

# **Cryopreservation of Boar Semen**

**Studies on sperm viability in vitro and fertility**

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*Målet är ingenting -  
vägen är allt.*  
(Robert Broberg)

*To my parents and sisters*



# Abstract

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The world's pig population is consistently being upgraded through the international trade of superior genetics. The two major systems that are used for this purpose are the transport of live animals and the export of frozen boar semen. The main limiting factors for a wider use of frozen-thawed (FT) boar semen are low fertility levels of FT in comparison with liquid semen, and between-boar variation in freezing success. Consequently, there is a need for improved boar semen freezing methods. The main objective of this thesis was to develop a method for commercial freezing of boar semen, and the study of the effect of different factors on boar sperm survival in vitro and fertility after freezing-thawing in large, one-AI-dose containers. Semen was split-sample frozen in 5 mL Maxi-straws and in Cochette plastic bags. A computer-assisted sperm analyser (CASA) was used to evaluate sperm motility, while plasma membrane integrity (PMI) was assessed with fluorescent dyes. The fertilising capacity of the semen frozen in the two containers was assessed by inseminating (AI) gilts. The Cochettes yielded a significantly ( $P<0.05$ ) higher motility post-thaw, but the opposite was observed for PMI. No difference in fertilising capacity (pregnancy rate and number of viable embryos) could be seen. It was concluded that though proven feasible, the Cochette is not a suitable container for the commercial freezing of boar semen. The effects of different freezing and thawing rates on the post-thaw motility and PMI of boar spermatozoa, processed in either Maxi-straws or flat plastic packages called FlatPacks were studied. Values for percentage motile spermatozoa, sperm velocity and lateral head displacement, were significantly higher for samples frozen in FlatPacks than for those frozen in Maxi-straws ( $P<0.05$ ). The best post-thaw motility was obtained in semen frozen in FlatPacks at 50°C/min and thawed for 13 sec in a 50°C water-bath. No significant effect on post-thaw PMI was seen for the different freezing and thawing rates as well as package types used. Along with the freezing and thawing rates, extenders, packages and boars used in the present study, thawing rate had the greatest influence on post-thaw sperm survival followed by boar and freezing rate. The effect of holding time (HT) during cooling on PMI and motility before and after freezing as well as the in vitro penetration ability of boar spermatozoa post-thaw were investigated. Before freezing, the HT used had no significant ( $P>0.05$ ) effect on either PMI or percentage of motile spermatozoa. Post-thaw, PMI was significantly higher for a 10 h and a 20 h HT than for 3h, while the percentage of motile spermatozoa decreased significantly with a 20 h HT, as opposed to a HT of 3 h and 10 h ( $P<0.05$ ). In terms of in vitro penetration ability, there was no significant difference among HTs. Based on these results, there is no reason to change the current protocol of a 3 h HT. However, a prolonged HT may be used if needed for practical reasons (e.g. sperm transport after collection for freezing, and more convenient time schedules, etc). In an insemination trial using boar semen frozen in FlatPacks and exported for artificial insemination (AI) to overseas nucleus herds, a mean farrowing rate (FR) of 73% (308 litters from 421 inseminations) and a mean total number of piglets born (TNB) of 10.7 were obtained. The results for AI with FT vs. liquid semen, in a within-sow analysis of purebred Landrace (L) and Yorkshire (Y) animals, showed a lower FR (-6.5%) and TNB (-0.3 piglets) for the FT semen (both differences non-significant). Although a significant boar effect on the in vitro sperm parameters could be seen ( $P<0.05$ ), the between-boar variations in post-thaw sperm quality were relatively small. These are encouraging results, especially considering the fact that they were achieved without any pre-selection, based

on semen freezability, of boars or ejaculates. Collectively, the results show that the present freezing-thawing protocol and the FlatPacks maintain high sperm viability and fertility post-thaw, indicating they are a reliable alternative for freezing of boar semen under commercial AI conditions.

*Key words:* semen, cooling, cryopreservation, thawing rate, freezing packages, freezing rate, holding time, in vitro oocyte penetration ability, motility, plasma membrane integrity, field fertility, boar.

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# Appendix

## List of original papers

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals **I-IV**:

- I.** Eriksson, B.M. and Rodríguez- Martínez, H., 2000: Deep freezing of boar semen in plastic film “Cochettes”. *J. Vet. Med., A*, 47: 89-97.
- II.** Eriksson, B.M. and Rodríguez-Martínez, H., 2000: Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws. *Anim. Reprod. Sci.* 63: 205–220.
- III.** Eriksson, B.M., Vasquez, J.M., Martínez, E., Roca, J., Lucas, X. and Rodríguez-Martínez, H., 2000: Effects of holding time during cooling and type of package on plasma membrane integrity, motility and *in vitro* oocyte penetration ability of frozen-thawed boar spermatozoa. *Theriogenology* (accepted for publication).
- IV.** Eriksson, B.M., Petersson, H. and Rodríguez-Martínez, H., 2000: Field fertility with exported boar semen frozen in the new FlatPack container. (Submitted for publication).

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# Introduction

## Background

The world's pig population is constantly being upgraded to meet the demands for high-quality pork meat. This is done at a regional or national level mainly using artificial insemination (AI) with liquid boar semen preserved for up to 3–5 days for pork production and breeding, and through the transport of live animals or frozen semen for the international trade of superior genetics. The purpose of the latter is to widen the base of selection at the breeding pyramid. Following the discovery of the protective effect of glycerol on spermatozoa (Polge et al 1949), and the impact this finding had on cattle breeding, it was anticipated that the freezing methods developed for bull semen could be adapted to freeze boar spermatozoa. Quite early, it was apparent that this would be a difficult task, and it is as yet partly unsolved (Polge 1956).

During the 1950s and 1960s, the studies on the preservation of boar spermatozoa by deep-freezing, using procedures originally designed for bull semen, generally resulted in some motile spermatozoa post-thaw but no fertility (for a review, see Graham et al 1978, Bwanga 1991, Johnson et al 2000). Although some few reports claiming successful fertility with frozen boar semen appeared in the literature before 1970, the results could not be repeated. In 1970, the first study describing a method for successful fertilisation with frozen-thawed (FT) boar semen was reported (Polge et al 1970). However, fertilisation was only achieved with oviductal (i.e. surgical) insemination. Fertility with FT boar semen, inseminated via the cervix, was reported by three different groups in 1971 (Crabo and Einarsson 1971, Graham et al 1971, Pursel and Johnson 1971). These publications put an end to a long period of uncertainty as to whether it is possible at all to deep-freeze boar spermatozoa with retained fertility.

Following these initial studies, efforts began to aim to improve and adapt the procedures for routine use. Today still, freezing in straws (Westendorf et al 1975) and pellets (Pursel and Johnson 1975), or a modification of these, are the most commonly used methods. Although fertilisation has occurred, followed by the birth of live piglets, the fertility results have been substantially lower than those for extended, liquid semen (Johnson 1985). Therefore, along with the attempts to solve the problems concerning FT boar semen, there has been a parallel development of methods of preserving liquid semen, which for reasons of practicality, low cost, and fertility levels similar to those of natural breeding, are now widely used around the world with good results. Consequently, it seems unlikely that deep-frozen semen will ever replace the use of fresh semen, even if the fertility levels were similar. Nevertheless, there are strong arguments for the role FT semen could still play in certain segments of the pig industry. Frozen boar semen offers several advantages since it –

- enables planned matings which are essential at the top of the breeding pyramid for optimal progress in every gestation, thanks to the infinite life span of the frozen semen gene bank;

- allows selection of genetics from all over the world, defying temporal or geographical boundaries;
- facilitates preservation of top quality genetic lines which may be used in future breeding programmes; and
- offers an extra health safeguard afforded by the ability to store frozen semen until the completion of any health test specified by a country or breeding organisation. This is optimal from a herd health perspective, if proper measures for disease control are taken.

Currently, the utilisation of FT boar semen prepared for AI is estimated to be less than 1% of all inseminations world wide (Wagner and Thibier 2000). The limitations for any wider use have not changed through the years (Reed 1985, Almlid and Hofmo 1996), namely –

- low fertility levels compared with those achieved by liquid semen, with a wide range (40–70%) of farrowing rates (FRs) (about 20–30% units lower than with liquid semen) and litter sizes of 7–10 piglets born (about 2–3 piglets fewer than produced with liquid semen) (Johnson 1985, Almlid and Hofmo 1996);
- a large, between-boar variation in freezing success, measured as both *in vitro* sperm viability and *in vivo* fertility post-thaw;
- requirements for a high number of spermatozoa per insemination dose ( $5\text{--}6 \times 10^9$ ) compared with liquid semen ( $2\text{--}3 \times 10^9$ ),
- higher costs, the freezing-thawing of semen being an expensive procedure, in terms of both labour and laboratory equipment;
- the lack of reliable laboratory tests for the accurate assessment of semen quality, becoming an even larger problem when trying to relate *in vitro* viability with the fertility outcome *in vivo* (see below); and
- the critical timing for AI. The “window” of optimal fertility of FT boar spermatozoa is substantially shorter than that for liquid semen.

Consequently, there is a need to develop and improve the methods of freezing and thawing boar semen.

Boar semen differs in several respects from the semen of other domestic animals. It is produced in large volumes and is extremely sensitive to sudden cooling immediately following collection (the so-called “cold shock”). The success of freezing boar semen depends on our understanding of how several factors influence the capacity of spermatozoa to survive freezing and thawing while maintaining their ability to fertilise. These factors are either internal, such as the inherent characteristics of spermatozoa and the existing differences among boars and ejaculates, or external, such as the composition of the extenders, type and concentration of the cryoprotective agent applied, rates of extension and cooling or equilibration, and the method of freezing and thawing of the semen (Johnson et al 2000). Unfortunately, only these external factors can be modified in order to optimise the freezing protocol.

## Freezing packages

In early studies, boar spermatozoa were frozen in glass ampoules (Polge 1956) or glass tubes (Settergren 1958) using low cooling rates together with relatively high glycerol concentrations. Later, the technique of freezing bull semen as pellets on dry ice (Nagase and Niwa 1963) was adapted to boar semen, allowing decreased glycerol concentrations to 1–3% levels, thanks to the fast cooling achieved (Pursel and Johnson 1975). Boar spermatozoa have also been frozen in 5 ml Maxi-straws (Westendorf et al 1975), 4–5 ml aluminium packages (Waide et al 1975), 0.5 mL Medium-straws (Fazano 1986, Baron 1986, Leps 1988), 1,7–2,0 mL flattened Maxi-straws (Leps 1988, Moura 1988, Ewert 1988, Stampa 1989, Wegmann 1990, Simmet 1993), and 0.25 mL Mini-straws, as well as in different types of 5 mL plastic bags (Larsson et al 1976, Bwanga et al 1991b, Mwanza and Rodriguez-Martinez 1993, Rodriguez-Martinez et al 1996). All package forms have their own advantages and drawbacks. Pellets and the small, 0.25 and 0.5 mL, straws have a cryobiologically suitable shape with a large surface-to-volume ratio. With pellets, however, the identification of the doses becomes difficult. Also, there is a risk of cross-contamination during storage and the thawing procedure is rather complicated. As such, the fact that you need to thaw 10–20 small straws, or 2–3 flattened straws, to obtain one insemination dose is a major obstacle to routine application. The 5 mL Maxi-straw contains one insemination dose but has a relatively small surface-to-volume ratio, which constrains optimal freezing and thawing throughout the sample (Weitze et al 1987). The plastic bags tested so far allow even more homogeneous freezing and thawing and also contain a whole insemination dose, but they are not suited for storage in standard liquid nitrogen containers, and are therefore not in use.

Despite a relatively low sperm survival post-thaw, the 5 mL Maxi-straw appears to be more widely used for AI than is the pellet method (Almlid and Hofmo 1996), mainly because of hygienic reasons and of easier thawing under field conditions. Several authors have demonstrated a higher sperm viability post-thaw in small, Mini- or Medium-, straws, flattened straws or plastic bags, compared with Maxi-straws, measured in terms of a higher percentage of motile spermatozoa with intact acrosomes (for a review, see Simmet 1993). The poor post-thaw viability in the Maxi-straw has been attributed to sub-optimal freezing and thawing rates, affecting the spermatozoa located in the centre of the straw. Concerning fertility, the results are inconclusive, with no major differences in fertility between Maxi-, Medium- or flattened Maxi-straws reported by some authors (Fazano 1986 and Stampa 1989). Despite data showing a higher fertilisation rate and a higher number of accessory spermatozoa in the zona pellucida when inseminating with semen frozen in flattened straws (Simmet 1993) or in 5 ml plastic bags (Bwanga et al 1991b), compared with Maxi-straws, none of these methods has been widely used. Since flattened containers are cryobiologically suitable, further research on the effect of large, one-AI-dose, freezing packages (e.g. plastic bags) on the post-thaw viability and fertility of boar spermatozoa is therefore needed.

## **Freezing and thawing rates**

Cells undergoing freezing are challenged not for their ability to endure storage at very low temperatures but rather, by the lethality of an intermediate zone of temperature which they must traverse, both during cooling/freezing and during thawing. There is an optimal freezing and thawing velocity for every cell type. According to the two-factor theory proposed by Mazur et al (1972), cell damage or cell death can result as consequence of either exposure to high concentrations of solutes (the so-called “solute effect”) occurring when the freezing rate is too low, or the formation of intracellular ice, when the freezing rate is too high. During thawing, the events are reversed. In theory, if the cooling/freezing rate is high, the thawing process should also be fast, that is, speaking in relative terms, and depending on the cell type. The effects of freezing and thawing are interrelated with the concentration of the cryoprotectant included in the extender. Since boar spermatozoa are sensitive to glycerol, comparatively low levels are usually used, which requires the spermatozoa to be frozen rapidly (Mazur 1977). Optimal freezing rates, with regard to the maintenance of motility and acrosome integrity, therefore appear to be 30°C/min, with 3% glycerol, for 0.5 mL straws (Fiser and Fairfull 1990), or 50°C/min, with about 1.5% (0.2 M) glycerol, for 0.25 mL straws (Woelders and Den Besten 1993). When freezing spermatozoa in 5 ml Maxi-straws using 3.3% glycerol, the best post-thaw sperm survival is, however, reached at a slower freezing rate, of 16°C/min (Pursel and Park 1985). A relatively quick thawing rate likewise seems to be beneficial to boar spermatozoa. This was shown both by Fiser et al (1993), who found the optimum thawing rate to be 1,200°C/min using 0.5 mL straws, and Westendorf et al (1975), who used Maxi-straws. Bwanga et al (1991a) studied the effect of different freezing rates in straws and plastic bags. Besides this study, detailed investigations on the effect of different freezing and thawing rates on boar sperm viability post-thaw when using large plastic bags as packaging have not been published.

## **Cold shock and holding time during cooling**

Polge noticed, as early as 1956, a loss in the motility of boar spermatozoa during cooling. This phenomenon is particularly pronounced when the temperature of the semen after collection is quickly lowered to below 15°C, and further to 5°C and to 0°C, the so-called “cold shock”. Among the spermatozoa of domestic animals, boar spermatozoa show the highest sensitivity to cold shock, a sensitivity that is suggested to be caused by the special composition, in phospholipids and cholesterol, of their plasma membrane (Watson and Plummer 1985). Boar spermatozoa can acquire resistance to cold shock if incubated at low extension rates, for 1–5 h at ambient temperatures (Pursel et al 1972, Pursel et al 1973). Based on these findings, most freezing protocols for boar semen include a holding time (HT) of a few hours, at or above 15°C. If the incubation time is increased to 16 h (Tamuli and Watson 1994) or 24 h (Zorn 1987, Weber 1989)

before decreasing the temperature to below 15°C, the proportion of spermatozoa surviving cold shock increases dramatically. This incubation or HT appears to be more important than the exact temperature level (Weber 1989). The beneficial effect of an extended HT, of 20 h instead of 4 h, during cooling on post-thaw sperm viability (Kotzias-Bandeira 1997) and on *in vivo* fertility (Simmet 1993) has been reported. In all the above studies, different measures of sperm viability were, however, used. Therefore, it would be of interest to investigate the effect of a prolonged HT during cooling, in terms of both sperm viability and fertilising ability.

### **Fertility and *in vitro* tests**

Frozen-thawed semen has a lower fertilising capacity, when used for AI, than does fresh semen or semen preserved in liquid form. This is due both to a loss of viable spermatozoa during the freezing-thawing process, and, apparently, to more subtle changes in the surviving sperm population (Watson 2000). Because of the latter, the lower fertility of FT semen cannot be completely compensated to the same level as achieved with fresh semen, by inseminating equal numbers of “live” spermatozoa. Cooling, freezing and thawing induce capacitation-like changes which cause FT semen to have a shorter survival time in the female genital tract compared with its fresh counterpart (Watson 1995). For FT boar semen, optimal fertility is achieved when inseminating in the interval 4–6 h before ovulation until ovulation occurs (Larsson 1976, Waberski et al 1994), as compared with during the 12–28 h preceding ovulation for liquid semen (Waberski et al 1994, Nissen et al 1997). These figures may differ considerably among females (Stevenrink et al 1997).

Spermatozoa need to possess certain attributes or characteristics, expressed in a correct temporal sequence, to achieve normal fertilisation (Amann and Hammerstedt 1993). For this reason, the evaluation of freezing protocols includes the assessment of different sperm parameters such as motility, viability and acrosomal status, in order to monitor the number of surviving spermatozoa post-thaw. Correlations of such *in vitro* semen quality parameters with field fertility data are, however, generally low in pigs (Flowers 1997). This is not surprising since the complex processes leading to successful fertilisation require the analysis of most or even all the necessary characteristics present in a large population of spermatozoa to reach a more reliable estimate of the fertilising capacity of a semen sample. Furthermore, there are inherent limitations of both the parameters assessed *in vitro* and the field fertility data in pigs (Woelders 1991). The use of *in vitro* tests, in direct relation to the process of fertilisation, such as ZP-binding assays, sperm penetration assays and *in vitro* fertilisation (IVF), seems to provide some indication on the fertilising capacity of boar semen (for a review, see Larsson and Rodriguez-Martinez 2000). Whether such a relation would exist if boar semen is frozen in a more optimal way (such as using better freezing protocols and cryobiologically suitable packages) remains to be explored.

## Aims of the study

The main objective of this thesis was to develop a method for the commercial freezing of boar semen, and to study the fertility and the effect of different factors on boar sperm survival *in vitro* after freezing-thawing in large, one-AI-dose containers.

Specific aims were to –

- ↪ determine the feasibility of large, single-AI-dose flat plastic containers (Cochettes and FlatPacks) for freezing and thawing boar semen using Medium- or Maxi-straws as controls; and, in particular, to
- ↪ study the effects of different freezing and thawing rates on post-thaw motility and plasma membrane integrity (PMI) of boar spermatozoa;
- ↪ assess the effects of prolonged holding times (HTs) during cooling on the motility and PMI before and after freezing, as well as the *in vitro* oocyte penetration ability of boar spermatozoa post-thaw; and to
- ↪ determine the fertility of frozen-thawed (FT) boar semen packaged in FlatPacks, and used for AI in overseas nucleus herds as part of the export of selected porcine genetics.

## Materials and methods

### Animals

A total of 63 boars and 340 females (sows and gilts) were used in the study. In **Paper I**, 9 Yorkshire (Y) boars (1–2 y old) and 56 Y gilts in their second or third oestrus; in **Paper II**, 4 Y boars (10–31 mo old); in **Paper III**, 3 boars (1.5–2.5 y old); and in **Paper IV**, 18 Landrace (L), 20 Y, and 9 Hampshire (H) boars, FT semen, as well as 139 L sows (parity 2–12), 117 Y sows (parity 2–11) and 28 terminal crosses (TC females) (parity 1–9) were included. In **Papers I–III**, all the animals were kept at the Department of Obstetrics and Gynaecology, SLU, Uppsala, Sweden. In **Paper IV**, the boars were held at the boar station Norden Semin (Quality Genetics, Hållsta, Sweden) and the female pigs were located at three different farms, Lincou Farm (LF) in Taiwan (Tung Ying Co Ltd, Taipei, Taiwan) and New Inn (NI) and Causeway (C) farms in Ireland (Kerry Agribusiness, Tralee, Ireland).

### Semen processing

The sperm-rich fraction was collected with the gloved hand technique in an insulated thermos flask, separating the gel fraction through a gauze during collection. Single ejaculates were used in **Papers I, II** and **IV**, while in **Paper III**, the semen from three boars was mixed. The semen was processed according to the method described by Westendorf et al (1975) and modified by Bwanga et al (1990). Shortly after collection, the semen was diluted (1:1–1:3) with Beltsville Thawing Solution (BTS) (Pursel and Johnson 1975) at 30–35°C in a 250 mL centrifuge bottle. The semen was allowed to cool to 15°C for about 3 h in a cooling centrifuge (**Papers I–IV**). In **Paper III**, one of the split-sample aliquots (control) was cooled for 3 h while two others were held for 10 or 20 h at 17°C. Thereafter, they were all treated in the same way, being centrifuged at 800 g for 10 min, and the supernatant discarded. A further extension (about 1:1) was performed with a second extender (Extender II, 80 mL 11% lactose solution and 20 mL egg yolk) and the semen further cooled to 5°C for 2 h before being extended (2:1) with Extender III (89.5 mL Extender II, 9 mL glycerol and 1.5 mL Equex STM) (Nova Chemicals Sales, Inc., Scituate, MA, USA). The final sperm and glycerol concentrations were  $1 \times 10^9$  spermatozoa/mL and 3%, respectively. The semen was then filled into Maxi-straws (Minitüb, Tiefenbach, Germany) (**Papers I–III**), Cochettes (**Paper I**), and FlatPacks (**Papers II–IV**), all containing 5 mL, or into 0.5 mL Medium-straws (IMV, L'Aigle, France). The different packages were sealed at 5°C with plastic or metal balls (Maxi-straws), with heat (Auto-Seal 1202, Nitech AB, Sweden) (Cochettes, IMV, L'Aigle, France and FlatPacks, Dept. of OG, SLU through M&D Packaging AB, Uppsala, Sweden) or with PVC powder (Medium-straws), and put onto freezing racks. The doses were transferred to a programmable freezing machine (PTC-200, Planer Products Ltd, UK) (**Paper I**) or Mini Digitcool 1400 (IMV, L'Aigle, France)

(**Papers II–IV**), and frozen. The frozen doses were plunged into liquid nitrogen (LN<sub>2</sub>, -196°C) for storage.

### **Freezing and thawing rates**

The freezing programme (i.e. the chamber temperature) used in the different studies were as follows: in **Paper I**, 3°C/min from 5°C to -6°C, 1 min HT at -6°C, and 20°C/min from -6°C to -100°C; in **Paper II**, 3°C/min from 5°C to -6°C, 1 min HT at -6°C, and at different freezing rates 20, 50 or 80°C/min from -

38°C water-bath for 20 min before examination of motility. After incubation and mixing in the tube, 5 µl of semen were placed in a 10µm deep Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). Motility was measured both visually (subjectively, by phase-contrast microscopy) and by CASA (Strömberg-Mika Cell Motion Analyser; SM-CMA, MTM Medical Technologies, Montreux, Switzerland), at 38°C, in 4 (**Paper I**) or 8 (**Papers II–IV**) predetermined fields. In **Paper I**, the CASA analysis was done directly when the sample was in the microscope. In **Papers II–IV**, the eight fields were recorded for 15 sec each (total 2 min) and the CASA analysis was made from a video tape on a later occasion. The subjective evaluation of the percentage of motile spermatozoa was always made directly (**Papers I–IV**). The setting parameters for the SM-CMA program 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 a velocity of <10 µm/s was considered immotile and objects with a velocity of >25 µm/s were deemed motile. Spermatozoa deviating less than 10% from a straight line were designated “linearly motile” spermatozoa, and those with a radius of <25 µm were classified as “circularly motile”. The mean values of the following motility parameters were obtained from the SM-CMA: motile spermatozoa (CASA, %), linearly motile spermatozoa (%), non-linearly motile spermatozoa (%), and circularly motile spermatozoa (%); as well as straight linear velocity (VSL) (µm/s), average path velocity (VAP) (µm/s), curvilinear velocity (VCL) (µm/s), lateral head displacement (LHD) (µm), and beat cross frequency (BCF) (Hz).

#### *Sperm plasma membrane integrity*

Plasma membrane integrity was expressed as the percentage of spermatozoa with an intact plasma membrane (**Papers I–IV**).

#### Staining with carboxyfluorescein diacetate and propidium iodide

In **Paper I**, PMI was measured using the method originally described by Harrison and Vickers (1990) and modified for frozen boar semen by Ortman and Rodriguez-Martinez (1994). In brief, the spermatozoa (0.1 mL, diluted 1:4 in BTS) were added to 0.3 mL of staining medium (containing 1 mL saline medium, 20 µL formaldehyde, 20 µL propidium iodide [PI] [Sigma Chemical Co, St. Louis, MO, USA] and 20 µL 6-carboxyfluorescein diacetate [CFDA] [Calbiochem, Sweden]), and incubated in darkness for 45 min at 30°C. Sub-samples (5 µL) of the stained suspension were observed in random fields under 400x magnification with epifluorescence UV-illumination under a Leitz-Dialux 20 microscope (Enst Leitz Weztlar, Jena, Germany), and at least 200 cells were counted in each of two 5 µL aliquots. Particular attention was paid to the sperm head region. The counted sperm cells were classified into three groups, as follows: Group A – intact plasmalemma, stained green with CFDA and unstained with PI; Group B – damaged plasmalemma but intact acrosome, acrosome stained green with CFDA but the post-acrosomal region stained red with PI; Group C – both a damaged plasmalemma and damaged acrosomal membrane; cells were unstained with CFDA but stained red with PI.

#### Staining with calcein AM and ethidium homodimer

In **Paper II**, PMI was assessed with the membrane-permeant cytoplasmic esterase marker calcein AM (CAM) and the membrane-impermeant DNA marker ethidium homodimer (EthD-1) using a commercial kit (Live/Dead™ Viability/Cytotoxicity kit, Molecular Probes Inc, OR, USA) as described by Januskauskas et al (1996). Twenty  $\mu\text{l}$  of semen and 20  $\mu\text{L}$  of staining medium were mixed and incubated in the dark for 15–30 min at 33–35°C. Random fields were observed under 600x magnification with epifluorescence illumination at the warm stage (37°C) of a Laborlux-11 Leitz microscope (Ernst Leitz Wetzlar, Jena, Germany) using a 470–490 nm excitation filter, a 510 dichroic beam splitter and a 520 nm barrier filter. One hundred spermatozoa were examined in each of two 5  $\mu\text{L}$  aliquots from a stained sample. Three staining patterns could be discerned, (A) “live”, an intact plasmalemma stained entirely green with CAM, and unstained with EthD-1; (B) “moribund”, with the acrosome stained green with CAM, but the post-acrosomal region stained red with EthD-1; and (C) “dead”, both a damaged plasmalemma and a damaged acrosomal membrane, when the cells were unstained with CAM, but stained red with EthD-1. Only a sub-population of the spermatozoa belonging to category A were motile, but since they became immotile within a few seconds of illumination, no notice was taken of whether the spermatozoa were motile or not. All green spermatozoa were therefore deemed viable by this test.

#### Staining with SYBR-14 and propidium iodide

In **Papers III** and **IV**, PMI was evaluated post-thaw by applying a combination of the fluorophores SYBR-14 and PI (Fertilight® Sperm Viability Kit L-7011, Molecular Probes Inc, OR, USA), as described by Garner and Johnson (1975). The samples were evaluated under a Laborlux-11 Leitz microscope equipped with an objective lens set (ParaLens®, Becton Dickinson, Leiden, The Netherlands) with a 470–490 nm excitation filter, 510 nm dichroic beam splitter and 520 nm barrier filter. Aliquots of 50  $\mu\text{L}$  semen ( $\sim 50 \times 10^6$  spz/mL) were diluted in 150  $\mu\text{L}$  of m-TALP containing 3  $\mu\text{L}$  PI and 2  $\mu\text{L}$  of SYBR-14. Two hundred spermatozoa were examined in two 5  $\mu\text{L}$  aliquots from a stained sample, by two independent operators, and the mean values were then used for the analysis. The nuclei of SYBR-14-stained spermatozoa (“live”) were bright green, while spermatozoa with a damaged head plasma membrane stained red with PI. Only spermatozoa showing green fluorescence over the entire sperm head were considered to have intact head plasma membranes.

### **Temperature measurements**

In **Paper II**, temperature changes during freezing and thawing were measured in representative groups of Maxi-straws and FlatPacks with a type K copper-constantan thermocouple. One thermocouple was held in the centre and one, in the periphery of the Maxi-straw. With the FlatPacks, two thermocouples were

held in the centre, without touching the package wall, about 1 cm apart. When the packages were being filled prior to freezing the tips of the thermocouples were placed 3–6 cm from the end of the Maxi-straws or FlatPacks by means of plastic cushions. All thermocouples were connected to temperature recorders (Chessel<sup>R</sup>, Chessel Ltd, Worthing, West Sussex, UK) during freezing (Model 4180M) and to another (Model 324A) during thawing. The temperature changes in the semen were recorded repeatedly and from the printouts, the freezing and thawing processes were calculated.

### **Homologous *in vitro* penetration assay**

To measure the *in vitro* penetration ability of spermatozoa, a homologous *in vitro* penetration (hIVP) assay using immature pig oocytes was used in **Paper III**, as described previously (Martinez et al 1996, 1998). Immature oocytes were collected from medium-size antral follicles (2–5 mm in diameter) by slicing off ovaries with a single sterile blade into Dulbecco's phosphate-buffered saline (PBS) supplemented with 4 mg/ml BSA (Fraction V, Sigma Chemical Co., Alcobendas, Spain), 0.34 mM sodium pyruvate, 5.54 mM D-glucose, and 70 µg/ml Kanamycin (modified DPBS) at 37°C. The oocyte-cumulus complexes were washed three times with modified DPBS before insemination with FT spermatozoa. Thawed spermatozoa were diluted 1:1.5 in BTS and incubated for 30 min at 37°C. Spermatozoa were then rediluted to  $2 \times 10^8$  cells/ml in fertilization medium consisting of Medium 199 with Earle's salts (Sigma) supplemented with 12% heated foetal calf serum (Sigma), 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 2.92 mM calcium lactate, 50 IU/ml penicillin G and 30 µg/ml streptomycin sulphate (modified M199), and 2 mM caffeine. Groups of 15 immature oocytes were co-incubated with spermatozoa for 16–18 h in a 35 mm plastic dish containing 2 ml of fertilisation medium at 39°C under 5% CO<sub>2</sub> in air (final concentration:  $1 \times 10^6$  spermatozoa/ml). After co-incubation, the oocytes were stripped of cumulus cells and spermatozoa by pipetting, mounted on slides and fixed for a minimum of 24 h with ethanol:acetic acid (3:1 v/v), stained with 1% lacmoid and examined for evidence of sperm penetration under a phase-contrast microscope (x400 magnifications). Oocytes were considered to be penetrated if spermatozoa with swollen or unswollen heads were found in the vitellus. Oocytes with a broken oolemma or abnormal-looking cytoplasm were classified as "degenerated".

### **Electron microscopy**

In **Paper I**, aliquots from each sperm sample to be assessed with the fluorescent dyes were also fixed in a solution of 3% glutaraldehyde in 0.067 M cacodylate buffer (500 mOsm, pH 7.2), and representative samples were routinely processed for scanning electron microscopy (SEM), and examined under a Philips EM 420 electron microscope in SEM mode at 20 kV. The status (intact or damaged) of the plasma membrane of 100 spermatozoa per sample was assessed.

## Clinical investigations

### *Oestrous detection and insemination protocol*

In **Paper I**, oestrous detection was performed with the aid of a boar on two occasions daily, approximately 12 h apart. The first insemination with FT semen was done 24 h after standing oestrus was first observed and with fresh semen, 12 h after detected oestrus. If a gilt still showed standing reflex 12 h after the first insemination, she was inseminated a second time. All the gilts inseminated with fresh semen and 85% (39/46) of the gilts inseminated with FT semen received double doses. In **Paper IV**, when using liquid semen, oestrous detection was performed using a boar at LF and C farms twice daily, and at NI, once a day. Most animals were inseminated twice during each oestrus. For the inseminations with FT semen, oestrous detection was performed twice daily, in the morning (08:00–09:00) and in the evening (17:00–18:00). The first insemination with FT semen took place 24 h after the sow had first shown standing reflex, and the second insemination was performed about 9 h (if the standing oestrus was first seen in the morning) or 15 h (if the standing oestrus was first seen in the evening) after the first. Double inseminations were used if the sow was still in oestrus on the second occasion, which occurred in 96% of the cases.

### *Pregnancy diagnosis*

In **Paper I**, the gilts were checked for signs of oestrus from day 17 to day 24 after insemination and examined by A-mode ultrasound (Preg-Tone, Ysta-Maskiner, Ystad, Sweden) 28–30 d after insemination. In **Paper IV**, signs of a possible return to oestrus were monitored for all inseminations, and pregnancy diagnosis was performed by Doppler ultrasonography at 28–35 d post-insemination. Records of farrowings (farrowing rate, FR) and litter size (total number born piglets, TNB; piglets born alive, BA; and stillborn, SB, piglets) were collected at the farms by the personnel.

### *Examination of the female genital tract after slaughter*

The sows presumed to be pregnant in **Paper I** were slaughtered 30–38 d after insemination. The genital organs were examined after slaughter, and the number of viable embryos (normal size and development) and corpora lutea were counted.

## Statistical analyses

Analyses were performed using the Statistical Analysis System software package (SAS<sup>®</sup>, Cary, NC, USA, 1994 [**Papers I–III**] and 1996 [**Paper IV**]). The level of statistical significance was set to  $P < 0.05$ . In **Paper I**, data on motility, PMI and embryonic survival were analysed using analysis of variance (ANOVA) (GLM procedure), including the effect of boar and type of package. Pregnancy results were analysed using Fischer's exact test. In **Paper II**, data on post-thaw motility and PMI were analysed by ANOVA (GLM procedure), and

included the effect of boar, freezing rate, thawing rate, and the interaction between the freezing and thawing rates. In **Paper III**, data on motility measurements and head PMI, as well as the hIVP assay, were analysed by a repeated measurement ANOVA using the MIXED procedure. The statistical model used for samples analysed before freezing included the fixed effect of temperature, HT during cooling, the interaction between temperature and HT, and the random effect of freezing operation (n=7). The model used for the samples post-thaw included the fixed effect of freezing package, HT during cooling, the interaction between freezing package and HT, and the random effect of freezing operation. In **Paper IV**, data for the *in vitro* tests post-thaw were analysed using a mixed model, with breed as fixed effect and boar as random effect. Reports with data on all services, returns and farrowings were sent regularly to Pig International Breeding AB (PBI) to be used for breeding evaluation.

Two analyses of the fertility data, using the PROC MIXED method, were done, one for FR and the second for measures of litter size (TNB, BA and SB). In the second analysis, a measure of the number of piglets born per sow per year was also included. Only data on purebred L and Y services, either with liquid or with FT semen, rendered during the respective time period when FT semen was used in the different herds, were included in the analyses. For the analysis of FR, all sows that had been inseminated with FT semen were included. Concerning litter size analysis, only sows that had produced a litter with FT semen were included. The statistical model for both analyses included the fixed effect of farm, breed, season (warm: May–October, or cool: November–April), semen type (liquid or FT), parity (2, 3–4, 5–6, 7–8, >8), sow nested within breed and farm, and the interaction between breed and semen type as well as the interaction between semen type and parity. The random effect of boar nested within breed and farm was also included. In addition, the interactions season\*service type and service type\*farm were included in the analysis of the litter size measures only. Correlations (Pearson's) were used to calculate the relationships between sperm parameters *in vitro* and field fertility for the boars that had been used for at least ten inseminations with FT semen.

## Results

### Effect of freezing package (papers I–III)

#### *Temperature measurements during freezing and thawing (Paper II)*

The single major difference noticed between packages during freezing was the longer freezing point plateau (FPP) depicted with Maxi-straws, as compared with FlatPacks. For both types of package, the FPP increased at a slower freezing rate. Differences in the length of the FPP, seen among measurement points within the freezing packages, were most pronounced in the Maxi-straw (longer FPP in the centre vs. the periphery), but were also noted in the FlatPacks. After the FPP was completed, the freezing rates (between  $-5^{\circ}\text{C}$  and  $-60^{\circ}\text{C}$ ) within both packages were practically identical. By contrast, there were remarkable differences in the course of thawing, within the interval of  $-20^{\circ}\text{C}$  to  $0^{\circ}\text{C}$ , both within the Maxi-straw and between the two packages. In this temperature interval, the thawing rate was about 4–5-fold higher in the FlatPacks than in the centre of the Maxi-straw, and 3–4 times faster in the periphery than in the centre of the Maxi-straw. Owing to the very quick course of thawing in the FlatPacks, the temperature difference that could be measured between different locations was negligible.

#### *Post-thaw motility*

Overall, motility rates post-thaw were consistently higher in the flat containers than in the straws. Motility was higher ( $P<0.05$ ) for spermatozoa stored in Cochettes than for those stored in Maxi-straws, both at 30 min (47% vs. 40%) and at 120 min (32% vs. 26%) (**Paper I**). The FlatPacks yielded significantly better post-thaw motility than did the Maxi-straw ( $P<0.05$ ), both measured subjectively and with CASA (**Paper II**). The percentage of motile spermatozoa was significantly higher for FlatPacks (47%) than it was for Maxi- or Medium-straws (34% and 36%, respectively,  $P<0.05$ ) (**Paper III**).

Also, the analyses of motility patterns measured with CASA showed clear differences between packages. Spermatozoa frozen in Cochettes displayed a higher VCL than did those frozen in Maxi-straws, but the difference was only significant at 30 min post-thaw. There was no significant difference in the percentage of linearly motile spermatozoa between Maxi-straws and Cochettes at either 30 or 120 min post-thaw (**Paper I**). FlatPacks gave a significantly higher VSL, VAP and VCL and LHD post-thaw than did Maxi-straws ( $P<0.05$ ). FlatPacks also showed a significantly higher percentage of linearly motile, and lower percentage of circularly motile spermatozoa ( $P<0.05$ , **Paper II**). However, FlatPacks and Maxi-straws yielded significantly fewer circularly motile and non-linearly motile spermatozoa than did Medium-straws ( $P<0.05$ ) although mean VSL, VAP, VCL and LHD were all significantly higher for FlatPacks than for Maxi- or Medium-straws. Medium-straws yielded a significantly higher VAP,

VCL and LHD than did Maxi-straws, but no difference was noticed for VSL (**Paper III**).

#### *Plasma membrane integrity*

More spermatozoa with intact plasma membranes were present in the semen FT in Maxi-straws than in that FT in Cochettes ( $P<0.05$ ) (**Paper I**). There were no significant differences in PMI between Maxi-straws and FlatPacks ( $P>0.05$ ) (**Papers II and III**), but PMI in both was higher ( $P<0.05$ ) than in Medium-straws.

#### *Fertility*

No significant differences in pregnancy rate or in numbers of viable embryos, 30–38 d post-insemination, were found when comparing Maxi-straws and Cochettes (**Paper I**). The hIVP rates were significantly higher for spermatozoa FT in FlatPacks than for those processed in Maxi- and Medium-straws, in terms of both oocyte penetration rates and number of spermatozoa per penetrated oocyte (**Paper III**).

### **Effect of freezing and thawing rates (Paper II)**

#### *Post-thaw motility*

There were significant effects of freezing rate, thawing regime and boar on the number of motile spermatozoa post-thaw, either estimated visually or with CASA. Among cooling rates, 50°C/min gave better results than did 20 or 80°C/min; while for the different thawing regimes, there was an advantage for FlatPacks, which yielded significantly better post-thaw motility than did the Maxi-straws. Within package, there was an improvement in motility when a higher temperature was used for thawing. More motile spermatozoa ( $P<0.05$ ) were found in the FlatPacks when thawing took place at 50°C and 70°C than when it was done at 35°C. In the Maxi-straw, however, post-thaw subjective motility (but not CASA) increased with rising thawing temperature ( $P<0.05$ ) (**Paper II**).

More spermatozoa displayed linear motility in the samples frozen in FlatPacks and thawed at 50° and 70°C, than in Maxi-straws ( $P<0.05$ ). The FlatPacks contained a smaller ( $P<0.05$ ) proportion of circularly motile spermatozoa, compared with the other thawing regimes. In Maxi-straws, sperm velocity increased in relation to higher thawing temperature ( $P<0.05$ ). This could also be seen when comparing 35° and 50°C in the FlatPacks, but not between 50° with 70°C. The different freezing rates did not affect sperm velocity.

There was a significant increase in LHD when using a higher thawing temperature within package, the FlatPacks yielding higher values than the Maxi-straw. Freezing rate, thawing regime and boar all had a statistically significant effect on the proportion of linearly motile spermatozoa, whereas only thawing

rate and boar influenced the proportion of those circularly motile ( $P<0.05$ ) (**Paper II**).

#### *Plasma membrane integrity*

Neither freezing rate nor thawing regime had any significant influence on PMI, although there was a tendency for improvement when thawing at 70°C in both Maxi-straws and FlatPacks (**Paper II**).

### **Effect of holding time during cooling (Paper III)**

#### *Effect on sperm motility parameters and plasma membrane integrity*

##### Temperature

Both the percentage of spermatozoa with intact head plasma membranes and the progressive motility decreased significantly when cooling from 32°C to 5°C. The absolute difference for PMI was, however, very small. There was a significantly lower proportion ( $P<0.05$ ) of spermatozoa with a linear motility pattern at both 15°C and 5°C, than there was at 32°C. At the same time, the frequency of spermatozoa with circular motility increased significantly at the lower temperatures compared with 32°C. At 15°C and 5°C, the mean VSL and VAP parameters were significantly lower than at 32°C. The VCL showed a tendency to increase at lower temperatures, the difference being significant only between 32°C and 15°C. Mean LHD was also significantly higher at 15°C and 5°C than at 32°C ( $P<0.05$ ).

##### Holding time

The different HTs had no significant effect on either the proportions of PMI or motile spermatozoa. The frequencies of linearly motile spermatozoa decreased and those of spermatozoa with circular motility increased significantly for a 10 h and 20 h compared with a 3 h HT. Mean VSL values were significantly lower ( $P<0.05$ ), and VCL and LHD values significantly higher ( $P<0.05$ ) for 10 h and 20 h than for a 3 h HT.

##### Interaction between temperature and holding time

There was significant interaction between temperature and HT during cooling for linear, circular, VSL and VCL parameters. At 32°C, there were no statistically significant differences between the HTs for any of these parameters. On the other hand, at 15°C and 5°C compared with 32°C, there was a significant decrease ( $P<0.05$ ) in the percentage of linearly motile spermatozoa and VSL, and a significant increase ( $P<0.05$ ) in the proportion of circularly motile spermatozoa and VCL. These changes were amplified with 10 h and 20 h HTs compared with a 3 h HT.

### *Effects on motility, PMI and in vitro penetration ability post-thaw*

The percentage of spermatozoa with PMI was significantly higher for a HT of 10 h and 20 h than for a 3 h HT, while a 20 h HT yielded a significantly lower portion of motile spermatozoa than did 3 h and 10 h ( $P<0.05$ ). There was a significant decrease in the number of linearly motile spermatozoa, and consequently, more circularly motile spermatozoa for 10 h and 20 h ( $P<0.05$ ). The parameters for mean VSL and VAP decreased significantly for 20 h compared with a 3 h HT. There was no significant difference, between the HTs, in the *in vitro* hIVP assay, in terms of percentage of penetrated oocytes and number of spermatozoa per penetrated oocyte.

## **Field fertility (Paper IV)**

### *Semen parameters before freezing*

In the ejaculates of L, Y and H boars frozen for export, the mean frequency of subjectively assessed motile spermatozoa after initial dilution ranged from 81% to 83%. From the average total number of spermatozoa (range 45–55 $\times 10^9$  per ejaculate), 6.7–8.2 doses were produced containing 4.6–4.8 $\times 10^9$  spermatozoa, respectively. Mean sperm loss during centrifugation averaged 20–25% (range 2–37%) per ejaculate.

### *Sperm quality post-thaw*

Although considerable variation was present in PMI and sperm motility (percentage of motile spermatozoa and different motility patterns assessed with CASA) post-thaw among ejaculates, there were only minor, non-significant (NS) differences in the mean values for these parameters between the L, Y and H ejaculates. The mean percentage of PMI post-thaw was 60% for all three breeds and the percentage of motile spermatozoa ranged from 49% to 53%. Only three ejaculates (1.9%) had <45% of PMI spermatozoa and two ejaculates (1.3%) showed <40% motile spermatozoa post-thaw. A significant within-breed boar effect was seen for all FT sperm parameters explored *in vitro*.

### *Relationships between in vitro parameters and in vivo fertility*

None of the *in vitro* post-thaw parameters studied (PMI, motility, and CASA motility patterns) correlated significantly with fertility (FR and TNB) (boars used with more than ten inseminations).

### *Fertility trial*

From a total of 308 litters derived from 421 inseminations using FT semen, a farrowing rate (FR) of 73% and litter size of 10.7 piglets (measured as TNB) were obtained. The purebred, L and Y, inseminations yielded a FR of 72% (254 litters/352 inseminations), while in TC females (including twelve gilts), inseminations with FT semen yielded a FR of 76% (39/51) and a mean litter size of 10.5 TNB and 9.8 BA piglets at NI farm. Out of the latter, the H semen

yielded an 82% FR (23/28) and a litter size of 11.0 TNB piglets. For the non-purebred inseminations, the FT semen on L and Y sows gave a FR of 8/8 and 7/9, with TNBs of 11.4 and 12.9 piglets, respectively.

For the purebred inseminations with liquid and FT semen, farm had a significant effect on FR, TNB and BA, as well as on the number of BA piglets per sow/year, ranging from 64.4% to 85.6%; 9.8 to 12.5; 8.6 to 11.3 and 20.4 to 26.0, respectively. Litter size (TNB and BA) at the LF farm, in Taiwan, was significantly smaller ( $P<0.05$ ) than what was recorded at the two Irish farms. No significant interaction ( $P>0.05$ ) was found for farm\*semen type for any of the outcome parameters. Parity had a significant effect on FR, TNB, BA, and number of BA per sow per year, with the lower values for the higher (>8) parities. Stillborn figures did not increase with increasing parity. Boar (within breed) significantly affected only the number of BA piglets from inseminations with FT semen.

#### *Comparisons of fertility using frozen-thawed semen and liquid semen*

Use of FT semen resulted in a lower FR (-6.5%), TNB (-0.3) and BA (-0.1) compared with inseminations using liquid semen ( $P>0.05$ ) when using the same sows overseas. The number of piglets BA per sow/year did not differ significantly. There was a significant interaction between season and semen type for FR, where FT semen had a FR of 79% when inseminations were performed during the cool season, compared with 65% during the warm season ( $P<0.05$ ). However, FR did not significantly differ between seasons when liquid semen was used. For TNB, BA, and BA per sow per year, there was no significant interaction for season\*semen type. For FT semen, litter size (both TNB and BA) was 1.0 piglet larger ( $P<0.05$ ) for Y breedings than for L breedings. The increase of 4.8% units in FR in the Y breed compared with the L breed following insemination with FT semen was, however, not significant. For the females not farrowing after being inseminated with FT semen, the number of days from insemination until detected not pregnant was higher ( $P<0.05$ ) at the LF farm (mean=61 d), than at the NI and C farms (mean=33 d).

The fertility of the FT semen (from the inseminations done overseas with L and Y boars having been used for more than ten inseminations) appeared to be lower than that obtained with their liquid semen used in Sweden for AI. The FT semen of L and Y boars had, overseas, a 18–22% unit lower fertility (measured as FR;  $P<0.05$  for L boars) than that shown by their liquid semen when used for AI in Sweden (measured as expected fertility, EF), as well as a lower TNB (0.9–1.5 piglets) (NS).

## General discussion

### Freezing packages

In **Paper I**, when comparing samples frozen in Cochettes or Maxi-straws, motility (i.e. the percentage of motile spermatozoa and their velocity) at 30 min post-thaw was higher for Cochettes. The opposite was seen for PMI. The small insemination trial performed in the study indicated an equal level of fertility, measured as pregnancy rate, number of embryos and embryo survival on d 30–38, for the two packages.

The study showed that freezing of boar semen is feasible using Cochettes, but that this package also has serious drawbacks. Owing to the flexibility of the bag it is virtually impossible to get rid of all of the air inside. In the worst case, this could lead to bursting of the package during thawing, due to air expansion. Also, these physics could be responsible for the membrane damages noticed post-thaw, since they would lead to an inconsistent thawing at too slow a rate, and the occurrence of intracellular recrystallization (Ortman and Rodriguez-Martinez 1994). Therefore, the full benefit of a flat package, as opposed to the Maxi-straw, cannot be obtained with Cochettes. Furthermore, the advantage of being able to freeze, thaw and inseminate using the same package could not counterbalance the large amount of space that the Cochettes require during storage in commercial dewars. This problem must be solved before this kind of package can be considered as a viable commercial alternative to currently used package types such as Maxi-straws and pellets. It is concluded, therefore, that the Cochette is not a suitable package for the commercial freezing of boar semen.

In the following studies, a newly designed package, the FlatPacks, was used, which enables keeping a thin, 1 mm, film of semen throughout the package. The large surface-to-volume ratio provided is similar to that of small (0.25 mL) straws. From a cryobiological viewpoint, it is therefore attractive compared with the routinely mostly used 5 mL Maxi-straw, which has a diameter of 5.4 mm. Also, the FlatPacks hold enough semen for an entire insemination dose when used for AI and fit into the canisters of commercial storage dewars.

In **Papers II and III**, the semen samples frozen in FlatPacks consistently showed a higher percentage of viable, motile spermatozoa post-thaw than did the Maxi-straws. The CASA analysis of motility patterns revealed other differences between the two packages. The VSL, VAP and VCL were higher for the FlatPacks and so was LHD. In **Paper II**, the percentage of linearly motile spermatozoa was likewise higher for the FlatPacks. This suggests that FlatPacks provide conditions during freezing and thawing that are more advantageous for the retention of sperm motility. These results are in agreement with what has been reported elsewhere on the use of smaller or flatter containers, compared with Maxi-straws (for a review, see Simmet 1993). Whether the improved post-thaw quality of boar semen frozen in smaller packages is due to better freezing and/or thawing conditions is unclear (see further under “Freezing and thawing rates”).

In previous investigations, the percentage of spermatozoa with normal acrosome ridges (NARs) only occasionally showed differences between packages and the differences seen were relatively small. In fact, in one study, Simmet (1993) saw no differences in effect on the proportion of NARs between Maxi-straws and flattened (2 mL) packages in one study, but could see a significant improvement with the flattened straw in another trial, when using other boars. The percentage of NARs post-thaw was not monitored in the present work but the integrity of the head plasma membrane was assessed, as a more reliable measure of membrane and acrosome status (Rodriguez-Martinez et al 1997). Confirming many of the earlier studies, we found no significant differences in sperm PMI between FlatPacks and Maxi-straws (**Papers II, III**). Also, for spermatozoa of other species, motility has been shown to deteriorate before any change is seen in PMI when cryopreserved (McLaughlin et al 1992, Januskauskas et al 1996, Polcz et al 1998), exposed to anisotonic conditions (Liu and Foote 1998) or when cell-sorted by flow cytometry (Call et al 1997).

In **Paper III**, the penetration rates in the hIVP assay, in terms of both oocyte penetration rate and number of spermatozoa per penetrated oocyte, were significantly higher for FlatPacks than for Maxi-straws and Medium-straws. This is most likely a sequel to the better post-thaw motility seen with the FlatPacks. Sperm velocity, degree of LHD, and linearity were all reported to correlate with the outcome of human IVF results (Holt et al 1985, Liu et al 1991). When used for AI under standard field conditions, the samples frozen in FlatPacks yielded an overall fertility of 73% (measured as FR) and 10.7 TNB piglets (**Paper IV**). This was achieved with semen from 47 boars of the L, Y and H breeds, without any pre-selection for semen freezability. Considering the high sperm viability post-thaw (**Papers II–IV**) and the field fertility under commercial AI conditions (**Paper IV**), the FlatPacks, using the present freezing protocol, seem to be a reliable alternative for commercial freezing of boar semen.

The lower sperm survival seen in semen frozen in Medium-straws (0.5 mL) was most probably due to the unsuitable freezing programme used, which was designed for the large containers (i.e. 5 ml Maxi-straws). Whether sperm viability post-thaw would have been higher using a more suitable programme is yet to be disclosed.

### **Freezing and thawing rates**

The ability to tolerate freezing and thawing is dependent on the velocities with which these processes are performed. Depending on different biological properties such as water permeability, the energy required for its activation and the size of the cell or, better put, its surface-to-volume ratio, there should be, for each cell type, an optimal rate of freezing (for a review, see Mazur 1985). The inverted U-shaped curve describing cell survival after freezing and thawing that has been presented for a number of cell types (Mazur 1984, Mazur 1985) appears to be also valid for boar spermatozoa (Fiser and Fairfull 1990, Woelders and Den Besten 1993). Due to their low tolerance to glycerol, the freezing of boar spermatozoa has to be relative rapid. In **Paper II**, the best post-thaw motility,

when using 5 mL packages (Maxi-straws or Flatpacks), was obtained with a freezing rate of 50°C/min (chamber temperature) with 3% glycerol, although the absolute differences were rather small when compared with samples frozen at 20°C/min or 80°C/min. This was close to the optimum freezing velocity reported when using 0.5 mL straws (30°C/min with 3% glycerol) (Fiser and Fairfull 1990) and for 0.25 mL straws (50°C/min with 1.5% glycerol) (Woelders and Den Besten 1993). Although the best cryosurvival in the two studies cited was at 30°C/min or 50°C/min, there were no dramatic differences in post-thaw sperm viability when using other freezing rates in a vast range between 10°C/min and 100°C/min. Therefore, it appears that both for small straws and for large freezing containers (Maxi-straws or FlatPacks), the optimum freezing rate for boar spermatozoa is in the range of 30–50°C/min. However, it also appears that boar spermatozoa tolerate a range of freezing rates around this optimum (Watson 1979). This has also been seen when semen from other species was frozen (Robbins et al 1976, Rota et al 1998), especially when a high thawing rate was applied (Woelders and Malva 1998). No significant effects could be noticed on PMI for the freezing rates used in **Paper II**. It has also been suggested that there is a variation among boars in terms of their sensitivity to sub-optimal thawing rates (Eriksson et al 1997a, b, Medrano et al 1998), which may account for some of the conflicting results concerning freezing rates reported so far.

The rate of thawing through the critical temperature range is an important factor affecting survival of spermatozoa and the optimal freezing rate clearly depends on the original freezing rate (Mazur 1985). In boar semen frozen at the optimum rate, both sperm motility and acrosome integrity improved with an increasing thawing rate, where 1,200°C/min appeared to be the optimum (Fiser et al 1993). Similarly, faster thawing rates were beneficial for boar semen frozen in pellets (Salamon et al 1973, Pursel and Johnson 1976). In **Paper II**, the best thawing regime for FlatPacks, in terms of post-thaw motility, was 50°C for 13 sec. This would represent a thawing velocity of about 900°C/min. It should be, however, borne in mind that thawing does not proceed in a linear fashion (see “Temperature measurements”). Sperm survival could not be further improved by using an even higher thawing rate (e.g. FlatPacks in 70°C for 8 sec), suggesting that there is a point at which thawing rate and sperm survival level each other. This is in agreement with what has been found by others when freezing boar (Fiser and Fairfull 1993), bull (Woelders and Malva 1998) and ram semen (Söderquist et al 1997). It suggests that there is a threshold value in thawing rate where the risk of recrystallization of minute ice crystals during thawing is minimised, after which no further improvement in sperm survival is seen. In terms of the relative importance of freezing and thawing rates for the protocol used in **Paper II**, it was the thawing rate that influenced the sperm survival the most.

The importance of the thawing rate was also substantiated in **Paper II** by the temperature measurements made during freezing and thawing. The most pronounced effects in the temperature courses were seen between –20°C and 0°C during thawing, with a 4–5-fold increase in thawing velocity when comparing

the centre of the Maxi-straw with that of FlatPacks. The difference between the centre and the periphery of the Maxi-straw, within the same temperature interval, was 3–4-fold. This is in agreement with findings by Weitze et al (1987), who noticed that the thawing rate between  $-20^{\circ}\text{C}$  and  $0^{\circ}\text{C}$  was 3.75 times slower in the centre than in the periphery of the Maxi-straw. This is due to the insulating effect of the already thawed extender in the periphery, which constrains an optimum thawing velocity in the centre of the straw. All these considerations taken together, the lower sperm survival post-thaw seen with Maxi-straws, as compared with smaller and flatter containers, appears to be due to an inadequate thawing. Together with the freezing and thawing rates, extenders, packages and boars used in the present study, thawing rate had the greatest influence on post-thaw sperm survival, followed by boar and freezing rate.

### **Holding time during cooling**

In **Paper III**, the effect of an extended HT in the presence of extended seminal plasma (SP) for up to 20 h, on sperm viability before and after freezing as well as on the ability of spermatozoa to penetrate *in vitro* homologous oocytes post-thaw, was assessed. The percentage of spermatozoa showing intact plasma membranes post-thaw increased concomitantly with HT (3–20 h) during cooling (**Paper III**). This is in agreement with the findings of Kotzias-Bandeira (1997). The mechanism behind the increased cryoresistance on the sperm head plasma membrane is not known, but above  $15^{\circ}\text{C}$ , the development of resistance to cold shock appears to depend more on the length of exposure to SP (which occurs during the HT), than on the actual temperature (Weber 1989). The role of the SP in connection with this extended HT, increasing the resistance to cold shock, has been debated. Pursel et al (1973) suggested that SP gives additional protection, whereas others found no positive effect of SP on resistance to cold shock (Tamuli 1993). In the results on FT boar semen, it has been indicated that SP does not appear to play an essential role for the increased cryosurvival seen with a longer HT (Kotzias-Bandeira 1997). However, it has been concluded that it is difficult to obtain entirely SP-free spermatozoa (Kotzias-Bandeira 1997). In contrast to these results, where a positive effect of a longer HT was also found on post-thaw motility, we saw a lower percentage of motile spermatozoa with a HT of 20 h than with a 3 or 10 h HT (**Paper III**). Individual differences among the boars used in these studies may provide an explanation for the conflicting results.

No significant effects could be noticed for the different HTs on the PMI or percentage of motile spermatozoa, assessed before freezing. However, an analysis of the motility patterns with CASA showed that more spermatozoa moved in a circular manner, which was accompanied by a lowering VSL and an increase in VCL and LHD, when cooled to  $5^{\circ}\text{C}$ , and also in the FT samples. These phenomena were amplified with a 10 or 20 h HT as compared with a 3 h HT. Some spermatozoa even displayed a pattern of movement similar to what has been described for hyperactivated boar spermatozoa (Suarez et al 1992). It has been suggested that cryopreserved spermatozoa are in a state resembling capacitation (Watson 1995). Whether the changes in motility pattern noticed in

our study (**Paper III**) are related to changes in the capacitation status (e.g. detectable with CTC staining or otherwise) of the spermatozoa will have to be studied further. In our study, the semen was not diluted at a high rate so that the concentration of SP-decapacitating factors (Dukelow et al 1967) may have been high enough to prevent any capacitation. The capacitation-like changes in boar spermatozoa caused by cell sorting are reversed by the inclusion of SP (Maxwell and Johnson 1999) and no penetration was observed with the presence of 10% SP in IVF medium (Suzuki, personal communication 2000). Collectively, these findings suggest that the changes in motility patterns observed in **Paper III** may not have been caused by capacitation-like changes.

The hIVP assay has been used as a means of testing the penetration ability of boar semen (Martinez et al 1996). The assay results have been correlated with the *in vivo* fertility of liquid boar semen (Gadea et al 1998, Martinez et al 1998). In **Paper III**, the different HTs used did not have any significant effect on the oocyte penetration ability, as assessed by the hIVP assay, but showed high rates of penetration with the freezing method used. If that is also the case with fertilisation and early embryonic development *in vivo* remains to be investigated.

The present results (**Paper III**), concerning the effect of a prolonged HT during cooling on the post-thaw sperm function, do not give any reason to change the current protocol with 3 h HT before passing 15°C and further cooling.

### **Frozen-thawed boar semen and field fertility**

Compared with liquid boar semen, the methods for cryopreservation of boar spermatozoa used in commercial AI are fairly ineffective. The number of insemination doses produced per ejaculate is substantially low. In **Paper IV**, a mean of about 7–8 frozen doses were obtained from each ejaculate when the boars had been collected frequently, most often 3–4 d prior to the semen freezing. This is about 35–40% of what would have been achieved with liquid semen under similar conditions of collection frequency. However, the efficiency in the production of frozen AI doses would be almost doubled if the boars were collected once a week, with few extra labour demands. This could not be achieved in **Paper IV**, because of the high demand for the boars used for liquid semen production. Owing to the high production costs, it would also be very valuable to use lower sperm numbers, below  $5 \times 10^9$  per dose, in connection with FT boar semen.

The fertility levels for FT boar semen are often reported to be substantially lower than for liquid semen (Johnson 1985, Almlid and Hofmo 1996). This is believed to be due to a lower viability post-thaw and to sub-lethal dysfunction in a proportion of the surviving cell population (Watson 2000). Therefore, the fertile life of FT boar spermatozoa in the female reproductive tract is shorter than that of liquid semen (Waberski et al 1994), and consequently, the insemination time in relation to time of ovulation becomes more critical with FT semen than it is with liquid semen. This is of particular importance in pig AI where a large variation among females occurs in the interval from onset of oestrus to ovulation,

which cannot be predicted beforehand (Weitze et al 1994, Kemp and Soede 1996).

In most instances, the lowering of fertility with FT boar semen, as compared with liquid semen, has only been estimated roughly, based on the expected fertility of liquid semen (Johnson 1985). The best comparison of the fertility levels for boar semen preserved in liquid or frozen form would be to run an insemination trial with liquid and frozen semen from the same boars, using the same females. This has, apparently, not yet been performed. The second best would be to perform a within-sow analysis of the fertility data when using liquid and FT semen originating from different boars. In one study of AI with FT semen, the TNB was reported to be 1.5 piglets lower than when liquid semen had been used in the previous parity (Almlid et al 1987). In **Paper IV**, a lowering of 6.5% units and 0.3 piglets, respectively, of the FR and TNB was noticed when using FT semen. These are promising results, especially as they were achieved without any pre-selection of boars based on their semen freezability. In the comparison between the fertility when using liquid semen in Sweden and FT semen overseas from the same boars, larger differences in fertility levels were noticed. However, in those analyses, fewer factors were controlled.

Moreover, it should be borne in mind that different boars were used for the liquid and the FT semen overseas. This and the selection of highly fertile females may have influenced the fertility results. The overall fertility obtained using FT semen, with a 73% of FR (308 litters from 421 inseminations) and a TNB of 10.7 piglets, is encouraging, in particular the results with FT semen with purebred Y inseminations, which yielded a litter size of 1.0 TNB piglets more than did the L inseminations. Also, the FR tended to be higher (4.8% units). It has been reported previously, in connection with FT semen, that the Large White breed gives better fertility results than the L breed (Johnson et al 1981, Paquignon and Courot 1976). It would be interesting to study whether this breed effect depends on mainly male and/or mainly female factors. In **Paper IV**, there were no differences between the L and Y breeds in the post-thaw semen parameters measured *in vitro*.

In the fertility trial (**Paper IV**), a more pronounced seasonal variation in FR was noticed when FT semen was used, compared with liquid semen. This deserves further investigation. Based on the results obtained in the fertility trial (**Paper IV**), no support was found for the suggestion that FT semen is associated with an increased incidence of early embryonic death (Salamon and Maxwell 1995). The reported occurrence of a late and irregular return to oestrus (Salamon and Visser 1973, Osinovo and Salamon 1976, Larsson and Einarsson 1976), which could be due to a number of different reasons, among them embryonic death, appears to be more related to different farm factors than to the use of FT boar semen *per se* (**Paper IV**).

In conclusion, the fertility of FT boar semen, as reported for the semen of most mammalian species, is lower than that of fresh or stored liquid semen. However, the use of an optimised freezing protocol in combination with good routines for oestrous detection and insemination showed that fertility levels were

not far from those recorded for liquid semen (**Paper IV**). The freezing protocol and package container (FlatPacks) used in **Paper IV**, with high sperm viability post-thaw and good fertility under commercial AI conditions, therefore indicate that this is a reliable alternative for freezing of boar semen.

### ***In vitro* tests**

The biggest loss of viable spermatozoa occurs during freezing and thawing, with only minor changes in motility and PMI during cooling (**Paper III**). This was also seen in some other studies (Paulenz et al 1995, Huang et al 1999), while some authors found the largest decrease in motility and PMI already to occur during cooling to 5°C (Maxwell and Johnson 1997).

During the course of these studies, the PMI for the sperm cells has been assessed using three different fluorophore combinations. Whether some of the differences in the results for this parameter may be explained by the different staining techniques remains to be studied.

There are several possible explanations for the discrepancy between the results for post-thaw motility and PMI but it appears that the head plasma membranes, measured as NARs or PMI, have a lower sensitivity to the stresses caused by freezing and thawing than what is seen for the structures in the tail which are responsible for the motility (Eriksson et al 1996, Ekwall et al 1997), probably owing to the different sub-cellular structure of these regions (Courstens and Paquignon 1985). It has been shown that boar spermatozoa can have intact head plasma membranes but damaged tail membranes, in which case they should be considered functionally dead (Nagy et al 1999). The status of the tail membranes was not assessed in this work but should be considered in future studies of the freezing of boar spermatozoa.

In practice, it is desirable to identify males with, and ejaculates of, inferior fertility. Different *in vitro* sperm quality tests are used in the hope of gaining some knowledge about the fertilising capacity of a certain semen sample. In **Paper IV**, none of the *in vitro* post-thaw parameters studied correlated significantly with fertility. This was not surprising considering the limitations of both the *in vitro* tests and the fertility measures (Amann 1989, Amann and Hammerstedt 1993). Typically, only a few inseminations are performed with FT semen from a particular boar. In the present study (**Paper IV**), semen from 23 frequently used boars was inseminated at only 10–20 inseminations, giving 7–16 litters, per boar. This low number inevitably makes the estimation of fertility at a boar level difficult. In a study with liquid boar semen, the percentage of motile spermatozoa was well correlated to fertility rates only when a sub-optimal semen dose was used (Tardif et al 1999). The sperm numbers per dose used in **Paper IV** may have been too high to reveal any correlations between the *in vitro* sperm parameters and fertility (Amann 1989). Holt et al (1997) found, on the other hand, significant relations between different CASA-derived motility patterns, conception rate and litter size using liquid boar semen. However, the authors mentioned some factors believed to be of importance for their success, such as a low sperm number per dose, combining CASA measurements with incubation in

conditions likely to induce capacitation, and the use of repeated measurements to assess temporal dynamics in the sperm population. In **Paper IV**, none of these factors was present, which may also explain why no such relations between *in vitro* sperm parameters and fertility could be seen.

With a better understanding of the dynamic changes spermatozoa undergo *in vivo*, a careful selection and combination of some sperm quality parameters (preferably measured on single spermatozoa but on a large number of them), and the application of more complex methods for data analysis (Abaigar et al 1999), it may be possible to derive more objective, function-based fertility tests for boar semen (Holt and Medrano 1997). The disadvantage of this logical approach to sperm function tests is that they may become too complex for implementation in routine laboratories.

### **Boar effect**

In a number of studies a significant boar effect on post-thaw sperm viability parameters (Larsson and Ersmar 1980, Almlid and Johnson 1988), *in vitro* fertilising capacity (Berger and Parker 1989, Martinez et al 1993) as well as on the fertility *in vivo* (Larsson and Einarsson 1976, Johnson et al 1981, Aumüller 1982, Almlid et al 1987) has been reported. The variation in post-thaw semen quality between individuals may have a genetic background (Thurston et al 2000). Significant boar effects were present in many of the *in vitro* parameters studied (**Papers I, II, IV**). In **Paper III**, the effect of different boars could not be assessed since in that study, pooled semen was used. Also, for the *in vivo* fertility measures (including number of viable embryos and embryo survival [**Paper I**], and number of piglets BA [**Paper IV**]), a significant boar effect was registered. There is a need for a better understanding of the factors governing variation among boars, both for the *in vitro* parameters and for fertility. Unfortunately, there is no strong relation between *in vitro* sperm quality and fertility, which makes it almost impossible to know which boars will have low fertility results with FT semen and should, therefore, not be used.

## General conclusions

- Using basically similar freezing protocols, the FlatPacks yielded better cryosurvival post-thaw than did the Cochettes. Cochettes do not appear, owing to the inconsistent, slow thawing and the large amount of space they need during storage in commercial dewars, to be a suitable package for the commercial freezing of boar semen. FlatPacks lack these drawbacks.
- The best sperm cryosurvival was obtained by freezing the semen in FlatPacks, with a freezing rate of 50°C/min, and thawing for 13 sec in a 50°C water-bath. Along with the freezing and thawing rates, extenders, packages and boars used in the present study, thawing rate had the greatest influence on post-thaw sperm survival, followed by boar and freezing rate.
- The routine freezing protocol for FlatPacks included a 3 h incubation (HT) during initial cooling. Prolongation of the HT was neither advantageous nor detrimental to *in vitro* sperm viability or fertilising ability post-thaw. Therefore, there is no reason for a change in the current protocol with 3 h HT. On the other hand, a prolonged HT may be used if needed for practical reasons (e.g. sperm transport after collection for freezing, more convenient time schedules, etc).
- The semen frozen in FlatPacks and exported for AI to overseas nucleus herds yielded a mean FR of 73% (308 litters from 421 breedings) and a mean TNB of 10.7 piglets. When compared with liquid semen (purebred L and Y), in a within-sow analysis, the FR was 6.5% and the TNB, 0.3 piglets lower for the FT semen (both differences NS). These are encouraging results, especially considering the fact that they were achieved without any preselection of boars or ejaculates, based on their semen freezability. Therefore, with the present freezing/thawing protocol, FlatPacks appear to be a reliable alternative for commercial freezing of boar semen.

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