by water sublimation. Sublimation removes crystalline ice and increases the contrast of the sample. The

temperature was then decreased to –130°C to stabilise the frozen preparation whose surface was thereafter coated with platinum/palladium to prevent charging and to obtain a good signal-to-noise ratio. The coated sample was then transferred to the microscope chamber where it was analysed at a temperature range of –125°C to –135°C.

Image analysis of cryo-SEM assessment (Papers I–III)

Digital images (seven to ten per container, at 600 x for image analysis and at 3–4,000 x for fine structural examinations) were collected at 5 KV and stored on a computer (JEOL SemAfore software; JEOL Skandinavisk, Sundbyberg, Sweden). Image analysis (EasyImage 3000 software, version 1.3.0; Tekno Optik, Huddinge, Sweden) of the areas occupied by the partially sublimated, solid-state free water ("ice lakes") and of the sperm-containing, concentrated extender veins was then performed. Results were expressed in μm^2 (**Paper I**). In **Papers II** and **III** three variables were retrieved per image to monitor the degree of hydration: the area of the frozen concentrated extender matrix (containing spermatozoa, e.g. veins), the area of the spaces occupied by the partially sublimated solid-state water (extracellular, outside the extender, e.g. lakes) and the degree of compartmentalisation in the sample (size and number of lakes per image).

Sodium dodecyl sulphate (SDS)-electrophoresis and Western blot analysis (Paper IV)

For Western blot analysis, sperm samples were homogenised in a mixer, briefly boiled and then centrifuged at 10,000 *g* for 15 min at 4°C. Western blotting was carried out on the resultant supernatants whose total protein content was calculated (Bradford 1976), after applying a commercial kit (Bio-Rad Laboratories, Hercules, CA, USA) to ensure a homogeneous loading of protein (20 µg) per lane. The immunological analysis was based on sodium dodecyl sulphate (SDS) gel electrophoresis (Laemmli 1970), followed by transfer to nitrocellulose membranes (Burnette 1981). Since the freezing extender contained a significant number of non-sperm proteins, the transfer of an equal quantity of sperm proteins to each electrophoretic lane was tested by previously staining each transferred membrane with Poinceau red (BioRad). Only those membranes holding nearly equal amounts of sperm protein/lane were subsequently subjected to the Western blot, being tested with the primary antibodies for GLUT-3 (an affinity-purified polyclonal antibody, 16 amino acid synthetic peptide, which recognises the facilitative GLUT-3 in mammalian tissues including spermatozoa) and GLUT-5 (a rabbit anti-GLUT 5 polyclonal antibody (twelve amino acid peptide) which reacts with GLUT-5 and does not cross-react with GLUT-1, 2, 3 or 4) (Chemicon Europe Ltd, Hampshire, UK). Detection of immunoreactivity was done with a peroxidase-conjugated anti-rabbit secondary antibody developed using the ECL Plus detection system (Amersham, Buckinghamshire, UK).

Immunocytochemical localisation of hexose transporters -3 and-5 (Paper IV)

Scanning electron microscopy: Smears were prepared by spreading sperm suspensions from each sampling point onto superfrost polylysine-coated slides which, immediately after being briefly air-dried, were fixed in buffered paraformaldehyde (0.5%) for 15 min at room temperature (RT) and further washed with phosphate-buffered saline (PBS), pH 7.2–7.4. The smears were then incubated with the same primary antibodies to GLUT-3 and GLUT-5 used for Western blotting, at a dilution of 1:500 (v/v) in PBS. The spermatozoa were further washed with PBS and incubated with goat anti-rabbit immunoglobulin (IgG)-15 nm gold (1:25) (Auroprobe; Amersham, Buckinghamshire, UK) for 1 h 50 min at RT. Silver enhancement (IntenSE^{TM} silver enhancement system for microscopy, code RPN 491; Amersham Biosciences, Buckinghamshire, UK) was then applied for 10 min and samples were air-dried for 24 h in the dark. The smears were coated with platinum/palladium (High Resolution Sputter Coater; Agar Scientific, Accessories for Microscopy, Stansted, UK) for 30 sec. Selected regions of the glass were cut and mounted onto stubs for observation under an electron scanning microscope (JEOL SEM 6320F; JEOL Ltd, Tokyo, Japan) at 5 kV of filament intensity.

Transmission electron microscopy: The spermatozoa were suspension-fixed in 0.5% (w/v) paraformaldehyde in a 0.1 M phosphate (Sorensen's) buffer, pH 7.3, for 1 h at 4°C and centrifuged at 800 x g for 10 min. The resulting pellet was dehydrated in graded alcohol solutions, and progressively embedded at 4°C in LR-White acrylic resin (Acrylic Resin; TAAB Essentials for Microscopy, Reading, Berkshire, UK), transferred to capsules (one block/capsule) and polymerised for 24 h at 50°C. Semi-thin (1–3 μm) and ultra-thin (60–80 nm) sections were obtained. The latter were mounted on 300-mesh nickel grids (Amersham, Buckinghamshire, UK) for immunocytochemical labelling with the specific primary antibody (GLUT-3 and GLUT-5; see above) at 1:500 dilution (v/v in PBS) for 2 h at RT. Non-specific binding was blocked by incubating the sections for 1 h at RT with 2% normal goat serum (NGS) in 1% BSA/PBS. Pre-immune rabbit serum and the specific antibody, pre-absorbed with saturating concentrations of the corresponding peptide, were used in the primary incubation step as negative stained controls. After extensive washing in phosphate-BSA buffer (PBB), pH 7.4, the sections were incubated with A-protein gold (AuroProbe^{TM} EM G 15 nm; Amersham Corporation, Louisville, KY, USA) at a dilution of 1:100 (w/v) in PBB for 1 h at RT. The sections were finally counterstained with uranyl acetate 2% (w/v) in distilled water for 30 min and in lead citrate 3% (w/v) in distilled water for 10 min (Reynolds 1963). Finally, the sections were examined using a transmission electron microscope (JEOL JEM-1230 Electron Microscope; JEOL Ltd, Tokyo, Japan) at 80 kV of filament intensity.

Immunocytochemical localisation of Aquaporin 7 (Paper V)

Epididymal spermatozoa, collected post-mortem by manual aspiration from the cauda epididymides, and ejaculated spermatozoa from the whole (n= 4) or from P1 (n= 4) and P2 (n= 6) of the ejaculate at different stages of cryopreservation (S1–S4) were suspended and fixed in 1% paraformaldehyde in PBS at RT for 1 h, followed by incubation for detection of AQP7. Sperm smears were prepared on polylysine-coated slides, air-dried and rinsed in PBS several times, finishing with distilled water. Subsequent blocking was done with 1% BSA/PBS for 10 min to reduce non-specific IgG(Y) binding, followed by incubation with the primary antibody (chicken polyclonal to AQP7) (ab15123; Abcam, Cambridge, UK) for 1 h at RT (1:500). A donkey-anti-chicken IgY(G) (1:100; H+L) conjugated with 4 nm colloidal gold (Jackson Immuno Research, West Grove, PA, USA) was used as secondary antibody. The binding was silver-enhanced with IntenSE^{TM}, washed and dried. From each slide, round pieces of the glass with the incubated smear were cut out with a diamond pen and mounted on a SEM stub coated with platinum/palladium (60 mA for 30 sec) (208HR High Resolution Sputter Coater; Ted Pella, Redding, CA, USA) to avoid charging of the sample. Analysis was performed with a JEOL SEM 6320F electron microscope using its backscatter detector (BSD) and/or secondary electron detector (SED). The images were recorded using JEOL Semafore software. The labelling across different domains and sperm sources was visually scored by the same operator in order to provide an overall, albeit semi-quantitative, view of the distribution of the immunostaining.

Statistical analyses

In **Paper I** the data (sperm motility and the relative mean surface area, in μm^2, of the areas of extra-cellular, unbound water, or ice lakes) were analysed using the General Linear Model (GLM) procedure of the Statistical Analysis Systems software (SAS Institute Inc., Cary, NC, USA). The model included packages (2) and freezing rates (3) for sperm motility and, in addition, locations within the package (2) for the areas of extra-cellular ice. Data derived from **Papers II–IV** were statistically examined using SPSS, version 14 (SPSS Inc., Chicago, IL, USA). The non-parametric Kruskal-Wallis test and Mann-Whitney U-test were used in **Paper II** to evaluate differences in cryo-SEM variables between boars ("good" and "bad" freezers) and groups. Spearman correlations were calculated to disclose relationships between post-thaw sperm quality variables and cryo-SEM measurements. In **Paper III** mean values for post-thaw CASA parameters and PMI were examined by analysis of variance (ANOVA) using the GLM procedure. The statistical model used included the effects of the boar, and the packaging system, and the interaction between boar and package. Differences in cryo-SEM variables between boars and packages were examined by ANOVA including the fixed effects of boar and package and the replicate as random effect. Pearson's correlations were calculated to disclose relationships between post-thaw sperm quality variables and cryo-SEM measurements. In **Paper IV** VIA was examined by a multi-analysis of variance (MANOVA). Student's *t*-test was used to compare least square means and a GLM was included to assess the differences among the various classes. For all studies, differences were considered statistically significant if $P<0.05$.

Results

Sperm cryosurvival post-thaw

Sperm viability post-thaw was primarily evaluated either by mapping PMI (**Papers II–IV**) (in **Paper II** also including mitochondrial membrane potential and acrosomal integrity) or by determining sperm motility by CASA (**Papers I–IV)**, in an attempt to disclose effects of freezing rate (**Paper I**), packaging container (**Paper III**) and the individual boar in terms of freezability potential (**Paper II**) and breed (**Paper IV**).

The proportion of spermatozoa with an intact plasma membrane post-thaw was significantly lower in the Entrepelado ($P<0.05$) than in the Lampiño Iberian breed (**Paper IV**). Regarding PMI, variation among males was seen, but not between containers, e.g. MFP and medium-straws (**Paper III**). Neither was there any interaction between boar and packaging system ($P>0.05$). Acrosomal integrity, PMI and mitochondrial activity were included as components of VIA and allowed categorisation of sires with regard to freezability potential (**Paper II**).

Freezing rate significantly ($P<0.05$) affected the number of motile spermatozoa, with cells cooled at a rate of 50°C/min showing the highest cryosurvival (44–54%) compared with 2°C/min (32–34%) and 1,200°C/min (0%) (**Paper I**). At 50°C/min, the FP yielded significantly better post-thaw motility than did the maxi-straw, for both total motile spermatozoa (52.8 v. 42.7%; $P<0.05$) and those motile spermatozoa depicting linear progressive motility (68.6 v. 50.2%; $P<0.05$) (**Paper I**). Total sperm motility post-thaw did not differ statistically between medium-straws and MFPs, with the exception of the percentages of LIN spermatozoa ($P = 0.036$) and VCL ($P = 0.02$). The MFPs displayed the highest percentage of linearly motile spermatozoa and the lowest VCL, and revealed no variation among boars (**Paper III**). The percentages of sperm motility were lowest post-thaw (compared with other steps of cooling) but showed no significant differences between the Entrepelado and Lampiño Iberian breeds (**Paper IV**).

In sum, PMI was consistent post-thaw, no matter which container was used, with significant variation between animals and breeds. Sperm motility, on the other hand, was clearly affected by extreme cooling rates and container types, the smallest containers (medium-straws and MFPs) depicting the highest proportions of linearly motile spermatozoa post-thaw, to the extent of masking boar and breed variation.

Cryo-SEM of frozen boar semen

Ultrastructure of the frozen semen

The appearance of the cross-fractured straws and bags frozen in extender with glycerol as CPA, followed by deep etching to remove crystalline ice, revealed two major domains, one with extensive extra-cellular ice formation (lakes), and a

complementary, homogenous, freeze-concentrated material (CPA and sperm-containing extender, e.g. veins). At higher magnification, the freeze-etched samples revealed that the spermatozoa had migrated into the freeze-concentrated material during solidification: spermatozoa were very rarely entrapped within the extra-cellular ice crystals, although a few sperm tails could be seen in these areas. Sperm details could be studied, with cells depicting an intact outer surface or inner structures fractured at different levels. Most spermatozoa appeared intact, without obvious signs of plasma membrane disruption, especially when smaller containers were considered and freezing had occurred at non-extreme speeds, e.g. 50ºC/min (**Papers I–III**).

At first sight, the distribution and shape of the veins differed between containers, being thinner and more compact, and apparently occupying less space in the FPs than in the maxi-straws and in the MFPs compared with medium-straws or the larger containers. Consequently, the size of the areas of ice was inversely proportional to the size of the veins, a finding that was later confirmed when quantifying the relative mean surface area of the ice lakes. Regarding shape and distribution, while in the FPs the shape of the icy areas was irregular and variable within the container, the "lakes" (and veins) in the maxi-straws formed radial patterns from the periphery towards the centre (**Paper I**). The distribution of components followed a similar trend, the area of the extra-cellular ice (i.e. the surface area of the "ice lakes") being larger in the periphery than in the centre ($P<0.05$), except for FPs frozen by LN_2 plunging (**Paper I**). Samples that were frozen at higher rates (1,200°C) had a freeze-concentrated material which occupied a much larger cross-sectional area (see below).

In the comparison of medium-straws from two classes of boars, divided into "good" or "bad" freezers (**Paper II**), the cryo-SEM showed that neither the number nor the distribution of ice lakes (or their counterpart veins) followed a uniform pattern, irrespective of the group of boars under consideration or the section in the straw (peripheral v. central). The relative size and shape of the lakes varied within the same straw. The complementary relative surface (in %) of the veins (frozen concentrated extender and included spermatozoa) showed a similar, and homogenous, width in both groups of boars but without a linear trend of solidification.

The MFPs showed larger lakes than did the medium-straws (**Paper III**), the latter showing a highly asymmetrical organisation of lakes and veins depending on the position of the section. Smaller lakes were seen in the periphery and centre of the medium-straw, while larger lakes were seen in intermediate areas (**Paper III**). The degree of dehydration seemed higher in the MFPs, since the areas of individual lakes appeared larger, thus leading to thinner/smaller veins (**Paper III**).

In sum, the ultrastructure of the frozen boar semen was clearly defined by two major domains, the frozen free water (lakes) and the concentrated, CPA extender including the spermatozoa (veins). The latter became more homogenous the slower the freezing rate applied, probably in relation to a higher degree of dehydration of

the components of the veins towards the lakes. There were clear differences between containers, particularly in comparison with the maxi-straws, which depicted major regional differences.

Quantitative differences between frozen areas

The difference in size of the "lakes" between locations was numerically larger in the maxi-straws than in the FPs (**Paper I**), probably owing to differences in the degree of dehydration of the spermatozoa and the extender. The area of the "ice lakes" was smaller the faster the rate of cooling, with a dramatic difference between 2°C/min and 1,200°C/min rates ($P<0.001$), being more conspicuous (area-wise) for maxi-straws than for FPs (**Paper I**). Numerical differences in the relative surface of the ice lakes between containers were only significant for the extreme speeds of cooling (**Paper I**). Spermatozoa frozen at 2°C/min or 50°C/min appeared very dehydrated, as did the surrounding extender in which they were embedded. By contrast, plunging into LN_2 (1,200°C/min) caused freezing of water both extra- and intra-cellularly **(Paper I**), with clear evidence of sub-cellular distortion caused by the presence of intra-cellular ice crystals in the peri-nuclear and peri-axonemal areas, owing to a lack of sperm dehydration during the process.

The overall relative mean percentage of the lake fraction (i.e. of solid-state free water in the frozen straws) was ~80% of the total surface while that of the veins was ~20%. There were no significant differences ($P>0.05$) between the two groups of boars (**Paper II**). In the "bad" sperm freezers the area of concentrated matrix (veins) averaged 17.7% (range 10.5–21.3%), and the area of ice (lakes) 82.3% (range 78.7–89.6%). The average number of lakes was 35.6 (range 16–64). The corresponding values measured in "good" sperm freezers were 18.5% (range 11.6–22.7%), 81.5% (range 77.7–88.4%) and 47.3 (range 12–111), respectively. None of the variables correlated significantly ($P>0.05$) with the outcome of post-thaw sperm quality (**Paper II**). However, significant inter-boar differences were detected for all the cryo-SEM measures ($P<0.0001$), with one sire showing a higher number of vein compartments, thus leading to a higher number of lakes per area (86.4±17.7, mean ± standard deviation [SD]).

The area occupied by lakes was significantly larger ($P<0.001$) in the MFPs (82%) than in the medium-straws (78%), and the converse was found for the area occupied by veins (18% and 22%, respectively; $P<0.001$), both variables having a low variation between replicates/ejaculates (**Paper III**). The mean number of lakes was lower in the MFPs (~50) compared with the medium-straws (61), but owing to the large variation between ejaculates there were no significant differences within medium-straws (**Paper III**). While none of the cryo-SEM variables correlated significantly with post-thaw sperm quality in the medium-straws, TSM was significantly correlated with cryo-SEM measurements, area of veins, and area of lakes in the MFPs (**Paper III**).

In sum, the size of the extra-cellularly frozen, free water (lakes) depends on the speed of freezing, being larger the slower the freezing, probably because slow

cooling allows water to leave the cells and the surrounding concentrated CPA-containing extender, thus decreasing the size of the veins. At a similar cooling rate range (40–50°C/min), following crystallisation, container size (and shape) influences the size of the lakes/veins, with the MFPs showing the highest homogeneity.

Expression of hexose transporters -3 and -5 in boar spermatozoa

The presence of hexose transporters GLUT-3 and GLUT-5 in boar spermatozoa was confirmed by Western blotting (**Paper IV**), with specific bands of about 50 KDa, which were mainly maintained during cooling (Steps A and B) and post-thaw (Step C). However, in almost all cases, the relative intensity of the GLUT-3 band in supernatants from sperm extracts decreased during the cooling and thawing steps (A–C) compared with equivalent fresh sperm samples, while that of GLUT-5 increased during Steps A–C compared with equivalent, freshly collected spermatozoa (**Paper IV**).

Immunocytochemistry of hexose transporters -3 and -5 in boar spermatozoa

The analyses of the SEM and TEM immunolabellings clearly showed that boar spermatozoa expressed the hexose transporters GLUT-3 and GLUT-5 in both the outer and the inner plasmalemma (**Paper IV**). The expression of the hexose transporter isoform GLUT-3, however, showed differences with GLUT-5 in terms of both location and concentration among spermatozoa, as well as during the various steps of the freezing-thawing protocol used (Steps A–C). The GLUT-3 immunoreactivity was apparently more conspicuous in the acrosome membrane of spermatozoa kept chilled at 17°C (Step A), especially in the anterior half of the head, clearly delimited by the equatorial segment, decreasing in the rest of the domains towards the mid-piece. This pattern of reactivity was maintained at 5°C (Step B) when glycerol was added. Post-thaw (Step C), the immunolabelling became, however, less clear in terms of both intensity and distribution (**Paper IV**). The immunolabelling of the hexose isoform GLUT-5 in spermatozoa kept chilled at 17°C (Step A) was mainly located in the apical and principal segments of the acrosomal region, with moderate labelling in the post-acrosomal region and strong labelling in the connecting piece, the mid-piece and the principal tail segment (**Paper IV**). Contrasting the GLUT-3 pattern, the immunolabelling was maintained at 5°C (Step B) and post-thaw (Step C), thus contrasting the GLUT-3 pattern. Controls were negative.

In sum, boar spermatozoa express the hexose transporters GLUT-3 and GLUT-5 in both the outer and the inner plasmalemma, mostly over the acrosome. The intensity of expression and the relative intensity of immunolabelling decreased during freezing-thawing for GLUT-3 but they were maintained throughout for GLUT-5.

Immunocytochemistry of Aquaporin 7 in boar spermatozoa

Controls were negative, while boar spermatozoa incubated with the AQP7 antibody showed differential immunolabelling depending on the sperm membrane domains considered (**Paper V**). The labelling was distinct in the tail region of most spermatozoa, particularly in spermatozoa from the cauda epididymides. The binding was most prominent over the mid-piece down to the annulus, with more intermittent or absent labelling along the principal piece and the end piece of the sperm tail. Cytoplasmic droplets, either proximally or distally located, were conspicuously immunostained. They were particularly visible in spermatozoa collected from the cauda epididymides, with a tendency for reactivity at the sperm neck region. The intact sperm acrosome domain did not show obvious labelling, but what presumably was unspecific immunostaining could be seen when the acrosome appeared exocytosed during processing (**Paper V**). Aquaporin 7 was more intensely localised in the entire tail and cytoplasmic droplets in spermatozoa from the cauda epididymides than in ejaculated spermatozoa, where it was intensely present only in the mid-piece and the principal tail piece, irrespective of the portion of the ejaculate that the spermatozoa had been collected from. Spermatozoa ejaculated in P1 showed very little labelling in the surrounding seminal plasma, while spermatozoa from P2 showed major background labelling.

Cryopreservation of the ejaculated spermatozoa modified the AQP7 immunolabelling, with a clear redistribution and increasing intensity following cooling and, particularly, freezing-thawing (**Paper V**). At RT (S1) after extension in BTS, and at 15°C (S2, extender 2), the immunostaining was mainly restricted to the sperm neck region and the mid-piece. At S3, when temperature decreased to 5°C and the extender contained the CPA glycerol, immunolabelling was maintained in the same regions. On the other hand, labelling increased post-thaw (at S4), both at the mid-piece and at the sperm neck, and also at the post-acrosomal region. Over the acrosome, a very intense immunolabelling (probably unspecific) was noted.

In sum, boar spermatozoa possess AQP7 at the plasma membrane, with specific domain location. The degree of immunolabelling is influenced by the site of origin (epididymal v. ejaculate), by interactions with seminal plasma (ejaculate fractions P1 v. P2) and by the stages of cryopreservation.

General discussion

Freezing of spermatozoa from various species of domestic and wild animals, and also from humans, has been done for decades, following the discovery that addition of egg yolk and rather simple CPAs (such as glycerol) allows spermatozoa to survive cooling and freezing, and be viable after thawing (Polge *et al* 1949; Smith & Polge 1950; Lovelock & Polge 1954). However, the success of cryopreservation using rather simple extenders and protocols is dependent on many factors, some of them still unknown. Perhaps the most striking of these factors is the difference between species (Holt 2000b). Spermatozoa from fowl or humans can be preserved relatively easily, while those from stallions or boars are more difficult, and marsupial spermatozoa are nearly impossible to recover as viable cells post-thaw (Watson & Holt 2001). There are also differences between individuals in the ability of their spermatozoa to survive freezing and thawing. For the porcine species, the frequency of spermatozoa from those males surviving the process of cryopreservation has, historically, been very low (Johnson *et al* 2000).

About 40% of the red meat consumed worldwide is currently provided by pigs, and increases in production/consumption of up to 25% are foreseen for the next decade (Rodriguez-Martinez 2007a). With such anticipated production figures, genetic selection of boars and sows for desired characters (growth, leanness, litter size, reproductive efficiency, health, etc) is regarded as a priority. In order to increase the dissemination of desired characters from the elite sires to the general breeding population, artificial insemination (AI) has been developed. The technique has grown exponentially since it started in the Soviet Union in the 1920s, when proper extenders to handle boar semen at RT were initially designed (Milovanov 1962; Serdiuk 1970). In the past 20 years, the use of boar semen preserved in liquid form for AI has increased more than threefold; in Europe >90% of the sows are now bred using AI with liquid semen. More than 99% of the approximately 30 million registered first AIs in the world are performed with liquid semen, while the remaining 1% utilise frozen-thawed semen (Wagner & Thibier 2000). Liquid semen is mostly used for genetic improvement at national or regional level while frozen-thawed semen is preferred for international trade, to avoid the movement of live animals.

The reason for this difference in the use of liquid-preserved v. frozen boar semen is the low sperm survival post-thaw. Those spermatozoa that survive usually show signs of a shortened lifespan, i.e. they succumb shortly after thawing, obviously leading to decreased fertility. To compensate for these losses (often ~50%), the total number of frozen-thawed spermatozoa contained in an AI dose is huge, usually in the order of 5 billion spermatozoa. Despite these excessive sperm numbers, fertility, particularly in terms of the number of piglets born after AI, is lower with frozen-thawed semen than with liquid-stored semen. The farrowing rates for cryopreserved spermatozoa are around 10–30% lower, and the litter sizes smaller by one to three piglets than those obtained with fresh, liquid semen stored at moderately reduced temperatures (i.e. 16–20°C) which, in turn, are similar to those reached after natural mating.

Research on freezing of boar semen has been carried out since the first successful inseminations with frozen-thawed boar semen in the early 1970s, leading to the design of the current semen cryopreservation techniques, which became commercially available shortly thereafter. However, these methods are still sub-optimal, and technically demanding. Consequently, boar sperm cryopreservation is currently limited to research, genetic banking and the export of semen for selected nuclei lines (for a review, see Johnson *et al* 2000). Moreover, the freezing procedure is expensive in terms of labour and laboratory equipment, as well as being time-consuming (for a review, see Roca, Rodriguez-Martinez *et al* 2006). Lastly, because of low sperm survival, the number of AI doses that can be prepared from a single ejaculate is low (<10). Taking these factors together, it seems unlikely that deep-frozen semen will replace the use of fresh semen on an extensive basis even if the fertility levels were similar. The procedure is, to put it simply, too expensive.

The major constraints to developing cryopreservation procedures for pigs are the inherent difficulties in freezing spermatozoa from this species (Holt 2000a; 2000b), and also, the sire-dependent cryosurvivability with the current process (Eriksson *et al* 2002; Holt *et al* 2005; Gil *et al* 2005; Waterhouse *et al* 2006; Hernandez *et al* 2006; Roca, Hernandez *et al* 2006). On the technical side, the methods currently used have been empirically designed, often simply extrapolating the techniques used in other species (mostly the bovine). In view of the lack of success, however, attempts are still being made worldwide to design novel cryopreservation methods and containers (Eriksson & Rodriguez-Martinez 2000; Kumar *et al* 2003; Saravia *et al* 2005; Woelders *et al* 2005). The goals of this research are to increase both cryosurvival (Peña *et al* 2003; 2004a, b; 2005, 2006; Roca *et al* 2004) and the number of AI doses per processed ejaculate (Bussiere *et al* 2000; Saravia *et al* 2005). These studies have had moderate success. Fertility post-AI is now substantially improved, in some cases approaching the rates seen with liquid semen (Eriksson *et al* 2002), even when using low sperm numbers. However, to achieve higher results, alternative sites for sperm deposition, such as deep intra-uterine deposition, have been used instead of the conventional, cervical AI (Bathgate *et al* 2005; Roca, Rodriguez-Martinez *et al* 2006). In any case, the problem of individual variability still remains. Methods are being modified (still empirically) in order to accommodate the differences in sperm survival between different sires. For instance, we use different containers to package the semen (from large maxi-straws to medium-straws, which represent a tenfold difference in size, with corresponding differences in cryogenic suitability), as well as different handling methods, new extenders (and CPAs) and different cooling and thawing rates. One way to resolve this confounding situation is to study how the freezing-thawing process affects sperm structures essential for VIA and sperm function. This knowledge may lead to the design of an ideal cryopreservation method, which could be used subsequently for all boars, in order to implement their selection, as is current practice with bull sires for AI.

Most groups dealing with cryopreservation of boar semen use similar extenders (often three, used stepwise), among which hexoses provide an energy source, egg

yolk functions as a cryoprotectant of the plasma membrane following modification (emulsification) with detergents, and glycerol is most commonly used as a CPA at low concentrations (~3%), owing to its toxicity. Most groups cool the semen in two steps, starting with a reduction from the temperature at collection (~ +35ºC), to +20ºC (RT), followed by a further decrease to +5ºC during extension and equilibration, and eventually to a seeding temperature (often –5ºC). This stage is followed by faster cooling (between 40 and 50°C/min or even higher) after the initial nucleation of ice. Packaging of the semen differs, from large containers (such as 5 mL maxi-straws or FPs) to smaller containers (such as 0.5 mL medium-straws or MFPs). Thawing procedures also vary, being adapted to the container in question (volume-wise).

The present thesis has studied the use of these techniques and their effects on the cryobiology of the frozen semen, with particular emphasis on the sperm plasma membrane, a structure that has been defined over the years as that mostly, and primarily, affected by cryopreservation (Watson 1981; Parks & Graham 1992). The sperm plasma membrane is responsible for maintaining the homeostasis of the spermatozoon, particularly regulating the transport of water and essential molecules (such as metabolic substrates). Moreover, it is the structure that interacts with the different environments the spermatozoa sequentially encounter during their migration from the caudal epididymis, along the female genital tract, until they meet the oocytes, with which they interact during fertilisation (Rodriguez-Martinez *et al* 2001). The plasma membrane is, therefore, responsible for destabilisation processes such as capacitation, which primes the spermatozoon for the fusogenic events that lead to the acrosome reaction upon subsequent contact between the sperm plasma membrane and the ZP (receptor-mediated, species-specific contact). Finally, fusion occurs between the oolemma and the post-acrosomal plasma membrane remnants during fertilisation (Yanagimachi 1994).

Most studies of the plasma membrane have focused on its integrity during or following cryopreservation (i.e. post-thawing). These studies employ either light microscopy with phase contrast optics, staining (particularly combining non-permeable dyes against a dark background, such as the classic eosin-nigrosin stain) or, more recently, the use of specific fluorophores, either penetrating or non-permeable (for a review, see Rodriguez-Martinez & Barth 2007). Other, recent studies have focused on determining the degree of (in)stability of the plasma membrane, particularly in relation to the lipid bilayer (Silva & Gadella 2006), which some researchers have linked to a "capacitation-like" phenomenon caused by cooling of the membrane (Green & Watson, 2001). However, more recent studies have indicated that the phenomenon is not linked to capacitation, but, rather, to subtle damage to the plasmalemma, which eventually (post-thaw) causes its disruption and, ultimately, cell death (Guthrie & Welch 2005a; 2005b; Saravia *et al* 2007). In the present studies (**Papers I–IV**), PMI and sperm motility were consistently lower post-thaw than pre-freeze, no matter which container or cooling speed was used, with significant variation between individual animals/breeds. These findings were expected, and they simply confirmed that the freezing method used was not optimal. However, differences were lower when the smallest

packaging containers (medium-straws and MFPs) were used, yielding the highest proportions of LIN spermatozoa post-thaw (**Papers II–IV**).

The present thesis studied the changes occurring during freezing, by examining processed boar semen in a frozen state, using cryo-SEM. The aim was to determine whether the appearance of the frozen material could be linked to VIA post-thaw, mostly recorded as membrane integrity and sperm motility. The methods used included determination of the degree of dehydration that the extender (including the CPA and the spermatozoa) was subjected to during freezing at different rates and in containers of different shapes and volumes.

The cryo-SEM and the accessories used (cryo-chamber, etc) constituted suitable instrumentation for examining these frozen samples, samples that were physically fixed and not subjected to excessive manipulation, thus avoiding the introduction of undesirable artefacts (Nijsse & Van Aelst 1999; Wyss *et al* 2002). In a conventional SEM microscope, placement of a fully hydrated specimen results in the removal of water from the specimen in the high vacuum of the microscope, causing the cells to shrink, deform and die (besides ruining the microscope). In the past, attempts to overcome this problem involved chemical fixation followed by freeze-drying or critical point drying in order to detect indirectly where ice crystals were, for instance (for a review regarding spermatozoa, see Rodriguez-Martinez & Ekwall 1998). These techniques were tedious, and liable to introduce unacceptable artefacts, either by the chemical fixation or through the methods used for dehydration and staining/fracturing. Cryo-SEM permits relatively quick assessment of non-chemically fixed, fully hydrated samples, providing instant views of the different components (such as extender and free water) and their ultrastructure, including internal structures if the fracture plane is favourable. Since the temperature of the sample can be controlled, it can be raised to allow water sublimation during the etching of the surface and thus expose the interface between free water in solid phase, such as extra-cellular ice, and the concentrated solutes, such as extender or spermatozoa. Alternatively, the temperature can be maintained at, or lowered back to, levels below –140°C, thus preventing changes in the original frozen state and allowing for extended examinations in vacuum, without water loss and distortions. The internal structure of cooled specimens can be sequentially revealed by several freeze fractures. Even the structure of highly hydrated cells can be exposed in this way, provided that care has been taken to minimise ice crystal growth. Moreover, cryo-SEM enables quantitative image analysis and, furthermore, X-ray microanalysis of the elemental contents of the sample (Rodriguez-Martinez & Ekwall 1998). Obviously, cryo-SEM would facilitate studies of the effects caused by different cooling rates, containers and location within frozen samples. These data would be beneficial to the development of new handling protocols for both freezing and thawing (Morris *et al* 1999; 2007).

The cryo-SEM ultrastructure of the frozen boar semen was clearly defined by the presence of two major domains, the frozen free water (lakes) and the concentrated CPA extender, which included the spermatozoa (the veins) (**Papers I–III**). These domains are formed during the process of supercooling of the CPA-treated sample, during which water changes phase from the liquid to the solid state, with the initial

formation of ice crystals in the extra-cellular milieu where free water is more abundant. Ice crystals grow and displace the solute-rich, cell-containing extender (the veins), which also loses water to the growing ice masses. These continue enlarging until an equilibrium is formed, either because the veins are dehydrated or because ice is also formed there. The duration of the process depends on the rate of cooling, and also on the volume of the sample to be cooled, the amount of CPA present, the composition of the extender and the number of spermatozoa involved. In the present studies (**Papers I–III**) some of these variables were tested for their effect on the morphology of the semen in a frozen state.

Since the organisation and relative surface of the frozen biological material (veins and lakes) could be easily registered by cryo-SEM, these complementary variables were measured and quantified by image analysis in samples from four different packages (FPs v. maxi-straws (**Paper I**), and medium-straws v. MFPs (**Paper III**)). The apparent organisation of lakes and veins differed between packages, with the MFPs depicting larger lakes than the maxi-straws and FPs, and tending to show a more homogenous distribution of lakes/veins compared with medium-straws. There seems to be a logical relation between lake/vein size and post-thaw viability, with the maxi-straws yielding the lowest proportions of surviving spermatozoa compared with the other containers. Following the classical theory, sperm damage is expected to occur during freezing, by the formation of intra-cellular ice (Mazur, 1984) or by the toxicity of the hyper-osmotic medium (Watson & Fuller 2001), and/or (as is considered today) at thawing, especially when re-warming is slow. The form and volume of the semen sample to be cooled/re-warmed, and the concentration of the contained cells, play a major role in cryo-injury during the most damaging interval in the processes, i.e. during the changes in phase of the extra-cellular water, when heat is either dissipated (during cooling) or incorporated (during re-warming) (Mazur *et al* 1972; Mazur 1984; Mazur & Cole 1985; 1989; Morris *et al* 1999). Maxi-straws and FPs, although holding the same volume, are constructed in different ways, the former being a cylinder and the latter a flat bag. Under current cooling/re-warming, the maxi-straws have longer heat exchange periods, and therefore presumably allow more damage to the cells than the FPs (**Paper I**), which have a larger surface-to-thickness ratio.

In a comparison of the smaller containers (medium-straws v. MFPs, **Paper III**), the cryo-SEM showed differences in the degree of *in situ* dehydration and the levels of homogeneity of freezing of the semen. The MFPs allowed for a more uniform dehydration of the spermatozoa, and higher cryosurvival, as determined by the proportion of LIN spermatozoa measured subsequently by CASA. The lakes in the medium-straws appeared highly asymmetrical, depending on their position in the section and their relative surface per section, with a smaller amount of free water (lakes) and, consequently, thicker veins in the medium-straws than in the MFPs. It was therefore concluded that MFPs allowed for a more homogenous dehydration of the spermatozoa/frozen extender compared with medium-straws, which may account for their somewhat better sperm quality post-thaw. Since the degree of dehydration and heat transfer is higher in MFPs than in straws, and the motility as well as PMI is superior in MFPs compared with straws, it can be concluded that

freezing in MFPs gives a higher proportion of viable cells after thawing, related to differences in surface-to-diameter ratio.

In **Paper I** three different cooling rates following ice nucleation (2°C/min, 50°C/min and 1,200°C/min) were tested for the largest containers. The results of post-thaw survival were dramatically different, the best sperm motility being seen in samples frozen at 50°C/min. These large packages can easily contain 5 mL volume and ~5 billion total spermatozoa, i.e. the customary AI dose for frozen boar semen. In these packages the ice lake area decreased inversely with increasing rapidity of cooling, being particularly evident with the extreme cooling speeds. Although the trend was similar for both maxi-straws and FPs, the differences were more obvious in maxi-straws, especially when comparing locations within the container. The ice lakes were larger in the periphery compared with the central compartment, except for the samples plunged into LN_2. The differences in the size of the lakes obviously relate to the possibility of water being transferred from the veins to the lakes, and therefore the quicker the freezing speed, the smaller the chance of vein dehydration.

The cryo-SEM observations also indicated that the ultrastructure of cryopreserved spermatozoa differed according to the rate at which the sample was cooled after nucleation. At higher magnification, the freeze-etched samples revealed that the spermatozoa had migrated into the freeze-concentrated material during solidification: spermatozoa were not entrapped within ice crystals, particularly when low cooling rates were used (2–50ºC). These frozen spermatozoa did not show evidence of major osmotic shrinkage or presence of ice voids within the sperm heads. On the other hand, some shrinkage was observed in the mid-piece and tail. It is, however, important to bear in mind that the cryo-SEM does not permit detailed observation of the inner structures, as seen with TEM, unless the cryo-fracture plane is suitable.

Samples cooled down at low rates (2–50ºC/min) had a freeze-concentrated material similar to that seen in human and stallion semen, where the overall composition of the veins has been reported to contain more than 60% glycerol, close to the eutectic concentration (Morris 2006; Morris *et al* 2007). At faster cooling rates (3,000ºC/min), in the same studies, the concentration of glycerol in the freeze-concentrated material was only approximately 20%. Although the semen frozen in the present study did not reach such high cooling rates, the sample morphology was similar at 1,200ºC. The present investigation has therefore provided significant insights into the effect of freezing on boar spermatozoa. In particular, the cryo-SEM has extended previous observations made by light microscopy (Holt *et al* 2005), and also by freeze substitution (Ekwall, Eriksson *et al* 1997), that spermatozoa and solutes migrate either entirely into the freeze-concentrated glycerol-rich matrix (veins) when cooled at low rates, or are entrapped in a looser matrix (containing more water) when higher temperature rates are applied (1,200ºC). These results agree with other observations in human and equine spermatozoa (Morris *et al* 1999; 2007).

Intra-cellular ice may be visualised and correlates with loss of boar VIA at thawing (Bwanga, Einarsson *et al* 1991; Eriksson & Rodriguez-Martinez 2000). Although intra-cellular ice has not been identified in human or equine spermatozoa, probably due to the low resolution used while conducting the studies (Morris 2006; Morris *et al* 2007), human/stallion spermatozoa possess more water in the sperm head than porcine spermatozoa do, as determined by the presence of uncondensed chromatin areas in the former species. Moreover, their spermatozoa, or those of primates, which hold a similar chromatin structure (Rutllant *et al* 2003), act as good osmometers, with a high permeability for glycerol (Gao *et al* 1993). By contrast, boar spermatozoa are not good osmometers, and show signs of glycerol toxicity at concentrations above 5% (Iida & Adachi 1966; Larsson 1985). Human spermatozoa can be frozen at very high cooling rates without inducing the death of all spermatozoa (Noiles *et al* 1993), while boar spermatozoa cannot be cooled at very high speeds without leading to cell death (Bwanga, Einarsson *et al* 1991). The CPA glycerol is added during cryopreservation in order to reduce ice crystal formation within the cell. However, glycerol is only effective when slow cooling rates are applied, which is usually the case with spermatozoa (Farrant *et al* 1977). With higher rates (see Bwanga, Ekwall *et al* 1991) intra-cellular ice crystals are formed in boar spermatozoa, although these rapidly cooled cells are apparently injured mainly during re-warming, since their ultrastructure is almost unaltered in the frozen state. Another possibility is that, at high cooling rates, the concentration of glycerol becomes too low in the vicinity of the spermatozoa, which may explain why samples frozen at such speeds usually die. Moreover, the viscosity of the frozen extender varies with the temperature, increasing at lower temperature ranges. The speed of cooling also contributes, not only to this viscosity, but also, to a concerted effect on the diffusion rate for water, which becomes limited and, in turn, restricts the osmotic loss of water from the cells (Morris *et al* 2006). Therefore, it can be concluded that freezing rates of 40–50ºC/min are suitable when boar semen is packaged in cryogenically effective containers, such as FPs, medium-straws or MFPs.

In sum, the size of the extra-cellularly frozen free water (lakes) depends on the speed of freezing. The lakes are larger the slower the freezing, probably since the slower rate allows water to leave the cells and the surrounding concentrated CPA-containing extender, thus decreasing the size of the veins. At a similar cooling rate range (40–50°C/min) following crystallisation, container size (as well as shape) influences the size of the lakes/veins. The MFPs show the most homogenous freezing of boar semen, which is consistent with the highest sperm survival post-thaw.

However, it should be remembered that another major factor affecting sperm recovery is the rate of change in solute concentration, which depends on the rate of cooling below 5ºC (Maldjian *et al* 2005) and, most importantly, on the rate of thawing (Saravia *et al* 2007). It has been observed by SEM that spermatozoa subjected to hypertonic conditions display exvaginations of the head membrane, resulting in a wrinkled surface (Hammerstedt *et al* 1990; Gao *et al* 1993). If this wrinkled surface results in a fusion of contiguous membranes under the influence of hypertonic stress, as has been described in other cell types (Homann 1998), then

an abrupt return to isosmolal conditions would possibly result in cell lysis. Thus, spermatozoa with a hypertonically stressed membrane during cooling would, upon return to isotonic conditions (after thawing), swell above their normal volume, and lyse (Lovelock 1953; Zade-Oppen 1968; Steponkus & Wiest 1979). It is at this point that the limitations to using cryo-SEM become clear. Thawing, which is now regarded as the most critical step in causing membrane disruption (Morris *et al* 2007), cannot, at least not at present, be studied using this instrumentation. Since the sample is in a vacuum, the containing water will sublimate from the sample if the temperature of the chamber is raised above –100°C. Therefore, since the extender and the cells contained therein also contain water (mostly as bound water), the sample will collapse when this water disappears, thus introducing damage not related to the process of thawing.

Moreover, cryo-SEM did not prove useful when studying differences in semen classified as coming from "good" or "bad" freezers (frozen at the same temperature, and using the same extenders and containers) (**Paper II**). As already mentioned, a major limiting factor for commercial cryopreservation of boar semen for AI is the large individual variation in cooling, where the degree of cell dehydration during ice (re)shaping appears to play a major role. Cross-sectioned medium-straws (0.5 mL) containing frozen semen from these boars were examined by cryo-SEM to determine whether differences between groups could be distinguished prior to thawing and would relate to differences in dehydration during cooling. The degree of hydration was monitored in relation to the areas of ice crystals formed extra-cellularly (lakes), the areas of frozen, concentrated extender (veins) where spermatozoa were located, and the degree of compartmentalisation (i.e. number of lakes) present. Irrespective of the region studied, the gradient of dehydration observed along the cross-section area of the straws was very irregular. Most spermatozoa were enclosed in the freezing extender matrix and no obvious signs of external membrane damage were observed. However, none of the cryo-SEM variables correlated significantly with post-thaw sperm parameters, thus invalidating the tested hypothesis. However, significant differences were detected between boars in all ultrastructural variables studied. This led to the conclusion that inter-boar post-thaw sperm differences are not related to the large variability in ice crystal formation during the conventional freezing process, once again disproving the hypothesis. However, thawing may still be the single most important process causing sperm damage and consequently, differences between boars may be visible only post-thaw.

Cold shock of the sperm plasma membrane is considered a predisponent factor for sperm damage (Watson & Morris 1987). Apparently, the sensitivity of the boar sperm membrane to cooling is strongly related to the characteristic lipid composition of this membrane (Watson 1995; Parks 1997), which reacts differently during freezing and thawing (Pettit & Buhr 1998). Moreover, any modifications of the structure of the plasma membrane, including protein deterioration or movement, could very well alter both the housekeeping metabolism of the cell and its ability to move. These effects could be caused by an altered capacity for energy sourcing (Cerolini *et al* 2001) if the proteins involved are related to the transport of substrates. Examples of these proteins are those belonging to the GLUT family,

which are mainly responsible for the transport of hexoses across mammalian sperm membranes (Burant *et al* 1992; Farooqui *et al* 1997; Angulo *et al* 1998; Kokk *et al* 2005). Other proteins may well play a role in the active transport of water, solutes or CPAs, such as the AQPs (Verkman *et al* 1996; Borgnia *et al* 1999; Izumi *et al* 2006). The presence and temporal localisation of representative proteins of these families, i.e. GLUT-3 and GLUT-5, and AQP7, respectively, were studied in boar spermatozoa at different stages during the process of cryopreservation (**Papers IV** and **V**).

Immunocytochemistry, i.e. the fundamental laboratory practice which uses antibodies to target antigenic receptors in order to determine the presence of certain proteins, is probably the technique of choice to determine the precise location of the above-mentioned sperm proteins (Griffits 1993). There are many methods available for performing immunological stains on cells and tissues, including the use of fluorescent or non-fluorescent indicators. With the first method, an antibody is used that is bound either directly to the antigen (marker) on a cell, with a fluorescent or coloured dye directly bound to the antibody, or indirectly, where the primary antibody binds directly to the antigen, and a second antibody, linked to a marker, binds to the primary antibody, thereby allowing the complex to be visualised. Such stainings are most often used for assessment with light microscopy, although the exact location of the antigen may be hard to determine because of the low resolution of the instrument. In order to increase the capacity for locating the antigens more precisely, for instance when trying to determine the sperm plasma membrane domain in which a certain protein is located, immunocytochemistry at the EM level can be performed using either SEM or TEM. The most commonly used method for detecting the antigen is by using a primary antibody which links to the protein of interest, followed by the binding of a secondary antibody that is conjugated with colloidal gold, which is then visible under the electron beam in the SEM or TEM. The technique can also be applied with light microscopy, with an additional incubation with silver that binds to the gold, the so-called "silver enhancement" (Griffits 1993).

Paper IV evaluated the effects of cooling, freezing and thawing on the PMI, and the kinetics and expression of two sugar transporters (GLUT-3 and GLUT-5) in spermatozoa from fertile Iberian boars of the Entrepelado and Lampiño breeds, two ancient pig breeds without pre-selection for sperm freezing. The expression of the proteins was determined by electrophoresis and Western blotting, while their precise localisation and temporal variations during cryopreservation were studied using immunocytochemistry at EM level. The results clearly confirmed that these breeds have not been selected for sperm freezing and that the method used for cryopreservation probably needs to be modified, since the post-thaw survival was very low. The study also showed that boar spermatozoa express both GLUT-3 and GLUT-5, these being localised in both the outer and the inner plasmalemma, mostly over the acrosome. The intensity of expression and the relative intensity of immunolabelling decreased during freezing-thawing for GLUT-3 (dramatically post-thaw) but were maintained for GLUT-5. The GLUT-3 is a very effective glucose transporter (Medrano *et al* 2006), while GLUT-5 is a fructose-specific transporter (Burant *et al* 1992). The specific distribution in the different sperm

domains may link their presence to different sperm metabolic events, either to the housekeeping metabolism (membrane activity, etc) or to sperm motility. Since boar spermatozoa show a very different affinity to utilise various monosaccharides such as glucose, fructose, sorbitol and mannose depending on the location and activity of these transporters, this finding indicates that the exact location of a specific hexose transporter would be the first regulatory step in the utilisation of a particular carbohydrate to obtain energy at a given moment (Medrano *et al* 2006). The relocation of the transporters links these proteins to the deterioration of function (particularly motility, but also membrane viability) in relation to cryopreservation. The maintenance of the GLUT-3 location in spermatozoa kept chilled at 5ºC suggests that the problem is not present up to this point, but that it appears during either sub-zero handling or at thawing.

The expression of GLUT-5 followed a different pattern, without undergoing any major changes in location, distribution or intensity of immunolabelling, despite the spermatozoa being damaged by the cryopreservation process. In mammals, fructose is mainly internalised from the seminal plasma by the GLUT-5 sperm membrane carrier, directly affecting the glycolytic activity of the mid-piece of the tail and, consequently, sperm motility (Grootegoed & Den Boer 1990). Fructose is produced and excreted by seminal vesicles. In boars it represents an important substrate, and it is usually stored for special situations (Sancho *et al* 2004; 2006). However, the affinity for, and rate of use of, fructose by boar spermatozoa is lower than that of glucose, probably owing to the differential substrate affinity depicted by hexokinases (Medrano *et al* 2006). The differential modifications of GLUT-3 and GLUT-5 may follow their association with different non-soluble sperm structures underlying the plasma membrane. For example, GLUT-5 may be linked to the mitochondria, and therefore to motility regulation (Silva & Gadella 2006). On the other hand, GLUT-3 may simply be linked to the plasma membrane, which explains its decrease when cells disrupt their plasma membrane.

Until recently, movement of solutes through the cell membrane was thought to occur only by slow, passive transport regulated by osmotic pressure differences. The discovery of AQPs, special proteins present in the cell membranes of several human organs (Agre *et al* 2002), was, therefore, a breakthrough. These proteins build water channels for active water transport, functioning with a tenfold to 100-fold higher capacity compared with diffusion. Of the two families of AQPs, the most interesting for cryopreservation of spermatozoa are the aquaglyceroporins (AQP3, 7, 9 and 10) since they are, apparently, involved in the transport of glycerol during lypolisis in fat tissue (Rodriguez *et al* 2006). Therefore, the presence and temporal localisation of a member of this family, AQP7, through which water movement appears to be bi-directional, was studied in boar spermatozoa, collected either from the caudae epididymides or from different fractions of the ejaculate (**Paper V**). The rationale behind using these two different sources was that epididymal spermatozoa survive handling and cryopreservation procedures better than ejaculated spermatozoa do (Lasley & Bogart 1944; Woelders & Den Besten 1993; Watson 1995; 2000). Spermatozoa collected in the first portion (P1) of the sperm-rich fraction of the ejaculate have also been proved to survive handling and cryopreservation procedures better compared with

spermatozoa collected in other portions of the seminal plasma (P2) or even compared with the whole ejaculate (Rodriguez-Martinez *et al* 2005). Survival to handling and cryopreservation is apparently related to the different amounts (rather than the kind) of seminal plasma proteins present in these fractions (Rodriguez-Martinez *et al* 2005; 2007; Saravia *et al* 2007). Lastly, the movement of water and cryoprotectants such as glycerol across the sperm plasma membrane plays a central role in sperm survival post-thaw. Therefore, if the rate of water transport or the replacement of water by glycerol, which occurs during cell cryopreservation, is modulated by AQPs, then these molecules rank highly among substances that can be used as markers for functional monitoring of this process.

Aquaporin 7 is present in both epididymal and ejaculated boar spermatozoa, thus confirming the hypothesis tested in **Paper V**. Aquaporin 7 was specifically and more intensely localised in the entire tail and the cytoplasmic droplets in cauda epididymidal spermatozoa compared with ejaculated spermatozoa, where it reached the same intensity only in the mid-piece and the proximal tail. The portion of the ejaculate the spermatozoa originated from did not influence the distribution of AQP7. Interestingly, AQP7 appeared in the plasma membrane domains where more water is intra-cellularly present (see **Paper III**) and where the cell dehydrates the most during cryopreservation, rather than in the sperm head, where the least water has been reported in boar spermatozoa (Ekwall, Eriksson *et al* 1997). The presence of seminal plasma from P2 contributed to extra-cellular labelling, probably in an unspecific manner. Processing of ejaculated spermatozoa modified the immunolabelling, with a clear redistribution and increasing intensity following cooling and freezing-thawing, indicating that the distribution of AQP7 was affected by these procedures. The acrosomal region was disturbed during processing of the samples and also post-thaw (during acrosome exocytosis) and caused an increase, probably unspecified, of immunolabelling over this particular domain. The role of AQP7 is controversial, since it has been related to fertility in the human (Saito *et al* 2004) but did not appear to be essential for fertility in AQP7 knockout (KO) mice (Sohara *et al* 2007). However, this does not necessarily mean that AQP7 is not involved in sperm function, since some of the other members of the family may have acted as back-up for the missing protein in the KO mice.

Why AQP7 was more strongly present in the epididymal caudal spermatozoa than in the ejaculated spermatozoa is not known. Boar caudal spermatozoa survive freezing and thawing better than ejaculated spermatozoa do, with less damage to the plasma membrane. Whether a higher amount of AQP7 is related to this higher resilience to cryopreservation remains to be studied. However, it is interesting that the labelling in the ejaculated spermatozoa decreased dramatically compared with the epididymal labelling. The ejaculated spermatozoa showed virtually the same labelling irrespective of whether they were collected in P1, P2 or the whole set of seminal plasma (i.e. the whole ejaculate), suggesting that there was no relation between surrounding proteins (epididymal or seminal) and the expression of AQP7. In the present studies, perhaps the most relevant link of AQP7 is with glycerol, since this is the CPA most frequently used when preserving boar spermatozoa. During the different steps of cooling, AQP7 binding was relatively higher when spermatozoa were cooled (5ºC) and exposed to glycerol (pre- and

post-thaw) (S2–S4), suggesting that it is during these stages that AQP7 becomes more visible (and perhaps active). Such observations of the distribution pattern of AQP7 and its temporal change may make it useful as a marker in studies of cooling and re-warming effects. Obviously, more studies are needed, including studies on other AQPs, to shed more light on their role in cryopreservation and hopefully facilitate the design of better cryopreservation protocols.

General conclusions

- The degree of dehydration of boar spermatozoa/frozen extender (measured as areas of extra-cellular, unbound frozen water) differed between single-dose (5 mL) containers (maxi-straws and FPs), the location of free water within the container and the cooling speed, explaining differences in post-thaw VIA, and suggesting that FPs permit a more homogenous freezing of boar semen.

- There was large variability among boars regarding ice crystal formation during the conventional freezing of medium-straws (0.5 mL), which was apparently unrelated to inter-boar post-thaw sperm differences.

- The MFP (0.5 mL) seemed to allow for a more homogenous dehydration of the spermatozoa/frozen extender compared with the medium-straw, which may explain the relatively better sperm quality observed post-thaw.

- The hexose transporters GLUT-3 and GLUT-5 were present in boar spermatozoa. Their pattern of expression was characteristic, in terms of location and concentration, although the expression was maintained only for the isoform GLUT-5 during cryopreservation steps, suggesting that cryopreservation affects the status of spermatozoa by altering the distribution of GLUT-3.

- Aquaporin 7 was present in the plasma membrane of boar spermatozoa, with differences in location between epididymal and ejaculated sources. Cryopreservation of ejaculated spermatozoa modified the AQP7 immunolabelling, which was most intense following thawing, thus suggesting that AQP7 could be an interesting marker for non-empirical studies of boar semen cryopreservation.

In summary, cryo-SEM is a valuable instrument to determine changes in the dehydration of boar spermatozoa during cryopreservation. The results clearly indicate that cooling rates, semen containers and boar influenced the amount of frozen free water (and, consequently, dehydration of the CPA-concentrated extender). Such differences affected post-thaw sperm survival, which was best using MFPs. Scanning electron microscopy and TEM immunocytochemistry was useful in determining the precise localisation of membrane molecules of relevance for sperm metabolism (hexose transporters GLUT-3 and GLUT-5) and glycerol transport/de(re)hydration, such as AQP7. Although EM techniques, particularly cryo-SEM, are expensive and require highly skilled personnel, they are a prerequisite to perform these in-depth studies.

References

Abaigar, T., Holt, W.V., Harrison, R.A., & Del Barrio, G. 1999. Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. *Biology of Reproduction 60*: 32–41.

Agre, P., King, L.S., Yasui, M., Guggino, W.B., Ottersen, O.P., Fujiyoshi, Y., Engel, A., & Nielsen, S. 2002. Aquaporin water channels – from atomic structure to clinical medicine. *Journal of Physiology 542*: 3–16.

Angulo, C., Rauch, M.C., Droppelmann, A., Reyes, A.M., Slebe, J.C., Delgado-López, F., Guaiquil, V.H., Vera, J.C., & Concha, I.I. 1998. Hexose transporter expression and function in mammalian spermatozoa: cellular location and transport of hexoses and vitamin C. *Journal of Cellular Biochemistry 71*: 189–203.

Armitage, W.J., & Mazur, P. 1984. Osmotic tolerance of human granulocytes. *American Journal of Physiology 247 (Cell Physiol 16)*: C373–C381.

Bathgate, R., Eriksson, B., Maxwell, W.M.C., & Evans, G. 2005. Low dose deep intrauterine insemination of sows with fresh and frozen-thawed spermatozoa. *Theriogenology* 63: 553–554.

Belbin, L. 1993. PATN, Pattern Analysis Package. Division of Wildlife and Ecology. Canberra, Australia: CSIRO.

Borgnia, M., Nielsen, S., Engel, A., & Agre, P. 1999. Cellular and molecular biology of the aquaporin water channels. *Annual Review of Biochemistry 68*: 425–458.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry 72*: 248–254.

Burant, C.F., Takeda, J., Brot-Laroche, E., Bell, G.I., & Davidson, N.O. 1992. Fructose transporter in human spermatozoa and small intestine is Glut 5. *Journal of Biological Chemistry 267*: 14523–14526.

Burnette, W.M. 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated proteins. *Journal of Analytical Biochemistry 112*: 195–203.

Bussiere, J.F., Bertaud, G., & Guillouet, P. 2000. Conservation of boar semen by freezing. Evaluation in vitro and after insemination. *32èmes Journées de la Recherche Porcine en France 32*: 429–432.

Bwanga, C.O., Einarsson, S., & Rodriguez-Martinez, H. 1991. Cryopreservation of boar semen. II. Effect of cooling rate and duration of freezing point plateau on boar semen frozen in mini-, maxi-straws and plastic bags. *Acta Veterinaria Scandinavica 32*: 455–461.

Bwanga, C.O., Ekwall, H., & Rodriguez-Martinez, H. 1991. Cryopreservation of boar semen. III. Ultrastructure of boar spermatozoa frozen ultra-rapidly at various stages of conventional freezing and thawing. *Acta Veterinaria Scandinavica 32*: 463–471.

Calamita, G., Mazzone, A., Bizzoca, A., & Svelto, M. 2001. Possible involvement of aquaporin-7 and -8 in rat testis development and spermatogenesis. *Biochemical and Biophysical Research Communication 28*: 619–625.

Carvajal, G., Cuello, C., Ruiz, M., Vazquez, J.M., Martinez, E.A., & Roca, J. 2004. Effects of centrifugation before freezing on boar sperm cryosurvival. *Journal of Andrology 25*: 389–396.

Cerolini, S., Maldjian, A., Pizzi, F., & Gliozzi, T.M. 2001. Changes in sperm quality and lipid composition during cryopreservation of boar semen. *Reproduction 121*: 395–401.

Courtens, J.L., & Paquignon, M. 1985. Ultrastructure of fresh, frozen and frozen-thawed spermatozoa of the boar. *In: Johnson, L.A., Larsson, K. (Eds.), Deep Freezing of Boar Semen*, vol. 1. Uppsala, Sweden: Swedish University of Agricultural Sciences, pp. 61–87.

Courtens, J.L., Ekwall, H., Paquignon, M., & Plöen, L. 1989. Preliminary study of water and some element contents in boar spermatozoa, before, during and after freezing. *Journal of Reproduction and Fertility 87*: 613–626.

Courtens, J.L., Ekwall, H., & Plöen, L. 1992. Comparison of nuclear hydration in boar spermatozoa before and after freeze-thawing: contrast analysis of cells embedded in bromide-labelled medium. *Microscopy Research Techniques 21*: 249–254.

Critser, J.K., Ameson, B.W., Aaker, D.V., & Ball, G.D. 1987. Cryopreservation of human spermatozoa. II. Post-thaw chronology of motility and of zona-free hamster ova penetration. *Fertility and Sterility 47*: 980–984.

Critser, J.K., Huse-Benda, A.R., Aaker, D.V., Ameson, B.W., & Ball, O.D. 1987. Cryopreservation of human spermatozoa. Effects of holding procedure and seeding on motility, fertilizability, and acrosome reaction. *Fertility and Sterility 47*: 656–663.

Curry, M.R. 2000. Cryopreservation of semen from domestic livestock. *Reviews of Reproduction 5*: 46–52.

Ekwall, H., Eriksson, B., Courtens, J.L., & Rodriguez-Martinez, H. 1997. Ice granulometry and cell damage in boar semen frozen in two types of containers. *Infusionstherapie und Transfusionsmedizin 24*: 376.

Ekwall, H., Eriksson, B., & Rodriguez-Martinez, H. 1997. Cryo-EM of frozen boar semen. *Proceedings of the Annual Meeting of the Royal Microscopical Society, "Low temperature microscopy and analysis"*, 6–9 July 1997, York, UK, p. 2.

Eriksson, B.M., & Rodriguez-Martinez, H. 2000. Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in large 5 ml packages (FlatPack). *Animal Reproduction Science 63*: 205–220.

Eriksson, B.M., Petersson, H., & Rodriguez-Martinez, H. 2002. Field fertility with exported boar semen frozen in the new FlatPack container. *Theriogenology 58*: 1065–1079.

Farooqui, S.M., Fakhri, A.I-B., O'Donnell, J.M., & Stout, R. 1997. Degenerative changes in spermatogonia are associated with loss of glucose transporter (Glut-3) in abdominal testis of surgically induced unilateral cryptorchidism in rats. *Biochemical and Biophysical Research Communications 236*: 407–412.

Farrant, J., Walter, C.A., Lee, H., Morris, G.J., & Clarke, K.J. 1977. Structural and functional aspects of biological freezing techniques. *Journal of Microscopy 111*: 17–34.

Gao, D.Y., Ashworth, E., Watson, P.F., Kleinhans, F.W., Mazur, P., & Critser, J.K. 1993. Hyperosmotic tolerance of human spermatozoa: separate effects of

glycerol, sodium chloride and sucrose on spermolysis. *Biology of Reproduction 49*: 112–123.

Gao, D.Y., Mazur, P., & Crister, J.K. 1997. Fundamental cryobiology of mammalian spermatozoa. *In: Karow, A.M., Crister, J.K. (Eds.), Reproductive Tissue Banking.* San Diego, CA: Academic Press, pp. 263–327.

Gil, M.A., Roca, J., Cremades, T., Hernandez, M., Vazquez, J.M., Rodriguez-Martinez, H., & Martinez, E.A. 2005. Does multivariate analysis of post-thaw sperm characteristics accurately estimate in vitro fertility of boar individual ejaculates? *Theriogenology 64*: 305–316.

Green, C.E., & Watson, P.F. 2001. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. *Reproduction 122:* 889–898.

Griffits, G. 1993. Fine structure immunocytochemistry. Berlin: Springer-Verlag, 449 pp.

Grootegoed, J.P., & Den Boer, P.J. 1990. Energy metabolism of spermatids: a review. In: *Hamilton, D.W., Waites, G.M.H. (Eds.), Scientific Basis for Fertility Regulation. Cellular and Molecular Events in Spermiogenesis.* Cambridge: Cambridge University Press.

Guthrie, H.D., & Welch, G.R. 2005a. Impact of storage prior to cryopreservation on plasma membrane function and fertility of boar sperm. *Theriogenology 63*: 396–410.

Guthrie, H.D., & Welch, G.R. 2005b. Effects of hypothermic liquid storage and cryopreservation on basal and induced plasma membrane phospholipid disorder and acrosome exocytosis in boar spermatozoa. *Reproduction, Fertility and Development 17*: 467–477.

Hammerstedt, R.H., Graham, J.K., & Nolan, J.P. 1990. Cryopreservation of mammalian sperm: what we ask them to survive? *Journal of Andrology 11*: 73–88.

Hernandez, M., Roca, J., Ballester, J., Vázquez, J.M., Martinez, E.A., Johannisson, A, Saravia, F., & Rodriguez-Martinez, H. 2006. Differences in SCSA outcome among boars with different sperm freezability. *International Journal of Andrology 29*: 583–591.

Hernandez, M., Roca, J., Gil, M.A., Vázquez, J.M., & Martínez, E.A. 2007. Adjustments on the cryopreservation conditions reduce the incidence of boar ejaculates with poor sperm freezability. *Theriogenology 67*: 1436–1445.

Holt, W.V. 2000a. Basic aspects of frozen storage of semen. *Animal Reproduction Science 62*: 3–22.

Holt, W.V. 2000b. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. *Theriogenology 53*: 47–58.

Holt, W.V., Medrano, A., Thurston, L.M., & Watson, P.F. 2005. The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. *Theriogenology 15*: 370–382.

Homann, U. 1998. Fusion and fission of plasma-membrane material accommodates for osmotically induced changes in the surface area of guardcell protoplasts. *Planta 206*: 329–333.

Iida, I., & Adachi, T. 1966. Studies on deep-freezing of boar semen. I. Effect of various diluents, glycerol levels and glycerol equilibration periods on deep-freezing of boar semen. *Japanese Journal of Zootechnical Sciences 37*: 411–416.

Ishibashi, K., Sasaki, S., Fushimi, K., Uchida, S., Kuwahara, M., Saito, H., Furukawa, T., Nakajima, K., Yamaguchi, Y., Gojobori, T., & Marumo, F. 1994. Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells. *Proceedings of the National Academy of Sciences, USA 91*: 6269–6273.

Ishibashi, K., Kuwahara, M., Gu, Y., Kageyama, Y., Tohsaka, A., Suzuki, F., Marumo, F., & Sasaki, S. 1997. Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea. *Journal of Biological Chemistry 272*: 20782–20786.

Izumi, Y., Sonoda, S., Yoshida, H., Danks, H.V., & Tsumukia, H. 2006. Role of membrane transport of water and glycerol in the freeze tolerance of the rice stem borer, Chilo suppressalis Walker (Lepidoptera: Pyralidae). *Journal of Insect Physiology 52*: 215–220.

Johnson, L.A. 1985. Fertility results using frozen boar spermatozoa: 1970 to 1985. In: *Larsson, K. (Ed). Deep Freezing of Boar Semen*, vol. 1. Uppsala, Sweden: Swedish University of Agricultural Sciences, pp. 199–222.

Johnson, L.A., Weitze, K.F., Fiser, P., & Maxwell, W.M. 2000. Storage of boar semen. *Animal Reproduction Science 62*: 143–172.

Karlsson, J.O.M. 2002. Cryopreservation: freezing and vitrification. *Science 296*: 655–656.

King, K.E. 2004. Blood and bone marrow pathology. *Transfusion 44*: 1119.

Kokk, K., Verajankorva, E., Laato, M., Wu, X.K., Tapfer, H., & Pollanen P. 2005. Expression of insulin receptor substrates 1–3, glucose transporters Glut-1–4, signal regulatory protein 1 alpha, phosphatidylinositol 3-kinase and protein kinase B at the protein level in the human testis. *Anatomical Science International 80*: 91–96.

Kumar, S., Millar, J.D., & Watson, P.F. 2003. The effect of cooling rate on the survival of cryopreserved bull, ram and boar spermatozoa: a comparison of two controlled-rate cooling machines. *Cryobiology 46*: 246–253.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*: 680–685.

Larsson, K. 1985. Boar semen viability after freezing and thawing. In: *Larsson, K. (Ed). Deep Freezing of Boar Semen*, vol. 1, Uppsala, Sweden: Swedish University of Agricultural Sciences, pp. 177–187.

Larsson, K., & Einarsson, S. 1976. Fertility of deep frozen boar spermatozoa: influence of thawing diluents and of boars. *Acta Veterinaria Scandinavica 17*: 43–62.

Larsson, K., & Ersmar, M. 1980. Laboratory studies on frozen-thawed boar semen in relation to contemporary fertility with liquid semen of AI boars. *Zuchthygiene 15*: 111–117.

Lasley, J.F., & Bogart, R. 1944. Some factors affecting the resistance of ejaculated and epididymal spermatozoa of the boar to different environmental conditions. *American Journal of Physiology 141*: 619–624.

Lovelock, J.E. 1953. The hemolysis of human red blood cells by freezing and thawing. *Biochimica et Biophysica Acta 10*: 414–426.

Lovelock, J.E., & Polge, C. 1954. The immobilization of spermatozoa by freezing and thawing and the protective action of glycerol. *The Biochemical Journal 58*: 618–622.

Maldjian, A., Pizzi, F., Gliozzi, T., Cerolini, S., Penny, P., & Noble, R. 2005. Changes in sperm quality and lipid composition during cryopreservation of boar semen. *Theriogenology 63*: 411–421.

Mazur, P. 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *Journal of General Physiology 47*: 347–369.

Mazur, P. 1984. Freezing of living cells: mechanisms and implications. *American Journal of Physiology 247*: C125–C142.

Mazur, P., & Cole, K.W. 1985. Influence of cell concentration on the contribution of unfrozen fraction and salt concentration to the survival of slowly frozen human erythrocytes. *Cryobiology 22*: 509–536.

Mazur, P., & Cole, K.W. 1989. Roles of unfrozen fraction, salt concentration, and changes in cell volume in the survival of slowly frozen human erythrocytes. *Cryobiology 26*: 1–29.

Mazur, P., Leibo, S.P., & Chu, E.H. 1972. A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells. *Experimental Cell Research 71*: 345–355.

Medrano, A., Watson, P.F., & Holt, W.V. 2002. Importance of cooling rate and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. *Reproduction 123*: 315–322.

Medrano, A., Garcia-Gil, N., Ramió, L., Rivera, M.M., Fernández-Novell, J.M., Ramírez, A., Peña, A., Briz, M.D., Pinart, E., Concha, I.I., Bonet, S., Rigau, T., & Rodríguez-Gil, J.E. 2006. Hexose-specificity of hexokinase and ADP-dependence of pyruvate kinase play important roles in the control of monosaccharide utilization in freshly diluted boar spermatozoa. *Molecular Reproduction and Development 73*: 1179–1194.

Milovanov, V.K. 1962. *Biology of Reproduction and Artificial Insemination of Animals.* Moscow: Selhozizdat, 696 pp.

Morris, G.J. 2006. Rapidly cooled human sperm: no evidence of intracellular ice formation. *Human Reproduction 21*: 2075–2083.

Morris, G.J., Acton, E., & Avery, S. 1999. A novel approach to sperm cryopreservation. *Human Reproduction 14*: 1013–1021.

Morris, G.J., Goodrich, M., Acton, E., & Fonseca, F. 2006. The high viscosity encountered during freezing in glycerol solutions: effects on cryopreservation. *Cryobiology 52*: 323–334.

Morris, G.J., Faszer, K., Green, J.E., Raper, D., Grout, B.W.W., & Fonseca, F. 2007. Rapidly cooled horse spermatozoa: loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation. *Theriogenology 68*: 804–812.

Nissje, J., & Van Aelst, A.C. 1999. Cryo-planing for cryo-scanning electron microscopy. *Scanning 21*: 372–378.

Noiles, E.E., Mazur, P., Watson, P.F., Kleinhans, F.W., & Critser, J.K. 1993. Determination of water permeability coefficient for human spermatozoa and its activation energy. *Biology of Reproduction 48*: 99–109.

Ortman, K., & Rodriguez-Martinez, H. 1994. Membrane damage during dilution, cooling and freezing-thawing of boar spermatozoa packaged in plastic bags. *Zentralblatt für Veterinärmedizin A 41*: 37–47.

Parks, J.E. 1997. Hypothermia and mammalian gametes. In: *Critser, J.K. (Ed.), Reproductive Tissue Banking.* San Diego, CA: Academic Press, pp. 229–261.

Parks, J.E., & Graham, J.K. 1992. Effects of cryopreservation procedures on sperm membranes. *Theriogenology 38*: 209–222.

Penfold, L.M., & Watson, P.F. 2001. The cryopreservation of gametes and embryos of cattle, sheep, goats and pigs. In: *(Watson, P.F., & Holt, W.V. (Eds.) Cryobanking the Genetic Resource.* New York, NY: Taylor & Francis, pp. 279–316.

Peña, F.J., Johannisson, A., Wallgren, M., & Rodriguez-Martinez, H. 2003. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Animal Reproduction Science 78*: 85–98.

Peña, F.J., Johannisson, A., Wallgren, M., & Rodriguez-Martinez, H. 2004a. Effect of hyaluronan supplementation on boar sperm motility and membrane lipid architecture status after cryopreservation. *Theriogenology 61*: 63–70.

Peña, F.J., Johannisson, A., Wallgren, M., & Rodriguez-Martinez, H. 2004b. Antioxidant supplementation of boar spermatozoa from different fractions of the ejaculate improves cryopreservation: changes in sperm membrane lipid architecture. *Zygote 12*: 117–124.

Peña, F.J., Saravia, F., García-Herreros, F., Nuñez, I., Tapia, J.A., Johannisson, A., Wallgren, M., & Rodriguez Martinez, H. 2005. Identification of sperm morphological subpopulations in two different portions of the boar ejaculate and its relation to post thaw quality. *Journal of Andrology 26*: 716–723.

Peña, F.J., Saravia, F., Nuñez-Martinez, I., Johannisson, A., Wallgren, M., & Rodriguez Martinez, H. 2006. Do different portions of the boar ejaculate vary in their ability to sustain cryopreservation? *Animal Reproduction Science 93*: 101–113.

Pettit, M.J., & Buhr, M.M. 1998. Extender components and surfactants affect boar sperm function and membrane behaviour during cryopreservation. *Journal of Andrology 19*: 736–746.

Polge, C., Smith, A.U., & Parkes, A.S. 1949. Revival of spermatozoa after vitrification at low temperatures. *Nature 164*: 666.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology 17*: 208–212.

Rigau, T., Rivera, M., Palomo, M.J., Fernandez-Novell, J.M., Mogas, T., Ballester, J., Peña, A., Otaegui, P.J., Guinovart, J.J., & Rodriguez-Gil, J.E. 2002. Differential effects of glucose and fructose on hexose metabolism in dog spermatozoa. *Reproduction 123*: 579–591.

Roca, J., Gil, M.A., Hernandez, M., Parrilla, I., Vazquez, J.M., & Martinez, E.A. 2004. Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. *Journal of Andrology 25*: 397–405.

Roca, J., Hernandez, M., Carvajal, G., Vázquez, J., & Martinez, E. 2006. Factors influencing boar sperm cryosurvival. *Journal of Animal Sciences 84*: 2692–2699.

Roca, J., Rodriguez-Martinez, H., Vazquez, J.M., Bolarin, J.M., Hernandez, M., Saravia, F.,Wallgren, M., & Martinez, E.A. 2006. Strategies to improve the fertility of frozen-thawed boar semen for artificial insemination. In: *Ashworth, C.J., Kraeling, R.R. (Eds.) Control of Pig Reproduction VII.* Thrumpton, UK: Nottingham University Press, pp. 261–275.

Rodriguez, A., Catalán, V., Gómez-Ambrisi, J., & Frübeck, G. 2006. Role of aquaporin-7 in the pathophysiological control of fat accumulation in mice. *FEBS Letters 580*: 4771–4776.

Rodriguez-Martinez, H. 2007a. Reproductive biotechnology in pigs: what will remain? In: *Wiseman, J., Varley, M.A., McOrist, S., Kemp, B., Paradigms in Pig Science.* Nottingham, UK: Nottingham University Press, pp. 263–302 (ISBN 978-1-904761-56-3).

Rodriguez-Martinez, H. 2007b. State of the art in farm animal sperm evaluation. *Reproduction, Fertility and Development 19*: 91–101.

Rodriguez-Martinez, H., & Ekwall, H. 1998. Electron microscopy in the assessment of cryopreserved spermatozoa viability. *Microscopy & Analysis* May: 11–13.

Rodriguez-Martinez, H., & Barth, A.D. 2007. In vitro evaluation of sperm quality related to in vivo function and fertility. In: *Juengel, J.I., Murray, J.F., Smith, M.F. (Eds.) Reproduction in Domestic Ruminants VI.* Nottingham, UK:. Nottingham University Press, pp. 39–54.

Rodriguez-Martinez H., Tienthai, P., Suzuki, K., Funahashi, H., Ekwall, H., & Johannisson, A. 2001. Oviduct involvement in sperm capacitation and oocyte development. *Reproduction. Suppl 58: Control of Pig Reproduction VI,* 129–145.

Rodriguez-Martinez, H., Saravia, F., Wallgren, M., Tienthai, P., Johannisson, A., Vázquez, J.M., Martínez, E., Roca, J., Sanz, L., & Calvete, J.J. 2005. Boar spermatozoa in the oviduct. *Theriogenology 63*: 514–535.

Rodriguez-Martinez, H., Saravia, F., Wallgren, M., Roca, J., & Peña, F.J. 2007. Influence of seminal plasma on the kinematics of boar spermatozoa during freezing. *Theriogenology (in press).*

Rutllant, J., Pommer, A.C., & Meyers, S.A. 2003. Osmotic tolerance limits and properties of Rhesus monkey (*Macaca mulatta*) spermatozoa. *Journal of Andrology 24*: 534–541.

Saito, K., Kageyama, Y., Okada, Y., Kawakami, S., Kihara, K., Ishibashi, K., & Sasaki, S. 2004. Localization of aquaporin-7 in human testis and ejaculated sperm: possible involvement in maintenance of sperm quality. *The Journal of Urology 172*: 2073–2076.

Sancho, S., Pinart, E., Briz, M., Garcia-Gil, N., Badia, E., Bassols, J., Kadar, E., Pruneda, A., Bussalleu, E., Yeste, M., Coll, M.G., & Bonet, S. 2004. Semen quality of postpubertal boars during increasing and decreasing natural photoperiods. *Theriogenology 62*: 1271–1282.

Sancho, S., Rodriguez-Gil, J.E., Pinart, E., Briz, M., Garcia-Gil, N., Badia, E., Bassols, J., Pruneda, A., Bussalleu, E., Yeste, M., Casas, I., Palomo, M.J., Ramió, L., & Bonet, S. 2006. Effects of exposing boars to different artificial light regimens on semen plasma markers and "in vivo" fertilizing capacity. *Theriogenology 64*: 317–331.

Saravia, F., Wallgren, M., Nagy, S., Johannisson, A., & Rodriguez-Martinez, H. 2005. Deep freezing of concentrated boar semen for intra-uterine insemination: effects on sperm viability. *Theriogenology 63*: 1320–1333.

Saravia, F., Hernandez, M., Wallgren, M.K., Johannisson, A., & Rodriguez-Martinez, H. 2007. Controlled cooling during semen cryopreservation does not induce capacitation of boar spermatozoa. *International Journal of Andrology (in press).*

Serdiuk, S.I. (1970) *Artificial Insemination of Pigs.* Moscow: Kolos, 144 pp.

Silva, P.F., & Gadella, B.M. 2006. Detection of damage in mammalian sperm cells. *Theriogenology 65*: 958–978.

Smith, A.U., & Polge, C. 1950. Survival of spermatozoa at low temperatures. *Nature 166*: 668–669.

Sohara, E., Ueda, O., Tachibe, T., Hani, T., Jishage, K., Rai, T., Sasaki, S., & Uchida, S. 2007. Morphological and functional analysis of sperm and testes in Aquaporin 7 knockout mice. *Fertility and Sterility 87*: 671–676.

Steponkus, P.A., & Wiest, S.C. 1979. Freeze-thaw induced lesions in the plasma membrane. In: *Lyons, J.M., Graham, D., Raison, J.K. (Eds.) Low Temperature Stress in Crop. The Role of the Membrane.* New York, NY: Academic Press, pp. 231–254.

Thurston, L.M., Watson, P.F., & Holt, W.V. 1999. Sources of variation in the morphological characteristics of sperm subpopulations assessed objectively by a novel automated sperm morphology analysis system. *Journal of Reproduction and Fertility 117*: 271–280.

Thurston, L.M., Siggins, K., Mileham, A.J., Watson, P.F., & Holt, W.V. 2002. Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. *Biology of Reproduction 66*: 545–554.

Thurston, L.M., Watson, P.F., & Holt, W.V. 2002. Semen cryopreservation: a genetic explanation for species and individual variation? *Cryo Letters 23*: 255–262.

Tsukaguchi, H., Shayakul, C., Berger, U.V., Mackenzie, B., Devidas, S., Guggino, W.B., Van Hoek, A.N., & Hediger, M.A. 1998. Molecular characterization of a broad selectivity neutral solute channel. *Journal of Biological Chemistry 273*: 24737–24743.

Verkman, A.S., Van Hoek, A.N., Ma, T., Frigeri, A., Skach, W.R., Mitra, A., Tamarappoo, B.K., & Farinas, J. 1996. Water transport across mammalian cell membranes. *American Journal of Physiology 270*: C12–C30.

Wagner, H.G., & Thibier, M. 2000. World statistics for artificial insemination in small ruminants and swine. *Proceedings of the 14th International Congress of Animal Reproduction, Stockholm, Sweden. Abstracts, 2: 15*:3.

Walcerz, D.B., & Karow, A.M. 1996. Cryopreservation of cells for tissue engineering. *Tissue Engineering 2*: 85–96.

Wales, R.G., & White, I.G. 1959. The susceptibility of spermatozoa to temperature shock. *Journal of Andrology 19*: 211–220.

Wang, F., Feng, X., Li, Y., Yang, H., & Ma, T. 2006. Aquaporins as potential drug targets. *Acta Pharmacologica Sinica 2*: 395–401.

Waterhouse, K.E., Hofmo, P.O., Tverdal, A., & Miller, R.R. Jr. 2006. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. *Reproduction 131*: 887–894.

Watson, P.F. 1981. The effects of cold shock on sperm cell membranes. *In: Morris, G.J., Clarke, A. (Eds.). Effects of Low Temperature on Biological Membranes.* London, UK: Academic Press, pp. 189–218.

Watson, P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reproduction, Fertility and Development 7*: 871–891.

Watson, P.F. 2000. The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science 60–61*: 481–492.

Watson, P.F., & Plummer, J.M. 1985. The responses of boar sperm membranes to cold shock and cooling. *In: Johnson, L.A., Larsson, K. (Eds.). Deep Freezing of Boar Semen*, vol. 1. Uppsala, Sweden: Swedish University of Agricultural Sciences, pp. 113–127.

Watson, P.F., & Morris, G.J. 1987. Cold shock injury in animal cells. *Symposia of the Society for Experimental Biology 41*: 311–340.

Watson, P.F., & Fuller, B.J. 2001. Principles of cryopreservation of gametes and embryos. *In: Watson, P.F., & Holt, W.V., (Eds.) Cryobanking the Genetic Resource.* New York, NY: Taylor & Francis, pp. 21–46.

Watson, P.F., & Holt, W.V. 2001. Organizational issues concerning the establishment of a genetic resource bank. *In: Cryobanking the Genetic Resource.* New York, NY: Taylor & Francis, pp. 113–122.

Weitze, K.F., Rath, D., & Baron, G. 1987. New aspects of preservation of boar sperm by deep freezing in plastic tubes [in German]. *Deutsche Tierärztliche Wochenschrift 94*: 485–488.

Westendorf, P., Richter, L., & Treu, H. 1975. Zur Tierfgefrierung von Ebersperma: Labor- und Besamungsergebnisse mit dem Hülsenberger Pailleten Verfahren (Deep freezing of boar sperm. Laboratory and insemination results using the Hulsenberger paillete method) [in German]. *Deutsche Tierärztliche Wochenschrift 82*: 261–267.

Woelders, H., & Den Besten, M. 1993. Cryopreservation of boar semen with small between-boar variation of post-thaw sperm survival. *Cryobiology 30*: 645.

Woelders, H., Matthijs, A., Zuidberg, C.A., & Chaveiro, A.E. 2005. Cryopreservation of boar semen: equilibrium freezing in the cryomicroscope and in straws. *Theriogenology 63*: 383–395.

Wongtawan, T., Saravia, F., Wallgren, M., Caballero, I., & Rodriguez-Martinez, H. 2006. Fertility after deep intra-uterine artificial insemination of concentrated low-volume boar semen doses. *Theriogenology 65*: 773–787.

Wyss, H.M., Hütter, M., Müller, M., Meier, L.P., & Gauckler, L.J. 2002. Quantification of microstructures in stable and gelated suspensions from cryo-SEM. *Journal of Colloid and Interface Sciences 248*: 340–346.

Yanagimachi, R. 1994. Mammalian fertilization. In: *Knobil, A., Neill, J.D. (Eds.)The Physiology of Reproduction.* New York, NY: Raven Press, pp. 189–317.

Yoshida, M. 2000. Conservation of sperms: current status and new trends. *Animal Reproduction Science 60–61*: 349–355.

Yudin, A.L., Gottlieb, W., & Meizel, S. 1988. Ultrastructural studies of the early events of the human sperm acrosome reaction as initiated by human follicular fluid. *Gamete Research 20*: 11–24.
Zade-Oppen, A.M. 1968. The effect of mannitol, sucrose, raffinose and dextran on posthypertonic hemolysis. *Acta Physiologica Scandinavica 74*:195–206.

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Populärvetenskaplig sammanfattning

Artificiell insemination på gris med färsk, utspädd sperma har ökat dramatiskt under senare år. Spermierna, späds med en väldefinierad buffert och kan sen lagras vid 16-20° C i flera dygn innan de deponeras i hongrisens könsorgan. Insemination med dessa färska spermier medför vanligen lika goda resultat när det gäller dräktighet och antalet födda griskultingar, som efter naturlig parning.

Konservering av galtspermier sker genom djupfrysning till -196° C (flytande kväve), där de kan förvaras i flera år, finns beskriven sedan 1970-talet. Djupfrysningsprocessen för spermier är dock lång och innehåller många steg med tillsats av olika ämnen vid olika temperaturer innan de slutligen överförs till flytande kväve för lagring. Frysning som metod, för att bevara galtspermierna för semin, är fortfarande inte optimal och de flesta spermier (ofta mer än 50 %) överlever inte proceduren eller skadas så mycket att de inte förblir befruktningsdugliga efter upptining. Dessutom finns det en stor variation mellan hangrisar, där vissa ”fryser” bra och andra dåligt.

När en cell utsätts för långsam nedkylning till temperaturer under 0° C fryser det omgivande vattnet till is även om man tillsätter frysskyddande medel som glycerol (jämförbart med glykol i bilkylaren!). Denna is blir allteftersom och med sjunkande temperatur omgiven av en ökande koncentration av salter, vilket påverkar det osmotiska trycket hos cellen. Cellerna, som önskar vidmakthålla det osmotiska trycket, svarar med att släppa ifrån sig vatten, vilket inte enbart leder till uttorkning (dehydrering), utan även till ökad saltkoncentration inuti cellen. Dessa förändringar sker även vid upptining av cellen fast åt motsatt håll. En snabb uppvärmning är därför nödvändig för att minimera de toxiska effekter som annars skulle uppstå, med celldöd som följd. Studier av frysning under olika hastigheter verkar behövas för att förstå graden av cellpåverkan. De flesta observationer har dock gjorts på upptinade prov och inte på prov som fortfarande varit frysta.

Galtspermier avsedda för semin har traditionellt frysts i stora förpackningar, s.k. maxistrån innehållande 5 ml och 5 miljarder spermier, vilket är ett det antal spermier som används för insemination av fryst sperma. Frysning i maxistrån har dock resulterat i en låg överlevnad och därmed ett lägre dräktighetsresultat. En rund förpackning, som denna, är inte kryobiologiskt optimal, eftersom kyl- respektive värmeöverföringen är låg, vilket förlänger nedkylnings- och upptiningsförloppet. Det är just under dessa faser som de flesta celler dör. Försök med att ändra formen på förpackningarna har gjorts i syfte att förbättra den kryobiologiska situationen. Vid SLU har en platt påse (s.k. FlatPack) testats, som innehåller samma volym och lika många spermier som maxistråna, men där spermierna överlever bättre och även resulterar i ett acceptabelt dräktighetsresultat efter insemination. Ännu högre överlevnad kan fås genom användande av s.k. mediumstrån eller de nyligen vidareutvecklade minipåsarna (s.k MiniFlatPack), som båda innehåller endast 0,5 ml och mellan 500 miljoner till 1 miljard spermier. Även om det är färre spermier som fryses i dessa förpackningar är det sannolikt att utvecklingen vad gäller svinsemin med fryst sperma går mot mindre spermadoser

som kan ge acceptabel fruktsamhet genom exempelvis deponering av sperman inne i livmodern. Den ökade överlevnadsgraden och dräktighetsresultatet som visats vid användning av dessa behållare förknippas med en mer effektiv frysning och uppvärmning, även om studierna som gjorts hittills huvudsakligen begränsats till undersökning av upptinade prover.

Vid studier av frysta celler har vikten lagts vid hur överlevnaden påverkar det fysiska svaret hos cellerna och då framför allt hos membranerna. Fram till idag är kunskapen om dessa skador inte så väldokumenterade, då det inte funnits metoder för denna typ av studier. Undersökningar med kryomikroskop på ljusmikroskopisk nivå är en metod som använts, men där kan man endast följa nedkylnings- och frysförloppet ned till 0° C, eftersom cellerna då fryser till is och det inte går att se detaljerna i dem. Fryssubstitution med efterföljande analys elektronmikroskopiskt är en annan metod som använts för att finna spår av iskristaller som bildats under frysningen, men metoderna är enormt tidskrävande och innefattar kemisk hantering.

Eftersom endast ett fåtal studier har redovisat hur ett fryst spermaprov ser ut under de olika faserna i infrysningen har man i denna avhandling istället använt sig av kryoscanning elektronmikroskopi (kryo-SEM). Metodiken innebär att provet hålls nedfryst vid en temperatur lägre än -120 C och analyseras i detta tillstånd, vilket ger fördelen att ingen kemisk eller annan process påverkat provet. Man kan således studera topografin hos provet och även fördelningen av fryst vatten jämfört med det biologiska materialet i fryst tillstånd. I kryo-SEM överförs det frysta materialet till en behållare, vilken innehåller flytande kväve, där förpackningen öppnas. Provet överförs sedan till en kryo-kammare som har samma vakuum som svep-elektronmikroskopet. Inne i denna kammare bryts provets yta med hjälp av en skalpell, för att blottlägga en fräsch yta. Därefter höjs temperaturen till -90° C, vilket är den temperatur där vatten övergår till gasform,. Förångningen av vatten sker tydligast utanför cellerna eftersom det är där huvuddelen av det icke proteinbundna vattnet finns. Proceduren bidrar till att motverka elektronuppladdningen av provet samt tydliggöra dess strukturer (ökar dess kontrast), vilka förstärks genom att provet därefter täcks med ett elektriskt ledande skikt av tungmetall som förångas över provet. Slutligen förs provet in i mikroskopet för analys. Denna avhandling undersökte hur galtsperma från olika galtar (även klassade som bra respektive dåliga ”frysare”) överlevde frysningen under olika förhållanden. Olika typer av frysförpackningar med olika volym, som kyldes och frystes med olika hastigheter, testades.

Dessutom undersöktes närvaron av proteiner för såväl socker- (energisubstrat), som vatten- och glyceroltransport genom spermiemembranet och hur dessa påverkades av frysning respektive upptining. De förstnämnda proteinerna (s.k. GLUT-proteiner) är ansvariga för att olika sockerformer, mestadels glukos, förs in i spermien. Spermien använder sockret för att utvinna energi för cellmetabolismen, men framförallt för sin rörlighet. Eftersom den största energikällan i spädningsvätskan för galtsperma utgörs av glukos är det av intresse att studera dessa proteiner under frysningsprocessens olika faser för att fastställa om deras funktion påverkas och därmed bidrar till den dåliga överlevnaden efter upptining

som ses idag. De andra proteinerna hör till familjen Aquaporiner, vilka är små proteiner som bildar kanaler genom vilka vatten och glycerol förs in/ut i cellen i hög hastighet. Tidigare trodde man att uttorkningen av cellerna skedde endast för att vatten lämnat cellen passivt genom membranet. Detta är dock en långsam process som inte håller som förklaring för de observationer man dittills gjort. Nu vet man att vatten passerar ett membran på två olika sätt. Dels sker detta genom en passiv transport på grund av det osmotiska trycket och dels genom en aktiv transport genom molekylära vattenkanaler bildade av Aquaporiner, med 100 gånger högre kapacitet än den osmotiskt drivna diffusionen. Funktionellt delas Aquaporinerna in i olika grupper beroende på deras uppbyggnad. En del av dem, såsom Aquaporin-7 (AQP7), kan även förutom vatten, transportera glycerol och andra molekylärt små proteiner. Vattentransporten verkar vara den drivande processen i samband med frysningen som orsakar torkningen. I samband med upptiningen som leder till ett tillbakaflöde av vatten till cellerna samt att glycerol är det mest använda frysskyddsmedlet vid frysning av galtspermier, verkade studier av AQP7 logiska. Frågeställningen var om det fanns AQP7, och i så fall var dessa fanns och om/hur de påverkades av frysning/upptining.

För att kunna undersöka dessa proteiner, som är för små för att kunna särskiljas med de flesta mikroskop, har immunocytokemi använts på elektronmikroskopisk nivå. I första hand har man försökt se om och var dessa proteiner fanns på den yttre spermieytan med hjälp av svepelektronmikroskopi (SEM). När det gäller GLUT-proteiner var man även intresserad av att se om/var de kunde finnas inne i cellen, och då har transmissionelektronmikroskopi (TEM) använts. Skillnaden mellan dessa procedurer är, att i den förstnämnda skannas endast ytan på cellen. I den påföljande snittas först cellen i ultratunna snitt som sedan bombarderas med elektroner för att kunna se var i snittet (utanför liksom innanför cellen) proteinerna finns. För att kunna lokalisera proteinerna av den rätta sorten krävdes dock att man först fäste ett antikroppsprotein till dessa som man märkt med ett elektrontätt material, exempelvis små guldpartiklar, som syns tydligt i elektronmikroskopet. På så vis kunde man lokalisera om, var och hur dessa proteiner varierade under en kryobehandling av galtspermier, antingen samlade från bitestikeln eller från olika delar av ejakulatet.

Resultatet från de fem studierna, som utgör stommen av denna avhandling, har redovisats i förhandsgranskade vetenskapliga tidskrifter, och kan sammanfattas på följande sätt:

Hypotesen som testats när man jämförde de fyra olika frysförpackningarna (5ml maxistrån, 0,5ml mediumstrån, 5ml FlatPack samt 0,5ml MiniFlatPack) var hur graden av torkning hos spermierna påverkades i de olika förpackningarna och hur olika nedkylningshastigheter inverkar på spermiernas överlevnad efter upptining. Kryo-SEM användes vid samtliga studier. Den platta typen av förpackning med 0,5 ml innehåll (MiniFlatPack) resulterade i den högsta frekvensen levande spermier, beroende på en snabbare torkning tack vare förpackningens bättre värmeutbyte. Olika fryshastigheter undersöktes och det visade sig, att 50° C/minut gav den högsta överlevnaden efter upptining. Vid långsamma fryshastigheter ansamlades

mer vatten runt den koncentrerade spädningsvätskan, som innehåller spermierna. Högsta fryshomogenitet hittades hos MiniFlatPacks.

Spermier, som frysts från olika galtar, uppvisar efter upptining olika grad av överlevnad. Troligen beror detta på skillnader i membranernas uppbyggnad, ändrat rörelsemönster, annorlunda sammansättning av seminalplasman eller andra egenskaper, vilket gör att man talar om ”bra” respektive ”dåliga” galtar ur frysbarhetssynpunkt. Studien av ”bra” respektive ”dåliga” frysare med hjälp av kryo-SEM, gav dock inget besked om orsaken då mätningar med hjälp av bildanalys inte visade på några signifikanta skillnader i organisationen i de strån som undersöktes. De förändringar som ger en ”bra” frysare dess egenskaper ligger troligtvis i en bättre tolerans hos membranerna vid kylning/upptining.

Förändringar i membranerna medför ändrad genomsläpplighet för exempelvis de sockerförande membranbundna proteiner som tillhör GLUT-familjen, vilka svarar för regleringen av spermiernas glukos- och fruktosinförsel till spermierna. Genom att använda elektronmikroskopi tillsammans med immunocytokemiska metoder kunde deras närvaro upptäckas. Dessutom kunde man påvisa att både GLUT-3 och GLUT-5 var lokaliserade till både ytter- och innermembranerna, mestadels över spermieakrosomen. Intensiteten i inmärkningen minskade för GLUT-3, men ökade för GLUT-5 efter upptining, vilket tyder på olika funktioner för dessa proteiner.

Slutligen fann man att Aquaporin-7 (kanalproteiner som förutom vatten kan transportera glycerol) förekom i både frysta/upptinade och färska galtspermier, där de framförallt var lokaliserade till spermiesvansens mittstycke. Hos spermier från bitestikeln återfanns samma mönster som hos ejakulerade spermier med tillägg för stark förekomst över den kvarvarande cytoplasmadroppen. Intensiteten av den immunocytokemiska märkningen skiljde sig mellan bitestikel- och ejakulerade spermier samt förändrades under frysning/upptining.

Sammanfattningsvis, kryo-SEM visade sig vara en utmärkt metod för att bedöma och studera frysta spermaprover som inte utsatts för någon bearbetning på kemisk väg. Med hjälp av denna metodik kunde man såväl bedöma som beräkna graden av torkning sperman drabbats av och hur pass homogen frysningen varit beroende på kylningshastighet och förpackningstyp. Med hjälp av elektronmikroskopi av immunocytokemiskt märkta prov kunde man påvisa närvaro av temporära förändringar under frysning/upptining av såväl sockerförande proteiner (GLUT-3 och GLUT-5), som reglerar införsel av energisubstrat (glukos respektive fruktos), som Aquaporin-7 (AQP7) i galtspermiernas plasmamembran. Sammantaget, kan dessa tekniker och markörer användas för framtida icke-empiriska och djupare studier. Även om utrustningen är dyr i inköp och drift, och kräver högt specialiserad teknisk personal, är det uppenbart att detta är en förutsättning för att kunna genomföra experiment av den här typen. Förhoppningsvis kan dessa experiment leda fram till en optimal metod för kommersiell frysning av galtsperma avsedd för insemination.