Cryopreservation of Boar Semen

Impact of the Use of Specific Ejaculate Portions, Concentrated Packaging, and Simplified Freezing Procedures on Sperm Cryosurvival and Potential Fertilising Capacity

Fernando Saravia
Faculty of Veterinary Medicine and Animal Science
Division of Reproduction, Department of Clinical Sciences
Uppsala

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Abstract

Generally, spermatozoa from most boars survive poorly the customary process of cryopreservation. Post-thaw (PT) survivors often show unstable membranes, short life-span, and low fertilising capacity when artificial insemination (AI) is used. This thesis aimed at improving this situation by (i) freezing high sperm numbers in novel packages (MiniFlatPacks™, MFP) and exploring their degree of PT-survival *in vitro* and fertility *in vivo* using deep intrauterine AI (DIU-AI). Moreover, it attempted to find out (ii) why particular portions of the ejaculate better resist cryopreservation and (iii) whether the process could be made attractive for routine use. Spermatozoa from the sperm-rich fraction of the ejaculate (SRF) were densely (2×10^9 sperm/mL) packed into MFP, frozen and studied post-thaw, alongside controls handled in plastic straws. Cells handled in MFP survived better *in vitro* than controls. *In vivo*, single DIU-AIs of these spermatozoa led, despite the very low volume inseminated, to pregnancies, fertilisation rates being higher in MFP than controls, when the interval between DIU-AI and ovulation was -8 to -4 h. Two specific portions of the ejaculate, namely the first 10 mL from the SRF (P1) and (P2) i.e. the rest of the SRF and the post-sperm fraction, were further tested for their resilience to extension, cooling and freezing-thawing *in vitro*, using a battery of tests for kinematics, membrane integrity/stability and chromatin intactness. Most often, P1-spermatozoa best sustained all handling procedures, revealing that capacitation-like sperm destabilisation did not occur during controlled cooling. Such *in vitro* resilience was not intrinsically sperm-related, but differently influenced by the seminal plasma (SP) of the portions (SP-P1, SP-P2). Pooled SP-P1 (with fewer and different proteins, as well as less bicarbonate, than SP-P2) was able to increase PT motility of cleansed P2-spermatozoa to P1 levels. Using all previous findings, a final study was designed to simplify cryopreservation, by freezing P1 in MFP with a shorter (from 8 to 3.5 h) freezing protocol (SF), excluding primary extension and removal of SP by centrifugation. The P1-SF-processed spermatozoa in MFP survived the process equally as well as SRF controls, but without variation among males or ejaculates, implying that this simpler freezing protocol could be advantageously applied to build genetic banks or commercialise frozen semen primarily for DIU-AI using P1. The P2 could still be used for production of conventional AI doses, allowing the AI enterprise to maintain routine management of commercially relevant stud boars.

Keywords: Semen cryopreservation, Artificial insemination (AI), Flow cytometry, Computer-assisted sperm analysis (CASA), Ejaculate portions, Bicarbonate, Seminal plasma (SP), Boar.

Author's address: Fernando Saravia, Division of Reproduction, Department of Clinical Sciences, SLU, Box 7054, 750 07 Uppsala, Sweden.
E-Mail: fernando.saravia@kv.slu.se

Dedication

A Francisca, María Isidora and Sebastián
Mis nenes

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Saravia, F., Wallgren, M., Nagy, S., Johannisson, A. & Rodríguez-Martínez, H. (2005). Deep freezing of concentrated boar semen for intra-uterine insemination: effects on sperm viability. *Theriogenology* 63(5), 1320-1333.

II Wongtawan, T., Saravia, F., Wallgren, M., Caballero, I. & Rodríguez-Martínez, H (2006). Fertility after deep intra-uterine artificial insemination of concentrated low-volume boar semen doses. *Theriogenology* 65(4), 773-787.

III Saravia, F., Hernández, M., Wallgren, M., Johannisson, A. & Rodríguez-Martínez, H. (2007). Controlled cooling during semen cryopreservation does not induce capacitation of spermatozoa from two portions of the boar ejaculate. *International Journal of Andrology* 30(6), 485-499.

IV Saravia, F., Wallgren, M., Johannisson, A., Calvete, J.J., Sanz, L., Peña, F.J., Roca, J. & Rodríguez-Martínez, H. (2008). Exposure to the seminal plasma of different portions of the boar ejaculate modulates the survival of spermatozoa cryopreserved in MiniFlatPacks. *Theriogenology* (In press).

V Saravia, F., Wallgren, M. & Rodríguez-Martínez, H. Freezing of boar semen can be simplified by handling a specific portion of the ejaculate with a shorter procedure and MiniFlatPack packaging. (Submitted for publication).

Abbreviations

AI	Artificial insemination
AN	Annexin-V
AQN	Alanine-Glutamine-Asparagine (heparin-binding) seminal plasma spermadhesin
ASMA	Automated sperm morphology analysis
AWN	Alanine-Tryptophan-Asparagine (heparin binding) seminal plasma spermadhesin
Bic-0	Extender without bicarbonate
BTS	Beltsville thawing solution
CASA	Computer-assisted sperm analysis
CF	Conventional freezing
DFI	DNA fragmentation index
DIU-AI	Deep intrauterine insemination
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
FC	Flow cytometer
FT	Frozen-thawed
GLM	General linear model
LEY	Lactose-egg yolk
LEYGO	Lactose-egg yolk, glycerol and Orvus-ES-Paste
LHD	Lateral head displacement
M-540	Merocyanine-540
MFP	MiniFlatPack™
MS	Medium-straw
P1	Portion 1, 10 first mL of the sperm-rich fraction of the boar ejaculate
P2	Portion 2, the rest of the sperm-rich fraction and the post-sperm fraction of the boar ejaculate

P1CENSP2	Centrifugation of P1 and addition of SP2
P2CENSP1	Centrifugation of P2 and addition of SP1
PI	Propidium iodide
PMI	Plasma membrane integrity
PS	Phosphatidylserine
PSF	Pre-sperm fraction of the boar ejaculate
PSP-I	Porcine seminal plasma protein I
PSP-II	Porcine seminal plasma protein II
PSRF	Post-sperm-rich fraction of the boar ejaculate
RT	Room temperature
SAS	Statistical analysis systems
SCSA	Sperm chromatin structure assay
SD	Standard deviation
SEM	Standard error of the mean
SF	Simplified freezing
SFP	SingleFlatPack™
sHOST	Short hypo-osmotic swelling test
SP	Seminal plasma
SP-P1	Seminal plasma from P1
SP-P2	Seminal plasma from P2
SR	Sperm reservoir
SRF	Sperm-rich fraction of the boar ejaculate
TUS	Transrectal ultrasonography
UTJ	Uterotubal junction
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight linear velocity
X-DFI	Mean DNA fragmentation index

Introduction

Artificial insemination (AI) is a crucial tool to improve global swine populations (Gerrits *et al.*, 2005); basically because it is the most developed and applied biotechnology in pig breeding [rev. by (Rodríguez-Martínez, 2007a)]. Globally, numbers of AIs increased almost exponentially from 6.6% at the start of the 1980s (Reed, 1982) to more than 80% 20 years later (Wagner & Thibier, 2000). Around 99% of those AIs are done with liquid, extended semen, briefly stored (few days) between 16 and 20°C (Wagner & Thibier, 2000). In the European Union (EU) ~84% of all sows/gilts are bred by AI (Feitsma, personal communication). Historically, and on a worldwide basis, the use of frozen-thawed (FT) semen has remained very low, from less than 0.5% in 1985 (Reed, 1985) to ~1% at the beginning of this century (Wagner & Thibier, 2000). Such levels persist (Thibier, personal communication), despite the keen interest of breeding companies in the further development of this biotechnology, since FT-semen could be used in situations in which the widely used fresh or chilled semen, cannot.

What are these anticipated uses? There are several, ranging from gene banking for conservation of genetic diversity and insurance for losses of males (in cases, for instance, of compulsory slaughter due to any infectious outbreak or the elimination of certain breeding lines that eventually result in losses of valuable genetics, or even in case of accidents with superior individuals having particular desirable traits) to commercial advantages. Among the latter, we can enumerate the incorporation of superior genetics to nucleus herds, or the commercialisation of genetics in the international exchange area. In this respect, sales of frozen semen, instead of the customary selling of livestock, imply a better sanitary safeguard of the material *per se*. Samples of semen can be kept frozen until the semen, or the boars that provided those samples, are tested for agents that could be transmitted via semen, thus providing a better warranty for the buyer and a

higher standard for the seller (Yoshida, 2000). However, these expectations are over-shadowed by problems associated with cryopreservation procedures and their post-thaw results, which underlie the restricted use of FT-boar semen. Compared with spermatozoa preserved suspended in liquid extender throughout, the FT-spermatozoa still show a lower survival rate, and many of those cells surviving the process show a shorter lifespan *in vitro* (Larsson & Einarsson, 1976; Waberski *et al.*, 1994; Johnson *et al.*, 2000; Kumar *et al.*, 2003). Obviously, AI with such spermatozoa has resulted in farrowing rates of ~70–80% under controlled breeding management conditions, which are lower than the >90% achieved by conventional AI of liquid semen (Eriksson *et al.*, 2002; Bolarín *et al.*, 2006). Further compromising the outcome for the common breeder are the smaller litter sizes after AI of FT-semen, 2–3 piglets fewer than when liquid semen is used (Johnson, 1985; Almlid & Hofmo, 1996). Additionally, the whole process of freezing an ejaculate is cumbersome, involving many steps and taking more than 8 h from collection to storage of the few doses (often less than 10 doses at 5×10^9 spermatozoa/dose) produced, implying that not many females are finally inseminated with one ejaculate (if two cervical AIs are to be performed per oestrus).

Procedures for cryopreservation of boar semen are periodically reviewed (Bwanga, 1991; Johnson *et al.*, 2000; Groβfeld *et al.*, 2008), enumerating the developments over time, including the resulting fertility of FT-semen. While several additives and changes in the speed of cooling and thawing have been tested, the overall procedure is basically still the same, most methods simply modifying the classical protocol reported by Westendorf more than 30 years ago (Westendorf *et al.*, 1975). Some modifications, which have shown impact *in vitro* [related to post-thaw sperm survival (Eriksson & Rodríguez-Martínez, 2000a) and oocyte penetration ability (Eriksson *et al.*, 2001)], as well as *in vivo* [in terms of on-farm fertility (Eriksson *et al.*, 2002)] involved the use of an alternative package (FlatPack™) which, containing 5×10^9 cells in 5 mL, represented a complete, single AI dose. Unfortunately, as remarked before, such single dosage contains about 10% of the total number of spermatozoa commonly processed per ejaculate.

Can the sperm number per AI dose be decreased? This question has been discussed in the past (Polge *et al.*, 1970; Krueger *et al.*, 1999; Krueger & Rath, 2000; Roca *et al.*, 2003), but considering that deposition of a large volume (~80–100 mL) containing 5×10^9 spermatozoa during cervical AI is usually followed by a reflux of the inseminate from the female genitalia,

there is a fear of decreasing the number of FT-spermatozoa used, risking birth of fewer piglets (Viring & Einarsson, 1981; Steverink *et al.*, 1998; Levis *et al.*, 2002). On the other hand, alternative techniques for AI have evolved, attempting the deposition of semen into the uterus, in lower volumes, and possibly containing fewer spermatozoa per dose. Such transcervical sperm deposition could either be done in the uterine body (Watson & Behan, 2002) or deeply in one uterine horn, the so-called deep intrauterine artificial insemination (DIU-AI) (Martínez *et al.*, 2001a). Either way, these doses could be less voluminous and contain fewer spermatozoa. Experiments done so far have shown pregnancies obtained with 20% of the usual sperm numbers (Roca *et al.*, 2003; Bolarín *et al.*, 2006). Spermatozoa can be frozen in small containers, such as plastic medium-straws (MS, 0.5 mL), but since sperm numbers packed per straw are customarily low, several straws must be pooled post-thaw to build a complete AI dose, even for DIU-AI (Roca *et al.*, 2006). Moreover, the volumes inseminated are larger than the volumes of the straws, usually 7 mL (Roca *et al.*, 2003; Bolarín *et al.*, 2006), thus requiring a re-extension of the sperm suspension, something that would cause sperm damage (Maxwell & Johnson, 1999), due to, for instance, variations in osmolarity. The question is, therefore, whether boar spermatozoa could be frozen in concentrated form, perhaps in numbers closer to those in the cauda epididymides, packed in small containers, and even inseminated in small volumes, thus avoiding re-extension and eventual damage. The rationale for such testing would be to diminish sperm damage and to enable preparation of more doses per ejaculate, packed, frozen, and thawed in optimal containers (using the basic design of the single FlatPack™) for eventual intrauterine deposition (DIU-AI), ultimately avoiding reflux of the inseminated semen.

The current status of boar semen cryopreservation is still considered poor-to-fair (Mazur *et al.*, 2008). Most problematic is that we still do not know, conclusively, the reasons for these sub-optimal results. As already mentioned, compared with freshly collected or extended counterpart spermatozoa, many, if not most, cells subjected to a cooling-freezing-thawing process have a short lifespan. This is presumably due to damage in the plasma membrane, caused, in turn, by differences in water efflux during freezing (dehydration) and the reverse rehydrating process during thawing (Mazur, 1985; Steponkus & Lynch, 1989; Eriksson & Rodríguez-Martínez, 2000b). However, such studies were done in pre-freeze or post-thaw samples, thus losing a view of the spermatozoa in the frozen state. Use of Cryo-scanning electron microscopy (Cryo-SEM), has proven valuable to determine the status of dehydration of spermatozoa and of regional

differences within different containers (Ekwall, 2008), with linkage to post-thaw survival. Among those FT-spermatozoa that survive the process of cryopreservation, some show changes in the plasma membrane [mostly of destabilisation of the lipid bilayer (Buhr *et al.*, 1994)] somewhat reminiscent of the process of sperm capacitation. Such findings have lead researchers to describe this phenomenon as "cryocapacitation" (Watson, 1995). The cryocapacitation concept argues that cooling and cryopreservation induce capacitation-like changes to spermatozoa from several mammalian species, including the pig (Maxwell & Johnson, 1997a), by altering the nature of the plasma membrane, thus leading to shorter lifespan and, consequently, to a reduced fertilising efficiency (Cormier *et al.*, 1997; Bailey *et al.*, 2000; Bailey *et al.*, 2003). A dissonant hypothesis was reported, albeit not referring to boar spermatozoa, claiming that the process of "cryocapacitation" was not equivalent to the physiological mechanism of sperm capacitation, the prerequisite for fertilisation in mammals (Thomas *et al.*, 2006), suggesting that the damage to the plasma membrane is basically caused by thawing. Since this is a very relevant question, boar spermatozoa should be monitored for changes of destabilisation of the plasma membrane, which might resemble capacitation or preclude membrane deterioration.

The boar ejaculate is a voluminous (200–300 mL) and particular mixture of aliquots of spermatozoa from the caudae epididymides [about 2–5% v/v, (White, 1958)] suspended in a fluid, the seminal plasma (SP, 95% v/v of the ejaculate) composed by epididymal cauda contents, and secretions of the accessory sexual glands. However, during the characteristic, relatively extense ejaculation process of the boar, this huge amount of fluid is not homogeneously released at once. Semen is expelled in jets in a fractionated way, usually with three distinct fractions, although some authors have reported the presence of four (Xu *et al.*, 1996). The fractions, artificially divided by their appearance, are called (i) the pre-sperm (PSF, dominated by the secretions of the urethral and bulbourethral glands, as well as the prostate), (ii) the sperm-rich (SRF, containing the vast majority of spermatozoa, and where the epididymal fluid in which they originally bathe is, after reaching the urethra, step-wise diluted with fluids derived from the seminal vesicles and the prostate) and, (iii) the post-sperm-rich [PSRF, that contains fewer and fewer spermatozoa over time, and where the fluid is primarily derived from the increasing secretion of the seminal vesicles, the prostate and, by the end of the ejaculation, the bulbourethral glands (Einarsson, 1971; Mann & Lutwak-Mann, 1981)].

Most ejaculated spermatozoa (80–90%) are in the SRF, and thus this fraction is the one customarily collected for semen processing (either for extension or cryopreservation). The number of spermatozoa expelled in the SRF is uneven, with most spermatozoa ejaculated in the first 10–15 mL of the SRF, and sperm numbers decreasing thereafter (Rodríguez-Martínez *et al.*, 2005). Studies reported in this particular paper suggested those spermatozoa in the first portion (Portion 1, P1) of the SRF ought to be considered, among the ejaculated spermatozoa, as those primarily colonising the sperm reservoir in the oviduct of the sow and, therefore, considered as those mainly (and potentially) involved in fertilisation (Rodríguez-Martínez *et al.*, 2005). Moreover, several studies have suggested boar spermatozoa from this P1 are those that best survive manipulation (including cryopreservation) (Sellés *et al.*, 2001; Peña *et al.*, 2003a; Peña *et al.*, 2004). Although reasons for such resilience were not fully provided, there is a suggestion that the SP of the particular portions have positive actions on the spermatozoa (Peña *et al.*, 2003a). In any case, there is a need to discover whether differences can be determined between spermatozoa from this particular P1-portion of the SRF and those contained in the rest of the ejaculate (called Portion 2, P2). Studies should include all possible steps of extension, cooling, and freezing-thawing, attempting to identify where changes in sperm kinematics and plasma membrane integrity or stability (all essential attributes for fertilisation) might diverge.

Boar SP (as bulk, without differentiation by portions) modulates sperm motility (Rodríguez-Martínez, 1991), the resistance to cold-shock (Pursel *et al.*, 1973), the stability of the plasma membrane (Maxwell & Johnson, 1999), and even the prevention of "cryo-induced" DNA damage (Fraser & Strzezek, 2007). Most of these studies and also those attempting to disclose effects of SP on sperm survival (Maxwell *et al.*, 1998; Maxwell & Johnson, 1999; Vadnais *et al.*, 2005; Maxwell *et al.*, 2007; Vadnais & Roberts, 2007; Okazaki *et al.*, 2008) have also used bulk SP as additive. However, there are differences in the amounts of total SP-protein content and of the different types of SP-proteins (Rodríguez-Martínez *et al.*, 2005; Hernández *et al.*, 2007) between portions of the boar ejaculate. The P1 contains less and somewhat different SP-proteins than P2 (Calvete *et al.*, 2005; Rodríguez-Martínez *et al.*, 2005). Therefore, differences in SP-proteins could well be linked to the different abilities of the spermatozoa bathing in the portions. For instance, such exposure might explain why sperm kinematics differs among spermatozoa in an ejaculate (defining heterogeneity) (Abaigar *et al.*, 1999; Abaigar *et al.*, 2005).

It is, however, not easy to unravel possible roles of the SP on cryopreservation of boar spermatozoa, mainly because of its complex composition, and the possible different roles *in vivo*. The SP-protein profile has been the most studied (Caballero *et al.*, 2004; Caballero *et al.*, 2005; Caballero *et al.*, 2006; García *et al.*, 2006; Jonakova *et al.*, 2007; Manaskova & Jonakova, 2008), despite the SP having other components (Mann & Lutwak-Mann, 1981). For instance, the SP contains ions of utmost importance for sperm function [(such as Ca^{++} or bicarbonate (Einarsson, 1971; Rodríguez-Martínez *et al.*, 1990; Rodríguez-Martínez, 1991)]. Whether these ions are differentially represented in the various portions of the ejaculate and whether, ultimately, they affect cryopreservation, is unresolved. Attempts should be made to determine whether the SP of the various portions/fractions could influence variables indicating sperm resilience to cryopreservation, such as sperm kinematics, plasma membrane activity, and chromatin integrity; the first-named essential for fertilisation and the last, for early embryo development. Moreover, it would be important to determine which component of the SP might be involved.

The current protocol to cryopreserve boar semen has — as already mentioned — not been substantially modified for the past 33 years, although some progress in sperm survival has been made, mostly by changes that have not simplified the process, rather, the opposite. Even with these better survival rates, the major drawback of freezing boar semen, for example, the need to employ at least 8 h to process an ejaculate makes the process unattractive for routine use. Therefore, attempts should be made to shorten the procedure, making it more simple and less depending on heavy equipment, such as refrigerated centrifuges, while guaranteeing acceptable cryosurvival.

Aims of the study

The overall aim of the present thesis was to improve available methods of cryopreserving ejaculated boar spermatozoa, to the extent that the method could be commercially applied.

The study specifically aimed to test whether:

- boar spermatozoa could be frozen, concentrated (2×10^9/mL), in a novel flat container, the MiniFlatPack™ (MFP, 0.5–0.7 mL), potentially usable for deep intrauterine AI,
- frozen-thawed boar spermatozoa highly packed in MFP or MS could be inseminated in low volumes via deep intrauterine AI and lead to pregnancies,
- cooling would affect plasma membrane architecture or sperm motility of ejaculated boar spermatozoa temporally present in two different sub-sets of seminal plasma (SP),
- the SP content in different portions of the boar ejaculate would affect sperm attributes (kinematics, membranes, and chromatin) during cryopreservation, and whether
- the methodology for cryopreserving boar spermatozoa could be simplified, using experimental findings and methodological changes.

Material and methods

Animals

Ejaculated spermatozoa were collected from 20 sexually mature boars (aged 1.5 to 5 years) of three breeds (Swedish Yorkshire, Swedish Landrace, and Norwegian Landrace) that were solely selected by clinical normality, acceptable semen quality, and proven fertility after AI of their liquid-preserved semen. No boar was pre-selected for semen freezability. All boars were kept on straw beds in individual pens, with sows in the vicinity. Multi-parous crossbred sows (Swedish Landrace × Swedish Yorkshire, n= 42) with a lactation of 5 weeks and a mean parity of 3.5 farrowings (range: 2–5) were purchased from a commercial herd and brought directly after weaning to the Division of Reproduction, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. Boars and sows were fed according to Swedish standards (Simonsson, 1994) and were provided with water *ad libitum*. The experimental protocols had previously been reviewed and approved by the Ethical Committee for Experimentation with Animals, Uppsala Sweden.

Collection of semen

Semen was collected with regular rest periods of 6–7 (**Papers I–III**) or 3–4 (**Papers IV** and **V**) days between collections. Each boar was allowed to mount a dummy and semen was manually collected with the gloved-hand technique. Different portions/fractions of the ejaculate were selectively collected, depending on the experiment in question, for example, as the sperm-rich fraction (SRF) (**Papers I–II** and **V**) and as Portions 1 and/or 2 (**Papers III–V**). The SRF and P2 were collected in a plastic bag inside an insulated thermos flask, and the P1 in a pre-warmed 12 mL-plastic tube.

Only ejaculates with at least 70% motile spermatozoa and 75% morphologically normal spermatozoa were used. Immediately post-

collection, the semen was held at room temperature (RT, +20 to +22°C) in its own SP (as portions or as SRF) for 60 min (**Papers I** and **II**) or 30 min in the dark (**Paper V**), before extension with Beltsville Thawing Solution (either BTS+®, L'Aigle, France [(**Papers I** and **II**, **IV** and **V**, Exp III) or self-made extender (**Paper V**, Exp II)] or directly extended 1:3 (P1) or 1:1 (P2) with BTS+® (**Paper III**).

In **Paper IV**, P1 and P2 were split. One aliquot was kept as P1 or P2 for 60 min in the dark at RT. The other aliquot was centrifuged twice (800 × g for 10 min) to separate the SP (supernatant, SP-P1 or SP-P2, see above) from the spermatozoa (pellet). The sperm pellets from each portion were thereafter mixed 1:9 with thawed, pooled (mixed aliquots of SP from the same boars included in the study) SP from P2 (SP-P2), or alternatively, with thawed, pooled SP from P1 (SP-P1) from the same boars and kept in the dark for 60 min at RT, a treatment referred to as "P1CENSP2" or "P2CENSP1", respectively.

Seminal plasma separation

Seminal plasma (SP) from P1 and P2 was used as a replacement "additive" prior to freezing in **Paper IV**. Aliquots from P1 and P2 were centrifuged five times (3,000 g × 20 min each time), removing the supernatant SP to a clean tube before each consequent centrifugation. After the last centrifugation, the harvested SP was separately filtered through disposable filters of 0.2 μm diameter (Filtropur S 0.2; Sarstedt AG & Co, Nümbrecht, Germany), passed to 10 mL tubes, and stored at -20°C, separately for each boar, ejaculate, and portion until use.

Determination of seminal pH and bicarbonate concentrations (Paper V, Exp I)

Semen, as portions P1 or P2, and also as SRF, was (in different opportunities) collected from five mature stud boars, either in plastic 12 mL tubes (P1) or into an insulated thermos flask (P2 and SRF). Determinations of seminal pH and its relative concentrations of bicarbonate were done immediately after collection using a hand-held blood analysis system with a cartridge CG8+ for acid-base analyses of pH and bicarbonate (i-STAT®, Abbott Laboratories, Abbott Park, IL, USA).

Sperm motility changes during liquid storage of semen in extenders with various concentrations of bicarbonate (Paper V, Exp II)

Immediately after collection of P1 and P2, each specific portion from each male was pooled, sperm concentration adjusted to 50 × 10^6 spermatozoa/mL, and extended in test extenders with a common base [glucose, 205.3 mM/L; sodium citrate dihydrate, 20.39 mM/L; potassium

chloride, 5.4 mM/L; ethylenediaminetetra-acetic acid (EDTA), 3.35 mM/L; penicillin-G Na 0.6 g/L and dihydrostreptomicin 1.0 g/L]; where the concentration of sodium bicarbonate was adjusted to 0, 5, 10, or 15 mM/L (Bic-0 to 15). BTS+® (IMV, L'Aigle, France), containing 22.5 of bicarbonate mM/L, was used as control extender. The extended semen (as 100 mL) was kept at 16–17°C in the dark. The temperature of semen incubation was checked daily before motility assessments. Sperm motility was evaluated using a computer-assisted sperm analysis (CASA) instrument, immediately after collection (at time 0), and every 24 hours (at time 0 and after 30 min of incubation, both at 38°C) until day 5 (120 h). Additionally, semen as P1 or P2 extended in Bic-0, were incubated until 144 h and one aliquot was re-exposed to control BTS+®. Sperm motility was then assessed at time 0 and after 30 min of incubation, both at 38°C.

Sperm cryopreservation

Semen was processed using basically the same protocol throughout **Papers I–IV,** with a simplification for **Paper V**. Particular modifications as reported in each paper will be referred to when pertinent. Generally (**Papers I–IV**), semen (as SRF or portions) was immediately (**Paper III**), or following an SP-holding time (see above), extended with BTS+® (1:1 for SRF and P2, or 1:3 for P1) and kept at +15°C for 3 h. After this period, the semen was centrifuged twice at 800 × g for 10 min (**Papers I, II** and **IV**). After centrifugation, the supernatant was discarded and sperm concentration (Bürker haemocytometer) determined, before spermatozoa were re-suspended in a lactose-egg yolk (LEY) extender [80 mL (80%, v/v, 310 mM) of β-lactose + 20 mL hen's egg yolk] to reach the following final sperm concentrations for the different containers used: SFP: 1 (**Paper I**); MS: 2 (**Papers I** and **II**); MFP: 2 (**Papers I** and **II**), or 1×10^9 spermatozoa/mL (**Papers III–V**). After thorough mixing, the semen was further cooled to +5°C for 2 h, after which the semen was slowly mixed with a LEYGO extender (89.5 mL LEY extender, 1.5 mL of Equex STM (Nova Chemicals Sales Inc., Scituate, MA, USA), which is equivalent to Orvus Es Paste (Graham et al., 1971) and glycerol to yield a final concentration of glycerol of 3%), and packed. In the simplified method (**Paper V**), the semen was — following the 30 min pre-freezing SP-incubation at RT — mixed with LEY and cooled down to +5°C within 1.5 h, after which the semen was slowly mixed with LEYGO and packed.

Semen was packed at +5°C inside a cool cabinet (IMV, L'Aigle, France) in different packages; 0.5 mL volume plastic medium-straws (MS, Minitüb, Tiefenbach, Germany) (**Papers I and II**), 5 mL plastic single FlatPack™ (SFP, **Paper I**) and/or MiniFlatPack™ (MFP, i.e., four 0.7 mL (**Papers I** and **II**) or 0.5 mL (**Papers III–V**) volume segments of a SFP). The MS were sealed with PVC powder, while heat-sealing was used for the Packs

(SFP: end-sealed; MFP: segment- and end-sealed) and placed in conventional (MS) or specially designed freezing racks (Eriksson & Rodríguez-Martínez, 2000b).

All racks were transferred to the chamber of a computer-controlled freezer (Mini Digitcool 1400, IMV, L'Aigle, France) initially set at +5°C. The cooling/freezing rate, equal for all packages, was as follows: 3°C/min from +5°C to -5°C, one min for crystallisation, and thereafter 50°C/min from -5°C to -140°C (**Papers I–V**). The samples were then plunged into liquid N_2 (-196°C) for storage. The different packages were thawed in a water bath, with different temperature/time rates depending on the package: The MS were thawed at 35°C for 20 sec (**Paper I**) or 50°C for 12 sec (**Paper II**); SFP (**Paper I**) were thawed at 50°C for 13 sec and MFP at 35°C for 20 sec (**Papers I–V**).

Sperm evaluation

Assessment of sperm kinematics

In different stages during cryopreservation (S1= after collection, suspended in BTS+®; S2= at 15°C, suspended in LEY; S3= at 5°C, suspended in LEYGO; S4= post-thaw, in **Papers III** and **IV**) and after thawing (**Papers I, II** and **V**), the semen suspension was extended with 20–22°C BTS+® + LEY extender (95 mL BTS+® and 5 mL LEY) at a 1:20 ratio, to give a sperm concentration of ~50×10^6 spermatozoa/mL, which was considered optimal for the CASA equipment (SM-CMA; MTM Medical Technologies, Montreaux, Switzerland). The inclusion of the LEY extender prevented the spermatozoa from sticking to the chamber glass during motility evaluation.

The re-extended, thawed semen was placed into 38°C incubation for 30 min (**Papers I–V**) and/or up to 120 min (**Paper V**). Five microlitres of semen were placed in a pre-warmed 10 μm-deep Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and warmed to 38°C. Sperm motility was assessed in a microscope equipped with 38°C microscope stage and phase contrast optics (200×, Optiphot-2, Nikon, Japan), both subjectively (**Paper I**) and using the CASA instrument (**Papers I–V**) at 0 (**Papers I**, **IV** and **V**), 30 (**Papers I–V**), 60 (**Paper I**), and/or 120 min (**Paper V**).

For each sample, eight predetermined optical fields around the central reticulum of the chamber were used to count a minimum number of 200 spermatozoa per sample. Besides the percentage of total motile spermatozoa, the following motility patterns were recorded: linearly motile spermatozoa (%); non-linearly motile spermatozoa (%); circularly motile spermatozoa (%); locally motile spermatozoa (%); straight linear velocity (VSL, μm/sec);

average path velocity (VAP, μm/sec); curvilinear velocity (VCL, μm/sec); lateral head displacement (LHD, μm). The parameter settings for the SM-CMA software were: 32 frames with a spermatozoon present in at least 16 in order to be counted; time resolution 20 ms (50 Hz). An object with VAP <10 μm/sec was considered immotile and objects with a velocity >25 μm/sec were deemed as motile. Spermatozoa deviating <10% from a straight line were designated as linearly motile, and those spermatozoa with a radius <25 μm were classified as circularly motile.

Assessment of sperm plasma membrane status

Sperm plasma membrane stability and integrity measurements (**Papers I** and **III–V**) were carried out with an LSR flow cytometer (FC, Becton Dickinson, San José, CA, USA) equipped with a HeCd ultraviolet (UV) laser (325 nm, 8 mW), an argon ion laser (488 nm, 20 mW) and a HeNe laser (633 nm, 17 mW) as excitation sources. The FC was used at low flow rate (6–24 μL/min).

Plasma membrane integrity (PMI) was assessed (**Papers I**, **IV** and **V**) using the LIVE/DEAD® Sperm Viability Kit L-7011 (Molecular Probes Inc., Eugene, OR, USA) (Garner & Johnson, 1995) where aliquots (one mL) of the sperm suspension were loaded with SYBR-14 and propidium iodide (PI) fluorophores, incubated at 38°C for at least 10 min before either FC (**Papers I** and **IV**) or epifluorescent microscopy (**Paper V**) were used for evaluations. FC-data from at least 10,000 gated events per sample were collected in list mode, and spermatozoa classified as live (SYBR+/PI-), dying (SYBR+/PI+), or dead (SYBR-/PI+). In **Paper V** (Exp III), PMI was assessed using a Dialux 20 microscope (Leitz, Wetzlar, Germany) at 40× magnification. Two experienced operators counted 200 spermatozoa per sample each, accepting a maximum of 10% counting differences between operators. Spermatozoa were classified as live (SYBR+, green) or dead (SYBR-/PI+, red). Additionally, in **Paper I**, *in vitro* membrane functionality was evaluated using the rapid hypo-osmotic swelling test (sHOST) as previously described for boar spermatozoa (Perez-Llano *et al.*, 2001), on the same semen doses as for FC-analysis. In brief, an aliquot (100 μL) of post-thaw semen was immediately added to BTS+®, adjusted with distilled water to obtain a hypo-osmotic test solution (75 mOsm) and incubated at 38°C for 5 min. Following incubation, 200–300 μL of the hypo-osmotic suspension was fixed in the same adjusted BTS-solution plus 5% formaldehyde. Two trained operators counted 100 spermatozoa each, using documented guidelines (Jeyendran *et al.*, 1984) at 40× magnification in a microscope equipped with phase-contrast optics (Laborlux 12, Leitz, Jena, Germany). The outcome was accepted only if a <10% difference was encountered between operators and averaged.

Early sperm plasma membrane destabilisation was checked by exteriorisation of the phospholipid phosphatidylserine (PS) at different stages during cryopreservation (S1-S4, **Papers III** and **IV**). PS-exteriorization was detected by an Annexin-V-FITC apoptosis detection kit I and II (Pharmigen, San Diego, CA, USA), using previous protocols for boar semen (Peña *et al.*, 2003b) with slight modifications, mainly for better exclusion of no-sperm events using Hoechst 33342 (Invitrogen, Carlsbad, CA, USA). Staining and incubation procedures were conducted at RT. FC-acquisitions were stopped after recording 50,000 H33342-positive events. For the gated cells, the percentages of viable spermatozoa with a stable plasmalemma [Annexin-V (AN)-negative/PI-negative (PI–)], spermatozoa with an unstable yet intact plasma membrane (AN+/PI–), and membrane-damaged cells (AN–/PI+), as well as double positive (AN+/PI+) cells were evaluated based on quadrants determined from single-stained and unstained control samples.

Merocyanine-540 (M-540) was used to evaluate the appearance of early lipid scrambling in the sperm plasma membrane at different stages during cryopreservation (S1-S4, **Paper III**). The staining protocol used for M-540 was according to Januskauskas *et al.* (2005). FC-acquisitions were stopped after recording 10,000 H33342-positive events. Dot plots regions were set to differentiate three populations: viable with low M-540, viable with high M-540, and dead cells (Yo-Pro-1 stained).

Sperm chromatin structure assay (Paper IV)

Chromatin structure was assessed using the *in situ* denaturation procedure originally designed by Evenson *et al.* (1980) and described in detail by Januskauskas *et al.* (2001). Prepared samples were analysed using a FACStar^{PLUS} FC (Becton Dickinson Immunochemistry Systems, San José, CA, USA) equipped with standard optics and an argon-ion laser (Innova 90; Coherent, Santa Clara, CA, USA) tuned at 488 nm and running at 200 mW. At least 10,000 events were measured at a flow rate of ~200 cells/sec. Equivalent instrument settings were used for all samples. Data collection was carried out using CellQuest, version 3.3. Further calculations were performed using FCS Express version 2 (De Novo Software, Thornhill, Ontario, Canada). Sperm DNA fragmentation index (DFI) was established by calculating the amount of red fluorescence divided by the total (red plus green) fluorescence, indicating the amount of denatured sperm DNA over the total DNA present. For each sample analysed, the procedure generated a mean DFI (X-DFI), a standard distribution of DFI (SD-DFI) expressed in channel numbers 1–1,024, and a percentage of spermatozoa with detectable DFI (% DFI); all calculated from the DFI frequency histogram.

Assessment of acrosome integrity (**Paper V**)

Acrosome morphology was evaluated immediately after thawing, fixing spermatozoa from either freezing protocol with a solution of 2% buffered formalin (Hancock, 1957), and kept at RT in the dark until analysis. Two hundred spermatozoa were counted on wet smears with a light microscope equipped with contrast phase optics (Laborlux 12, Leitz, Jena, Germany) at 1000× magnification, per sample. A very experienced operator evaluated the samples, disclosing whether a spermatozoon had either normal or deviating acrosome morphology, including breakage, as described by Bane (1961).

Artificial Insemination (**Paper II**)

Detection of oestrus and ovulation

Oestrous detection started from day two after weaning. The sows were observed for signs of proestrus every 12 h, primarily by inspection of the vulva for reddening and swelling. When the sows revealed some signs of oestrus, the detection was performed every 4 h until the end of oestrus. Sows that revealed standing reflex to back pressure were considered to be in oestrus. The time of onset of oestrus was defined as the first time a sow revealed a back-pressure response, minus 2 h. The moment when oestrus ended was defined as the last time a sow revealed a standing response plus 2 h. The same procedures for oestrus detection were applied in the first and second oestrus post-weaning. Transrectal ultrasonography (TUS) of both ovaries was performed with an annular array sector scanner probe (5 MHz) (Scanner 250, Pie Medical b.v., Maastricht, The Netherlands) 18 h after the onset of the 1st and 2nd oestrus post-weaning, the ovaries scanned every 4 h until completion of ovulation. At each scanning, the estimates of follicle numbers and the average diameter of presumptive ovulatory follicles (i.e., >4 mm) were recorded. Ovulation time was defined as the first scanning when no presumptive ovulatory follicles were seen, minus 2 h. If the follicular count was not zero, but lower than previously, the ovulation was assumed to have just started, and called time 0 (Soede *et al.*, 1992).

Deep intrauterine insemination technique

Deep intrauterine-artificial inseminations (DIU-AI) were performed in the 2nd oestrus post-weaning, in relation to the expected spontaneous ovulation (Mburu *et al.*, 1995). Sows were randomly allotted for DIU-AI in one of three groups: (1) single DIU-AI, 8 h before expected ovulation, (2) single DIU-AI, 4 h before expected ovulation, and (3) double DIU-AI, 12 and 4 h before expected ovulation. Occurrence of ovulation was confirmed by TUS and recorded. The DIU-AIs were performed as described by Martínez *et al.* (2002) with a flexible intrauterine catheter (Firflex®, University of Murcia, Spain). Sows were inseminated without sedation or immobilisation. FT-

semen from MFP or MS was then infused into the flexible catheter by use of a temperated syringe. Since the volume of the intrauterine AI-catheter inner lumen was previously measured to approximately 2 mL, the same volume of extender was used to flush the catheter clean of the previously infused semen. The degree of easiness of the DIU-AI was recorded for each sow. In those sows not returning to oestrus, TUS was performed at day 28 from onset of oestrus, to confirm pregnancy.

Statistical analyses

Data, as mean values [using angular (Bliss), logarithmic, or square root transformation, where necessary] or as least square means for post-thaw sperm parameters, number of foetuses, number of spermatozoa in the oviductal sperm reservoir, and of accessory spermatozoa per oocyte, membrane and chromatin measurements were examined by analysis of variance (ANOVA) using the Statistical Analysis Systems software (SAS Institute Inc., Cary, NC, USA, 1994) (**Papers I–V**), using the general linear model (GLM) or the PROC MIXED procedures. Flow cytometry–derived data were first tested using a Kolgomorov-Smirnov test to determine the normality of data distribution. For non-normally distributed data (**Papers III–V**) the non-parametric Mann-Whitney U-test or non-parametric procedures (NPAR1WAY) were used. The statistical models used included the effects of boar, the packaging system, the interaction between boar and package (**Paper I**); the number of AIs and the interval AI-to-ovulation (**Paper II**). In **Paper III**, the statistical model included the fixed effects of stages and males; and the interaction between stages and males within each portion separately. In **Paper IV**, the statistical model included the fixed effects of male, stage, and treatment, and their interaction. In **Paper V**, the statistical models (Exps I–III) included the fixed effects of male, batch, time, treatment, and their interaction. Differences were considered significant at $P < 0.05$ (**Papers I–V**) or $P < 0.1$ (Exp II, **Paper V**).

Results

Spermatozoa used in the experiments included in this thesis were derived from a total of 160 ejaculates (as SRF or in portions) from 20 sires. Only two ejaculates (both from the same boar) were discarded, owing to an occasional low sperm concentration (**Paper I**).

Freezing of highly concentrated semen in MFP and MS (Paper I)

The statistical model showed no interaction between males and packaging system. Sperm motility post-thaw did not differ between the packages with higher sperm concentration (MS and MFP) and the control (SFP), either in terms of overall sperm motility or sperm velocity (VSL, VAP, or VCL). The percentage of motile spermatozoa which depicted a linear trajectory differed significantly, with the control group (SFP) having the highest proportion (31.6% ±3.9%, mean ±SEM) of spermatozoa with linear motility and the MS the lowest (13.7% ±1.5% mean ±SEM). While the sHOST did not shed significant differences in membrane functionality between either package or boar, the use of fluorophores showed that, compared to MS, spermatozoa frozen in SFP or MFP depicted the highest percentages of PMI. Consequently, more spermatozoa, in absolute numbers, maintained PMI post-thaw in the MFP (494.5 ±13.5 × 10^6/spermatozoa, mean ±SEM) than in the MS (381.5 ±22.1 × 10^6/spermatozoa, mean ±SEM). These results indicate that freezing semen in highly concentrated low-volume doses did not cause any extra damage to spermatozoa, compared to controls.

Fertility after DIU-AI of highly concentrated sperm doses (Paper II)

All 42 sows used in the fertility trial showed normal signs of oestrus, including occurrence of spontaneous ovulation. The rest of the reproductive parameters of the females, during the oestrous cycle, AI, and pregnancy were within the normal ranges for the species. The DIU-AI could be performed in all sows, but took longer (>5 min) in 11.9% (5/42) of the females, indicating some degree of difficulty. Four out of these 5 sows

returned to oestrus. The overall pregnancy rate averaged 35% and was clearly related to the interval between DIU-AI and ovulation, being highest (60%, 12/20) when spontaneous ovulation occurred between -8 and -4 hours before AI. Fertilisation rates were highest with MFP-packed semen. Thus, pregnancy could be obtained using highly concentrated low- volume sperm doses and DIU-AI, but the interval AI to ovulation appeared crucial for fertilisation rates.

Changes in sperm viability and membrane stability during various steps of cryopreservation: differences between ejaculate portions (Paper III)

Total proportions of motile spermatozoa were always significantly higher in P1 than in P2 (S1= P1: 86.2%, P2: 78.4%; S2= P1: 89.5%, P2: 85.3%; S3= P1: 91.6%, P2: 83.9%, and S4= P1: 66.3%, P2: 54.2%) for all stages of cryopreservation. Within portions of the ejaculate, total motility did not vary during cooling (P1: 86.2% to 91.6%; P2: 78.4% to 85.3%), but was clearly reduced post-thaw (P1: 66.3%; P2: 54.2%). The percentage of spermatozoa depicting linear motility was proportionally similar between ejaculate portions, remaining around 20–30% during cooling, to significantly increase post-thaw (checked after 30 min of incubation at 38°C) in both portions; to 45.4% and 44.3%, P1 and P2 respectively. Sperm velocities (μm/sec) were, immediately post-collection, higher in P1 than in P2 (VCL: 103.2 vs 84.9; VSL: 65.3 vs 53.3 and VAP: 68.4 vs 55.6), a difference not maintained during the rest of the process.

When spermatozoa were incubated under non-capacitating conditions (low bicarbonate level, ungassed medium), >90% of live spermatozoa had a stable membrane (low M-540 fluorescence). This particular sperm population decreased significantly post-thaw, similarly for both ejaculate portions, without showing high M-540 fluorescence (which indicates instability of the plasma membrane), but merely increasing the proportions of dead spermatozoa (P1: from 8.5% to 40.8%; P2: from 12.5% to 43.9%). However, those M-540 low-fluorescent spermatozoa (i.e., with stable membrane) could react to conditions that are used to induce sperm *in vitro* capacitation (high bicarbonate level, gassed medium), this time by clearly increasing the proportions of high M-540 fluorescent spermatozoa, except post-thaw (S1= P1: from 1.5% to 64.3%, P2: from 1.4% to 42.9%; S2= P1: from 0.9% to 9.6%, P2: from 1.2% to 21.2%; S3= P1: from 0.45% to 3.0%, P2: from 0.6% to 4.3% and S4= P1: from 1.3% to 1.7%, P2: from 1.6% to 1.6%). Such response to bicarbonate challenge varied among stages of the cooling process and ejaculate portions (being highest in newly collected semen than in semen from the following stages of cooling and thawing), and being numerically higher in P1 (64.3 ±4.3%) than in P2 (42.9 ±6.3%) (mean ±SEM). Monitoring of the plasma membrane PS-exteriorisation by the Annexin-V/PI assay showed similar trends as for M-540. During

cooling and irrespective of ejaculate portion, most spermatozoa were live-AN- (S1: P1: 90.5%, P2: 92.3%; S2: P1: 89.8%, P2: 87.6% and S3: P1: 89.2%, P2: 85.4%), a proportion that dropped significantly post-thaw (P1: 58.3%, P2: 56.4%). Consequently, while very few live spermatozoa (<1%) had exteriorised their PS (AN+) during controlled cooling, they were dead post-thaw. When challenged with bicarbonate, the proportion of AN+ spermatozoa increased significantly in all stages and both portions, but remained below 4%. As before, bicarbonate challenge increased the proportions of dead spermatozoa in all stages and in both portions. Overall, the data suggest that plasma membrane stability, and barely motility, were not altered during controlled cooling and that the main damage inflicted by cryopreservation was recognised post-thaw as sperm death.

Effect of seminal plasma in cryopreservation (Paper IV)

The percentage of total motile spermatozoa post-thaw differed significantly among portions/treatments, with the highest values in P1- (65.8%) and in P2-spermatozoa re-exposed to pooled SP-P1 from several boars, (62.1%), while the lowest percentage was seen among P1-spermatozoa re-extended in pooled SP-P2 (49.8%). Such proportions were maintained during cooling but decreased significantly after thawing (S1= P1: 90.2%, P2: 79.6%, P1CENSP2: 76.1%, P2CENSP1: 88.7%; S2= P1: 90.7%, P2: 86.0%, P1CENSP2: 80.9%, P2CENSP1: 85.4%; S3= P1: 93.2%, P2: 84.1%, P1CENSP2: 81.3%, P2CENSP1: 83.4%, and S4= P1: 65.8%, P2: 56.2%, P1CENSP2: 49.8%, P2CENSP1: 62.1%). The highest percentages of spermatozoa showing linear motility were seen after the customary 30 min incubation post-thaw (38.0% to 44.5%). Linearity did not change between sperm source and treatment during any of the four stages of cryopreservation process (S1= 32.3% to 40.6%; S2= 32.3% to 35.3%; S3= 27.9% to 32.5%).

P1-derived spermatozoa had the highest percentage of PMI post-thaw (63.3%, $P < 0.05$). Re-exposure of either P1 or P2 to the opposite SP was able to modify PMI status assessed by SYBR-14/PI. On the other hand, neither Annexin-V/PI nor sperm chromatin structure assay (SCSA) were able to depict significant differences between ejaculate portions or treatments. For the latter, the values of post-thaw DFI were very low in spermatozoa from both P1 and P2 (<1.3%), and cleansing and re-exposure to the opposite pooled SP gave no substantial beneficial effect. A slight negative effect was seen when P1-spermatozoa were exposed to pooled SP from P2, as seen for DFI (1.2% to 2.5%), SD-DFI (22.1% to 28.4%) or X-DFI (216.0% to 225.1%). In sum, the SP from different ejaculate portions seems to modulate post-thaw sperm survival (as motility).

Bicarbonate concentrations and pH in different portions/fractions of the boar ejaculate (**Paper V**, Exp I)

Bicarbonate concentration in P1 was significantly lower (13.7 mM/L) than that of SRF, P2, or the whole ejaculate, whose relative concentrations were similar (19 to 23 mM/L). In comparison with P2, the pH of P1 was lowest (7.07 ±0.03), but there were no significant differences with either SRF or the whole ejaculate. The values of either parameter were statistically similar between males.

Sperm motility during storage in different bicarbonate concentrations (**Paper V**, Exp II)

From being equal at time 0, sperm motility changed when P1- and P2-spermatozoa were stored in varying concentrations of exogenous bicarbonate for up to 120 h at 16–17°C. Over time, and in relation to control, P1-spermatozoa sustained motility better than P2-spermatozoa (at 120 h, P1: 66.5–71.2%, control: 81.7%; P2: 33.6–58.3%, control: 61.5%). P1-spermatozoa stored in extender with 5, 10, or 15 mM/L of bicarbonate or in control BTS (22.5 mM/L of bicarbonate) maintained motility ~70% during 120 h, while P2-spermatozoa could not sustain those levels (33.6% to 61.5%). The relative absence of bicarbonate (semen extended with Bic-0 mM/L) clearly depressed sperm motility of either P1- or P2-spermatozoa to levels <30%. This low motility was clearly maintained during the storage period in P1-spermatozoa (48 h: 21.5%, 72 h: 27.6%, and 120 h: 30.2%, means), while P2-spermatozoa showed more erratic, albeit similarly low, values. When stored in presence of exogenous bicarbonate (5–15 mM/L), linearity increased within 24 h of storage, from levels below 10% (P1) or 10–20% (P2) to values between 30–50% (P1) or 25–45% (P2), being kept up to 120 h, steadily in P1-spermatozoa but more erratically in P2-spermatozoa (ns). Challenging P1- or P2-spermatozoa stored for up to 144 h in the relative absence of bicarbonate (Bic-0 extender), with a further re-incubation in the control (BTS+®) extender for 30 min, markedly increased both the percentages of total motile (P1: 3.6% to 44.3%, P2: 43.5% to 70.6%) and linear motile spermatozoa (P1: 0% to 47.3%; P2: 1% to 10.6%). Bicarbonate appears to modulate sperm motility, with an apparent threshold. Since P1-SP usually contains lower innate bicarbonate amounts than P2, the absence of extra bicarbonate in the extender (Bic-0) was clearly more detrimental for motility for P1- than for P2-spermatozoa.

Simplification of the freezing of boar semen (**Paper V**, Exp III)

Use of a simplified, shorter protocol (SF) seemed to yield similar post-thaw survival (as proportions of motile spermatozoa recorded by CASA 30 min of 38°C-incubation post-thaw, from either P1 or SRF) as the conventional control (CF) method (P1-SF: 68.9% ±2.4%, P1-CF: 65.2% ±5.4%, SRF-

SF: 55.8% ±3.1%, and SRF-CF: 64.4% ±2.7%, means ±SEM). Within those motile spermatozoa, the proportion depicting linear trajectories increased in all treatments from <10% at time 0 post-thaw to 34–51% (for P1-SF and SRF-CF, respectively) when incubated at 38°C for 30 min, and to a maximum by 120 min of incubation (46% to 66%, for P1-SF and SRF-SF, respectively). Consequently, the proportions of spermatozoa showing a non-linear motility pattern showed a "mirror" display. None of these patterns differed between freezing treatments. The VCL or LDH of post-thawed spermatozoa decreased during time of incubation, showing the same pattern for all freezing treatments assayed. The pattern was different for VAP and VSL. There was a significant increase after 30 min of incubation for the SF-treatments, while the CF-treatments had a slight but no significant increase. There were not significant differences in the two velocities after incubation at 120 min, except in treatment SR-SF, where VAP and VSL decreased. Few variables showed variation between males (VCL, including all treatments, and total motile spermatozoa; linear and non-linear motile spermatozoa for SRF-CF and -SF). There were no differences between ejaculates within male, for any variable.

The percentage of PMI-spermatozoa was lowest for the SRF-SF protocol (52.5% ±11.8%, mean ±SEM) and highest for the SRF-CF protocol (60.8% ±9.1%, mean ±SEM). Interestingly, these values did not differ significantly from those using P1-spermatozoa, either when using the simplified protocol P1-SF or the conventional one (P1-CF). For PMI, there was no variation either between males or between ejaculates within male. Most (60–70%) post-thawed spermatozoa maintained acrosomal morphology (and integrity) within normal limits. This variable did not show statistical differences either between freezing treatments or among/within sires. Freezing P1-spermatozoa with the simplified process (SF) yielded similar cryosurvival as the current protocol, but within a dramatically shorter processing time.

Discussion

One of the main drawbacks conspiring against a more extensive use of FT-boar semen is the inherent low freezability that spermatozoa from many boars show. Several factors may contribute to this problem. One of them is genetic (Thurston *et al.*, 2002); some boars produce spermatozoa with attributes (plasma membrane composition, metabolic capacity, etc.) that are insufficiently developed or have characteristics that make spermatozoa incapable of sustaining cryopreservation. Differences in freezability thus constitute a phenomenon that is most likely universal, as males from other species than the porcine also show such variation, often in relation to the relative phospholipid composition of the plasma membrane of their spermatozoa (Buhr *et al.*, 1994). Species such as avian (particularly chicken, Blesbois, 2007), human (Centola *et al.*, 1992), or bovine (Bailey & Buhr, 1994; Mathevon *et al.*, 1998; Zhang *et al.*, 2001) provide semen that freezes "easily", that is, has a steady, acceptable cryosurvival. In the case of the bull, one should, however, remember that the selection of sires has, over the past decades, included the use of a single, rather standardised freezing method. Bulls whose semen freezes well stay; the others… go. Boars also are often pre-selected for "freezability". They are pre-tested by freezing their semen, noting the outcome of alive (motile) spermatozoa, and determining which boars are "most suited" for freezing (Gil *et al.*, 2005). Such process is time-consuming. Furthermore, it hampers the use of boars with interesting genotypes, simply because the method used as tester for "freezability" is sub-optimal (Westendorf *et al.*, 1975; Bwanga, 1991; Johnson *et al.*, 2000; Roca *et al.*, 2006; Groβfeld *et al.*, 2008).

Another factor is the classical view that sows, owing to the anatomical characteristics of their genitals, in particular their large uterus, require a certain volume [often not less than 80 mL (Baker *et al.*, 1968)] in order to warrant an effective sperm transport from the site of semen deposition (cervix) towards the oviduct (Viring & Einarsson, 1981). Such volumes for an AI dose were considered prerequisite to harbour a certain number of suspended spermatozoa, in order to achieve an acceptable replenishment of the sperm reservoirs in the oviducts. In this manner, fertilisation of the newly ovulated oocytes could be assured by having enough sperm numbers

at the site of fertilisation (Hunter, 1981; Hunter, 1984). This is basically the rationale behind the use of liquid, extended semen for conventional AI, which holds 2.3–2.5 × 10^9 spermatozoa in an 80 mL dose. With regard to frozen material, unfortunately, since cryopreservation of boar semen usually kills a large proportion of the processed spermatozoa, their total number in an AI dose is usually double the figure used for liquid semen, for example, 5 × 10^9 spermatozoa in an 80 mL dose (Johnson, 1985; Johnson *et al.*, 2000). Several consequences arise from these large doses (in volume and sperm numbers). First, the huge sperm number per dose means that few, often not more than 10 doses can be prepared from a mean ejaculate. Second, the thawed semen usually contained in straws (either maxi-straws of 5 mL, or a series of 0.5mL MS) (Johnson *et al.*, 2000) is re-suspended in a large volume (80 mL) of extender. Such procedure has been considered to cause deleterious effects, often related to osmolarity changes, in the surviving sperm population (Larsson & Einarsson, 1976), and it probably contributes to the lower fertility of the FT-semen. Another contributing factor is the occurrence of reflux of the inseminated volume. Such reflux [that can account for ~35% of the total volume (Viring & Einarsson, 1981)] occurs shortly after cervical AI as a consequence of the myometrial activity that relates to the manual infusion of the inseminate, and that can, very well, impair fertility (Steverink *et al.*, 1998). Attempts have been made to diminish the sperm losses that such reflux causes, including AI with novel catheter designs (Gedis®, Michalak, 2005) or the use of GoldenBags™ (www.imv-technologies.com) hung from a stimulating saddle hooked on the back of the sow (for instance the EZ MATE, Hampshire, IL, USA), which allows the sow's own pelvic and uterine contractions to do the emptying of the AI volume, slowly infusing the contents of the AI dose without manual pressing, as it is customarily done using other containers (Belstra *et al.*, 2004).

Alternatives for the best use of FT-semen can also be listed. Cryobiologically best-suited containers for a 5mL dose (containing a total of 5 × 10^9 spermatozoa), such as the FlatPack™, have proven successful in terms of *in vitro* sperm survival and of acceptable fertility following two cervical AIs per oestrus (Eriksson & Rodríguez-Martínez, 2000b; Eriksson *et al.*, 2001; Eriksson *et al.*, 2002). However, the issues of the large AI volume and the large sperm number are still present. Alternative AI routes have been designed, such as the post-cervical AI, with the intention of lowering the dose in terms of volume and sperm numbers. Two types of uterine AI procedures have been developed, namely the post-cervical, with deposition in the uterine body (Watson & Behan, 2002) and the deep-intrauterine AI (DIU-AI) which deposits the inseminate far into one uterine horn (Martínez *et al.*, 2001a). Using either method, the volume and the sperm number per dose have been dramatically lowered and reflux minimised, leading to fertility using not only fresh semen (Martínez *et al.*, 2002; Watson & Behan, 2002), but also FT-semen (Roca *et al.*, 2003; Bolarín *et al.*, 2006; Bathgate *et al.*, 2008). In the present thesis, the results reported in **Paper I** revealed

that it was possible to successfully freeze boar spermatozoa, highly concentrated, in small volumes (0.5–0.7 mL). The rationale behind this study was to attempt the processing of highly-packed spermatozoa to numbers closer to those 3×10^9/mL found in the cauda epididymides (Einarsson, 1971), so that the viscosity of the sample would have also been increased, with consequences for freezing and also for counteracting reflux after AI. Moreover, those concentrated samples could be packed into a cryogenically proven container, with the same design as the FlatPack™, by portioning such a container into the now denominated MiniFlatPack™ (MFP, **Paper I**). After thawing, these spermatozoa presented survival/viability with respect to motility, PMI, and functionality comparable to that of semen frozen at lower concentrations and in a higher volume using FlatPack™, thus confirming the hypothesis tested; boar semen could be frozen in small containers, highly packed. Furthermore, compared to MS (of a similar volume and sperm concentration), the MFP yielded the highest number of post-thaw viable spermatozoa per dose.

Why such differences between maxi-straws, MS, and MFP? The packages differ mainly in shape: plastic maxi-straws and MS are cylinders, while the SFP and the MFP are flattened hexahedrons. They differ in thickness and, consequently, the ratio of surface-to-volume they express. Theoretically, depending on the thickness and the ability of the container to dissipate heat during cooling and thawing, cells would dehydrate mostly at the periphery, while ice damage would occur mostly in the centre of the sample (Courtens & Rety, 2001). Such results were reported in a study by Ekwall *et al.* (2007), attempting identification in the frozen state, i.e., *in situ*, of how much free water (extracellular "lakes") in extended boar semen could be frozen at different locations of MS and MFP packages against the size of the "dehydrated" areas (e.g., the frozen extender containing the spermatozoa, the so-called "veins"), by use of Cryo-scanning electron microscopy (Cryo-SEM). The Cryo-SEM methodology has proven most useful for disclosing such aspects in the assessment of boar spermatozoa (Rodríguez-Martínez & Ekwall, 1998; Ekwall, 2008). Interestingly, freezing of boar semen at high concentrations/volume in these two packages (MS and MFP), as done in **Paper I**, lead to differences in the structure of the frozen material (Ekwall *et al.*, 2007). The pattern of "lakes" and "veins" in the MFP was clearly homogeneous throughout a cross section of the container, while the MS had smaller "lakes" in the periphery of the straw than in the centre of the cross section, indicating that the degree of dehydration was different, higher in the centre and lower in the periphery. Most likely, the spermatozoa in the outer area would contain intracellular ice, owing to a hampered dehydration (Bwanga *et al.*, 1991). In contrast, the MFP showed larger "lakes" and thinner "veins" than the MS throughout, indicating the degree of dehydration was higher and more homogeneous (Ekwall *et al.*, 2007). In consequence, the number of spermatozoa surviving the process was higher in the MFP, which concurs with the findings of **Paper I**.

The reasons for the poor-to-fair success achieved until now when cryopreserving boar semen (Mazur *et al.*, 2008) are still not completely known. The relatively low success rate could be partly due to boar spermatozoa being very demanding cells to freeze, mostly owing to their reduced ability to sustain osmotic changes, during either freezing and/or thawing. Several factors, including the rate of freezing, cause osmotic changes in boar spermatozoa. Spermatozoa theoretically survive cryopreservation if a critical cooling rate for maximum survival is used, but such rate is yet to be found for the porcine species (Watson, 1979). Most often, we are far from these apparently optimal rates, and since use of a too-quick freezing (supra-optimal rates) leads to a drop in survival resulting from damaging intracellular ice formation (Bwanga *et al.*, 1991), high survival typically demands that the cooling rate is sufficiently low to avoid such internal freezing (Leibo *et al.*, 1978). Although rates low enough to prevent internal freezing injury are necessary for high survival, freezing at slow rates over a long time can cause "solution effects" injury resulting from the extreme concentration of extracellular solutes that may decrease survival as well (Critser *et al.*, 2002). Therefore, an optimal cooling rate must be slow enough to prevent intracellular ice formation, but sufficiently fast to avoid cryo-injury due to solution effects (Mazur *et al.*, 1972). The freezing rates used in this thesis were basically the same throughout, so that the structure differences in the frozen material reported by Ekwall *et al.* (2007), prepared using the protocol devised in **Paper I,** must –therefore– be a consequence of the packaging device used. On the other hand, a cell that has survived cooling to low subzero temperatures will ultimately not survive, if it is not thawed correctly. Thawing is also very traumatic to the sperm plasma membrane because of the highly destructive osmotic swelling that affects the spermatozoa during this process, where the range of osmolarity changes is often limited to 180–300 mOsm in the presence of extender (Gilmore *et al.*, 1998). Throughout the thesis, thawing rates were adapted to the various containers, volumes, and sperm concentrations, being ~900°C/min in SFP (**Paper I**) and ~700°C/min for MS and MFP (**Papers I–V**), in order to minimise membrane swelling (Fiser *et al.*, 1993).

In sum, the results of **Paper I** clearly showed that if the complete SRF were frozen in MFP, it could lead to the production of an average of 50–60 doses by ejaculate, with acceptable survival (~40%). But, are these spermatozoa fertile? **Paper II** intended to test the fertility, using DIU-AI of an AI dose containing highly concentrated spermatozoa frozen in a low volume. The obvious advantages seen for this treatment were the ability to easily store semen and to facilitate its handling post-thawing, without needing to re-extend the dose. However, could such small volume (0.5–0.7 mL) of spermatozoa be capable being efficiently transported, especially considering the DIU-AI is deeply done in one of the horns? Would spermatozoa fertilise oocytes ovulated from either ovary? Would fertility vary if one or two AIs were done per oestrus? Would fertility be related to the interval between AI and ovulation? Several of these variables have been explored for FT-boar

semen (Waberski *et al.*, 1994; Roca *et al.*, 2003; Bolarín *et al.*, 2006; Bathgate *et al.*, 2008) using cervical- or DIU-AI, but not using such sperm concentrations and such a low volume as the ones tested in **Paper II**. In any case, it was evident from the study reported by Martínez *et al.* (2006) that both uni- and bilateral fertilisations occur after DIU-AI, marking that sperm transport is effective enough to allow for bilateral fertilisations, even when their numbers were lower than those occurring unilaterally. Whether sperm transport was transperitoneal or transuterine, or both, is yet disputed (Martínez *et al.*, 2001b; Roca *et al.*, 2003; Martínez *et al.*, 2006). In **Paper II**, fertility was achieved (overall being 35%) after DIU-AI with concentrated low-volume FT-boar semen (0.5–0.7 mL) in spontaneously ovulated sows, without significant differences registered in pregnancy rates between the three AI schemes used (single DIU-AI at 4 or 8 h or double DIU-AI 12 and 4 h before expected ovulation). The DIU-AI could be performed in all sows, but insertion was difficult in about 10% of the animals, taking longer time than expected. The fact that 4 out of 5 of these sows returned to oestrus suggests that the ease of passing the catheter along the uterine cavity is of utmost importance. Excessive manipulation may be counterproductive by disrupting normal sperm transport. Post-thaw sperm motility averaged 40% [10 points over our threshold for FT-semen (Eriksson *et al.*, 2002)] for either packaging system and did not significantly change from thawing-to-AI. Such sperm viability suggests an average number of 400×10^6 live spermatozoa were deposited once or twice. Which dose is optimal for FT-boar semen has not yet been determined for DIU-AI. Using a dose as low as 250×10^6 spermatozoa led to acceptable fertility (50% pregnancy rate), in terms of farrowing rate (43%) and litter size (7.2) (Bathgate *et al.*, 2005). Use of 1×10^9 live spermatozoa yielded better results [70% farrowing rate and 9.25 $\pm$0.23 piglets born (Roca *et al.*, 2003)]. In **Paper II**, there were not significant differences in pregnancy rates between the three AI schemes used. However, when examined for the distribution of pregnancy and non-pregnancy through the different periods, it was evident that pregnancy rates clearly related to the interval between DIU-AI and ovulation, being highest (60%, 12/20) when spontaneous ovulation occurred between 8 and 4 h after AI, confirming that a minimal interval between AI and ovulation has to be respected, so that maximal fertilisation rate (Soede *et al.*, 1995a; Soede *et al.*, 1995b), farrowing rate, and litter size (Terqui *et al.*, 2000) can be obtained. Another finding was that there is no absolute need for a larger volume to be inseminated during DIU-AI; 0.5 mL seems to suffice for fertilisation to occur, as suggested by the number (as a range) of implantation sites obtained (**Paper II**). This indicates that sperm transport was effective enough, albeit not optimal, even when only such small volume was deposited in the tip of one horn. The spermatozoa are most likely being transported, by uterine contractions mechanically induced by the insertion and presence of the catheter, to the contra-lateral uterine tip to fertilise oocytes present at that side. Such findings have been reported earlier (Martínez *et al.*, 2002), but by using larger volumes and sperm concentrations than the ones used here. In conclusion, those low sperm

numbers in a very small volume, deposited deeply into one uterine horn, were able to fertilise oocytes resulting from spontaneous ovulations, even when numbers were variable and did not reach 100%. Moreover, fertilisations and the consequent pregnancy rates were clearly dependent upon the optimality of the interval between last AI and ovulation.

In any case, this methodology could be considered far from ideal. It follows a complicated protocol of freezing, where the step of concentrating the spermatozoa to 2×10^9/mL is an extra step in the cumbersome process of freezing the SRF. Surely, many more doses could be prepared per ejaculate, but would it not be possible to obtain more concentrated spermatozoa within the ejaculate, and thus simplify the procedure?

In this laboratory, already by 2000 (Sellés *et al.*, 2001) trials had been performed in order to determine the presence of different subpopulations in the boar ejaculate, a matter that has absorbed many researchers worldwide (Holt *et al.*, 1996; Abaigar *et al.*, 1999; Quintero-Moreno *et al.*, 2004; Abaigar *et al.*, 2005). Their work has confirmed that the ejaculate, in its heterogeneity, has spermatozoa with various degrees of motility, both before and, particularly, after freezing and thawing (Thurston *et al.*, 2003; Cremades *et al.*, 2005; Gil *et al.*, 2005). The boar ejaculate is voluminous, and produced *in vivo* in fractions, depending on the emission of secretory products from the accessory sexual glands to the urethra and the ejaculation of aliquots of spermatozoa from the cauda epididymides (Mann & Lutwak-Mann, 1981). Such fractions can be manually collected from a boar, and at least three are distinctly identified, namely the pre-sperm-rich fraction with a marginal number of spermatozoa and rich in electrolytes (Einarsson, 1971); the sperm-rich fraction (SRF), where most spermatozoa are located; and the post-sperm-rich fraction (PSRF), in which sperm numbers are minimal and which is mainly composed of protein secretions from the seminal vesicles, is rich in important ions (Na^+, Cl^-, Ca^{++}, Zn^+, bicarbonate) and sialic acid (Hartree, 1962; Einarsson, 1971). Sperm numbers are not evenly distributed in the SRF, since the greatest number of spermatozoa (about 25%, **Paper V**) is present within the first 10 mL, with sperm numbers slowly decreasing towards the end of the ejaculate. Since these first 10 mL can be retrieved while collection of the ejaculate is performed *as praxis*, studies were done on the spermatozoa contained in this first portion (P1, **Papers III–V**) with those present in the rest of the collected ejaculate (i.e., the rest of the SRF and the PSRF, the hereby named P2). Those studies revealed, using pooled ejaculates, that the spermatozoa present in P1 (earlier also named PI, Peña *et al.*, 2006) better sustained cooling and freezing-thawing compared to those fortuitously present in the bulk ejaculate (Sellés *et al.*, 2001).

The ejaculate portion was proven to have a significant effect on sperm membrane integrity, motility patterns, and capacitation-like changes (Peña *et al.*, 2003a; Peña *et al.*, 2003b; Peña *et al.*, 2004), changes that defined significant differences between fractions of the ejaculate in both fresh and frozen samples. Before freezing, more live cells were present in P1. The

freezing-thawing procedure impaired motility and sperm membranes in a different manner in each portion of the ejaculate, suggesting that spermatozoa present in P1 were less sensitive to the stress induced by the cryopreservation procedure. Although clear-cut results were seen, the above-listed experiments were performed using pooled semen samples. When individual males were compared, variation in the performance of spermatozoa derived from one of these two portions of the ejaculate was present among sires (Peña *et al.*, 2006). The reasons for the different results (Sellés *et al.*, 2001; Peña *et al.*, 2003a; Peña *et al.*, 2004) regarding variability among boars are still obscure, but differences in SP composition between males and ejaculate portions may be part of the explanation. Different morphological subpopulations have been described, also, as part of the explanation for the distinct boars' general inability to sustain cryopreservation (Thurston *et al.*, 2001). In relation to this, the morphometric study performed by Peña *et al.* (2006), using automated sperm morphology analysis (ASMA) software, revealed that spermatozoa present in P1 had significantly shorter and wider heads than those present in P2, for four of five boars studied. It is noteworthy, albeit solely anecdotal, that in this particular male, the cryopreservation of P2 was significantly better compared to P1. These studies suggest a possible male-effect should be considered in future studies, especially considering that inter-sire variation in semen freezability is of major concern (Thurston *et al.*, 2002). Disclosing which sires could have a higher resilience to freezing among the bulk of spermatozoa is, obviously, of practical interest. In particular, it would be interesting to determine whether a beneficial effect of a certain portion of the seminal plasma (SP) on sperm viability is present in each boar, or whether this effect also varies among sires.

Obviously, it is very difficult to believe that P1 or P2 spermatozoa are "chosen" from a population of ~60–80 $\times$ 10^9 spermatozoa in the ejaculate. A certain proportion of spermatozoa are just fortuitously present in P1, and largely and in a lapse of minutes from being ejaculated during natural mating, they reach the uterotubal junction (UTJ) and contribute to effectively building the sperm reservoir (Rodríguez-Martínez *et al.*, 2005). Those spermatozoa that had reached the sperm reservoir would, provided they progress from there in the "correct time" (i.e., in relation to ovulation) towards the ad-ovarian segment of the oviduct, be in physical and physiological conditions to interact with the oocytes and participate in the fertilisation process (Hunter & Rodriguez-Martinez, 2004). This is a purely mechanistic explanation for the "supremacy" of the spermatozoa contained in P1. But… could these spermatozoa be used to improve our management of the material to be frozen for AI? Concentration is obviously an advantage, since they are very concentrated already in the portion in which they are verted, but why do they sustain, at least in many boars, better freezing and thawing? Is there any relation to the surrounding SP?

The SP of the pig is formed by several components, the epididymal plasma with their particular proteins [often small proteins such as the inhibitor of

the acrosine (Dacheux *et al.*, 2003)], but also the concerted, albeit fractionated secretion of the accessory sexual glands with their varying contents (ions, sugars, proteins, etc.) (Mann & Lutwak-Mann, 1981). The SP is, therefore, a tremendously heterogenous mixture, and its composition strongly depends on which fraction of boar ejaculate we discuss. There are differences in the amounts and types of SP-proteins (Calvete *et al.*, 2005; Rodríguez-Martínez *et al.*, 2005). The portions P1 and P2 differ significantly in their relative contents of total protein being lower in P1 than in P2 (7.0 ±1.3 and 32.4 ±12.7 g/L, respectively), with clear differences in their distribution following electrophoresis on agarose gels (Wallgren *et al.*, unpublished results). Although most proteins in P1 and P2 are the same spermadhesins [particularly the porcine seminal plasma proteins I and II (PSP-I and PSP-II) but also the Alanine-Tryptophan-Asparagine proteins (AWNs) and Alanine-Glutamine-Asparagine protein 1 and 3 (AQN-1 and AQN-3) (Töpfer-Petersen *et al.*, 1998), which together represent over 90% of the total boar SP proteins (Dostalova *et al.*, 1994)], the P1 also contains some smaller proteins (less than 5.6 kDa) that most likely correspond, among others, to the inhibitor of acrosin (Calvete *et al.* unpublished). In sum, the P2 had almost exclusively spermadhesins, in particular PSP-I and PSP-II glycoforms (Nimtz *et al.*, 1999), while the P1 also contained these spermadhesins, but at lower concentrations and molecular weight. The PSPs have proven beneficial for sperm survival when boar spermatozoa have been highly extended, for reasons not yet clarified, but linked to a preservation of the sperm membrane stability (Caballero *et al.*, 2006; García *et al.*, 2006). The relevance of the other spermadhesins (AQNs and AWNs) for sperm function in the pig is less clear (Calvete *et al.*, 2005).

Another striking difference between the cauda epididymides spermatozoa and those in the ejaculate is the dramatic change in the amount of bicarbonate present in the surrounding fluid, which is basically 3 mM/L in the epididymides and >30 mM/L in the whole ejaculate (Rodríguez-Martínez *et al.*, 1990). Looking at the different fractions of the ejaculate, earlier reports have shown an increase towards the end of the ejaculate, starting with ~14 mM/L in the pre-sperm fraction, to ~17 mM/L in the SRF and increasing to >33 mM/L in the PSRF (Rodríguez-Martínez, 1991). The ion bicarbonate plays important roles in sperm physiology, both by maintaining intracellular pH (and the homeostasis of the cell), and by modulating sperm motility and membrane stability through its effects on the sperm adenylyl cyclase (Harrison *et al.*, 1996; Holt & Harrison, 2002; Litvin *et al.*, 2003). For instance, bicarbonate is responsible for the initiation of motility at ejaculation (Okamura *et al.*, 1985; Rodríguez-Martínez, 1991), and it is also considered the main effector of changes within the lipid bilayer of the sperm plasma membrane that are associated with sperm capacitation both *in vivo* (Tienthai *et al.*, 2004), as *in vitro* (Harrison *et al.*, 1996; Gadella & Harrison, 2000; Harrison & Gadella, 2005), in the pig.

Interestingly, the process of capacitation has been linked to the procedures of cooling and freezing. The number of spermatozoa that survives

cryopreservation is enormously reduced (~40-50% survivability). This surviving subpopulation has, moreover, a reduced *in vivo* lifespan, compared with the pre-freeze spermatozoa, showing a rather susceptible plasma membrane which, being markedly prompt to deterioration, leads to a loss of the fertilising capacity of the spermatozoon, if the encounter with the oocyte is delayed (for instance when spermatozoa are inseminated far from ovulation). These changes at the plasma membrane of cryopreserved spermatozoa (often seen during cooling) have been monitored by use of the antibiotic chlortetracycline (the so-called CTC-assay), where this fluorescent antibiotic yields different fluorescent patterns in the sperm head depending on the presence and location of Ca^{++}/Mg^{++} ions bound to the plasma membrane, and that could be linked to stable membranes, capacitated (unstable) membranes or even lost membranes (acrosome-reacted) (Tsien, 1989; Fraser *et al.*, 1995; Rathi *et al.*, 2001). That progression finally led to the concept of "cryocapacitation", since many of the surviving spermatozoa had the characteristics of unstable plasma membranes and likewise CTC-patterns of "capacitation" (Watson, 1995). However, the screening of these patterns was microscopically made, and it is therefore to be considered subjective. Moreover, because of its Ca^{++} dependency (Tsien, 1989; Rathi *et al.*, 2001), the assay supersedes other, often earlier steps of sperm capacitation, such as the lipid scrambling in the plasmalemma (Gadella & Harrison, 2002; Harrison & Gadella, 2005; Silva & Gadella, 2006). Changes in intracellular Ca^{++} occur during sperm cooling, and therefore CTC screening may reflect increases in intracellular Ca^{++} concentrations rather than capacitation (Guthrie & Welch, 2005a). So, the question remains, are boar spermatozoa induced to "capacitate" during cooling? Is this the reason for their post-thaw weakness?

An experiment was designed (**Paper III**), combining several aims, based in the consideration that boar semen frozen in MFP demonstrated better *in vitro* and *in vivo* results than with other containers, and that there are [albeit with inter-boar variation (Peña *et al.*, 2006)] differences between portions of the boar ejaculate. The goal was to freeze P1- and P2-spermatozoa in MFP and to compare the outcome, not only post-thaw, but during a series of well-controlled steps during handling and cooling. Spermatozoa were monitored throughout for kinematics, membrane (in)stability and membrane integrity. Moreover, spermatozoa in the different stages of the process of cryopreservation were explored for their capacity to respond to a challenge with bicarbonate at levels present in the site of fertilisation in the pig oviduct (Rodríguez-Martínez, 2007b). The hypothesis tested was that it was thawing, rather than controlled cooling, that seriously affected the plasma membrane architecture and sperm motility of ejaculated boar spermatozoa temporally present in two different sub-sets of seminal plasma P1 and P2). Moreover, we tested the hypothesis that these modifications were not related to "cryocapacitation-like" changes, defined as modifications of sperm plasma phospholipid stability and specific changes in sperm motility. The results showed that for both portions, although there was a

significant decrease in sperm total motility after thawing, motility was maintained over the cooling process, without significant variation among males. Similar results were previously reported (Eriksson *et al.*, 2001; Cremades *et al.*, 2005), but using the entire SRF. Other reports using a similar cryopreservation protocol but different semen manipulation, packages, and motility evaluation, found a sustained and continuous reduction in total motility during the cooling process (Maxwell & Johnson, 1997b). The stability of the plasma membrane was assessed by flow cytometry analyses with a combination of M-540 and Yo-Pro-1. The fluorophore M-540 changes the intensity of its fluorescence in direct relation to the degree of lipid disorder and permits differentiation of stable from unstable membranes while Yo-Pro-1 is a cyanine dye for marking DNA that can help identify changes in permeability of the plasma membrane that lead to membrane disruption and cell-death, accompanied by increases in Yo-Pro-1 staining (Martin *et al.*, 2004; Peña *et al.*, 2005). Early changes in plasma membrane, like the specific exposition of phosphatidylserine (PS, in normal conditions confined to the inner leaflet of the lipid bilayer of the membrane) to the outer leaflet of the plasma membrane were also examined with Annexin-V, while dead spermatozoa were monitored by using the DNA marker propidium iodide (PI). Under BTS-incubation conditions at RT, around 90% of the spermatozoa from either portion maintained their plasma membrane stability (low M-540 fluorescence), and during the controlled cooling process, that is, from RT to 5°C, less than 2% of spermatozoa showed lipid scrambling. A major change occurred after thawing with the same pattern for both portions, with around 40–45% of the cells appearing dead. Among those spermatozoa that survived, fewer than 2% depicted lipid disorder in their plasma membrane and less than 1% of them showed PS exteriorisation. These data show that, during controlled cooling, the boar sperm plasma membrane remained stable, and that early capacitation changes do not occur in this period of the lengthy process of cryopreservation. Most of the spermatozoa were alive (~90% for both portions) just before deep-freezing but a large number appeared dead after thawing. The reasons for this outcome are still not clear, but it seems unrelated to any subtle modification in plasma membrane that can be linked to capacitation-like changes; it most likely concerns lethal injury during thawing. This assumption confirms the hypotheses tested and also links to other findings (Guthrie & Welch, 2005b). In any case, the spermatozoa that were alive before cooling, during cooling, and post-thaw, were not capacitated, but could very well be induced to capacitate when exposed to bicarbonate at levels found *in vivo* in the pig oviduct, where fertilisation normally takes place (Rodríguez-Martínez, 2007b).

The most probable cause of damage to spermatozoa during freezing/thawing is the cellular dehydration/rehydration that causes disruption of the plasma membrane, owing to thermal, mechanical, and/or osmotic stresses imposed upon the membrane (Mazur, 1985; Steponkus & Lynch, 1989). Interestingly, the results reported in **Paper III** showed lack

of significant variation between the males, for which P1-spermatozoa sustained cryopreservation better than P2-spermatozoa. Such results differ from earlier trials (Peña *et al.*, 2006), with inclusion of different boars, thus calling for larger populations to be tested. In theory, the spermatozoa from P1 ought to be less or differently coated with SP-proteins than spermatozoa from P2 (Rodríguez-Martínez *et al.*, 2005), allowing the P1-spermatozoa to be better protected by the extenders simply because of a closer exposure of the plasma membrane to the extender. This might, although the argument is highly speculative, explain the slightly better performance of the P1-spermatozoa during cooling, compared to the P2-spermatozoa (**Paper III**), and, as we shall see later, even after cryopreservation (**Papers III–V**). But, would the surrounding SP cause the apparent higher resilience among P1-spermatozoa?

Incubation of boar spermatozoa with their native SP (usually from the SRF) has documented beneficial effects on avoidance of cold shock (Tamuli & Watson, 1994). Therefore, boar spermatozoa are, during the temperature decrease from 35–30°C at collection to room temperature (RT, 20–22°C), routinely incubated with their surrounding SP prior to the first extension with Beltsville thawing solution (BTS, Pursel & Johnson, 1976). This period of sperm-SP co-incubation (also called "holding time in SP") has ranged in our laboratory from minutes to hours (Eriksson *et al.*, 2001), but it has now been customised to 60 minutes (**Paper I**). But, again… are the differences seen in previous studies (such as **Paper III**) caused by the specific portioned SP?

In **Paper IV,** boar spermatozoa, primarily or secondarily (i.e., following cleansing and re-exposure) exposed to different well-defined portions of the ejaculated SP (P1 vs P2), were analysed for sperm kinetics using CASA at selected stages of extension, cooling, and freezing-thawing in MFPs. Moreover, spermatozoa were examined PT for plasma membrane intactness, membrane architecture, and chromatin (DNA) integrity, using specific markers/assays such as SYBR-14/PI, Annexin-V, and the sperm chromatin structure assay (SCSA), all with flow cytometry as monitoring instrumentation. The hypothesis tested was that the SP-P1 had a differential effect on the spermatozoa. Spermatozoa from P1 (incubated in their own SP) showed the highest percentage of motility during cooling and after thawing, compared to spermatozoa from P2 (bathing in SP-P2). When P1-spermatozoa were centrifuged to be cleansed from their original/native SP and were re-exposed to pooled SP from P2, there was a decrease in their motility during all stages checked. When the opposite situation was tested, that is P2-spermatozoa were centrifuged and re-exposed to SP from P1, their motility increased from base levels (i.e., while bathing in their original/native SP), to P1 levels, especially after thawing. These effects of SP on sperm kinematics were not ratified at the plasma membrane level or sperm chromatin structure.

The qualitative and quantitative differences in the SP-protein composition of P1 and P2 presented earlier could explain, at least in part, the better performance of P1-spermatozoa during cooling and after thawing (**Paper IV**). For instance, P1 still contains relatively large proportions of epididymal fluid components, and it is well documented that boar spermatozoa collected from the cauda epididymides sustain better routine freezing compared to ejaculated spermatozoa (Kikuchi *et al.*, 1998), either because of the presence of protective proteins or because the epididymal fluid contains other factors of interest. The positive effect of SP from P1 on total motility was not evidenced in variations at the plasma membrane level, and considering the negative effect of SP from P2 on motility, there is room for speculation that some particular component(s), such as SP-proteins from accessory glands, present in large amounts in P2, could be involved. Even peptides present in large proportions in P2 may interact with the sperm plasma membrane, reducing the motility.

Another factor that could influence boar spermatozoa after ejaculation is bicarbonate, the physiologically ubiquitous ion described earlier. Although indications of its relative presence and levels have been presented elsewhere (Rodríguez-Martínez, 1991), we performed some pilot measurements, reported in **Paper IV** (see discussion), where P1 had a two-fold lower bicarbonate concentration than P2, with a small variation among boars. Such preliminary assessments were tried again in **Paper V** (Exp I) to determine if the bicarbonate concentration (and the pH) of the P1 differed from the other portions or fractions of the boar ejaculate (mainly SRF and P2), and moreover (Exp II), whether the relative absence of bicarbonate in the extender promotes sperm survival. Bicarbonate concentration in P1 was significantly lower (13.71 mM/L, $P < 0.001$) than that of SRF, P2, or the whole ejaculate, which had similar concentrations (19–23 mM/L). The pH of P1 was 7.07, significantly lower than that of P2 (7.32), although there were no significant differences with either SRF or the whole ejaculate. The values of either parameter were statistically similar among males. Bicarbonate levels of ~10–15 mM/L in the extender sustained sperm motility the best over a 120 h-period at 16–17°C. Extension in a medium without bicarbonate (Bic-0) suppressed sperm motility of P1- and P2-spermatozoa. Their motility could, however, be restored by re-exposure to exogenous bicarbonate at ~20 mM/L level. Obviously, bicarbonate levels are relevant for boar sperm function.

Could it be that the P1-spermatozoa, which are in the portion of the ejaculate referred to as being the sperm subpopulation that mainly colonises the SR (Rodríguez-Martínez *et al.*, 2005), are gradually exposed *in vivo* (from cauda epididymes and after ejaculation to the sperm reservoir in the oviduct) to differential levels of bicarbonate, levels which, *per se* do not seem high enough to induce capacitation-like changes? (See **Paper III**). And, is this a possible reason for their resilience? Obviously, these spermatozoa are able to capacitate when exposed to bicarbonate concentrations similar to those in the site of fertilisation (**Paper III**). Being that the amount of

bicarbonate in the P2 clearly is higher than in P1, these spermatozoa might be more susceptible to these "capacitation-like" changes. However, none of these hypotheses has been tested, and all require, therefore, further studies.

Summarising the precedent work, it seems that we still have a step to cover: use the accumulated results to attempt the simplification of a cryopreservation protocol that has proven acceptable for some purposes (for instance, sperm motility post-thaw has increased throughout the studies in **Papers I–V**), but is as yet unacceptable in terms of the lengthy procedure involved in taking care of an ejaculate, in order to freeze AI doses. This sub-optimality regarding the cumbersomeness of procedures is highly relevant when commercial applications are considered.

Such a simplification of the freezing protocol was attempted in **Paper V** (Exp III) where P1-spermatozoa were held in "their" native SP for 30 min, mixed with LEY and cooled down to +5°C within 1.5 h, before being mixed with LEYGO and packed into MFP for customary freezing. The entire procedure, here named "simplified freezing (SF)" lasted 3.5 h compared to the "conventional freezing (CF)" that was used as control procedure, which lasted 8 h. As controls, spermatozoa from the SRF were compared to P1-spermatozoa.

The P1-SF-processed semen showed similar proportions of sperm motility (and kinematics), plasma membrane and acrosome intactness PT, to the SRF-semen frozen customarily (SRF-CF). Mean sperm motility post-thaw ranged from 56% to 69%, the highest percentages being among the P1-SF. Interestingly, there was barely any variation either between sires or within-sire for P1-derived variables, in contrast to SRF, independent of the handling method (CF or SF). The reason behind this maintained P1-sperm survival after this shorter freezing process is yet unknown, and we cannot rule out that differences between males still exist, since the number of boars was also small in this trial. However, the combination of P1 (SP or bicarbonate effects) and the use of the cryobiologically well-suited MFP was clearly beneficial. Obviously, the semen from many more males must be subjected to this methodology in order to further test these findings.

The practical application of SP on the processing of boar semen has been recently reviewed (Kirkwood *et al.*, 2008). An interesting, major role that emerged for SP is the protection of spermatozoa from a "spontaneous capacitation-like" reaction during thawing (Vadnais *et al.*, 2005). However, recent studies have reported that this protection was confined only to boars with good freezability, whose SP has documented improvement of the post-thaw motility of spermatozoa from boars classified as bad freezers (Hernández *et al.*, 2007; Okazaki *et al.*, 2008). However, there is no description of what causes these beneficial effects.

There are several advantages of using this simplified protocol, namely the exclusion of primary extension and the removal of an obviously beneficial SP by centrifugation. Such procedure allows for a quick handling of the

material and removes the necessity of an expensive refrigerated centrifuge. Moreover, while a boar effect was noted throughout the different trials (**Papers I–III**), such variation was minimised by use of P1-spermatozoa (**Papers IV** and **V**), a matter to be included among the advantages of using such a restricted portion of the ejaculate for freezing. Another major advantage of the inclusion of P1 as freezable spermatozoa is not only that these are the "best" spermatozoa to be cryopreserved, but also that it leaves the rest of the collected spermatozoa for liquid semen processing. This simpler protocol seems therefore be an interesting alternative for AI studs to freeze boar semen for gene banking or of AI doses for repopulation or commercial distribution, along with production of conventional semen doses for AI with liquid semen. This strategy would allow the pig industry to, without compromising the routine of the AI centre or the collection schedule of any boar, include in freezing those boars of major interest for breeding and thus increase business profit. Obviously, although all this information seems promising, and since we have studied only a restricted number of boars, more sires must be screened and their semen frozen in this manner and subjected to the ultimate testing for fertility, by running pertinent AI field trials.

Conclusions

- Freezing of highly concentrated boar spermatozoa, particularly in MFP, was possible, providing a suitable number of viable spermatozoa, in a single dose, that could be used for deep intrauterine AI.
- Deep intrauterine AI of highly concentrated spermatozoa in low volume MS or MFP once or twice during oestrus, at different intervals of expected spontaneous ovulation, led to pregnancies. Fertility was highest (60%) when DIU-AI was done -8 to -4h of spontaneous ovulation.
- Controlled cooling and freezing/thawing of spermatozoa from different portions of the boar ejaculate did not induce sperm capacitation-like changes *per se*. The P1-spermatozoa best sustained all processes of cryopreservation and showed the highest survival rates.
- The SP influence on sperm kinematics during conventional freezing was presumably related to different concentrations of either SP proteins or bicarbonate in the different ejaculate portions, being more advantageous for P1.
- Freezing P1 with a simplified protocol, maintaining a low bicarbonate level by excluding primary extension, and removal of SP by centrifugation dramatically reduced the time involved in the handling of the semen (from 8 to 3.5 h), and yielded similar proportions of PT-sperm motility, plasma membrane integrity, and normal acrosome morphology, as when using a conventional protocol. The process, pending testing of more boars *in vitro*, but particularly *in vivo* though field fertility trials, seems preliminarily suitable for commercial use.

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