Assessment of Sperm Attributes of Frozen-thawed AI Doses from Swedish and Estonian Dairy Bull Sires

With Special Reference to Pre-selection Through Swim-up, and the Influence of Age on Potential Fertility

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Abstract

The fertility of bull semen used in artificial insemination (AI) is essential for the effective use of this technology in bovine breeding. The laboratory evaluation of semen from healthy bulls, before and after freezing for AI, is largely based on subjectively scoring sperm motility, and on measurement of sperm concentration. Since the relationship between these parameters is barely indicative of the fertility of the semen samples, other, often more complicated methods are used to evaluate a battery of sperm attributes of importance for their fertilizing capacity. This thesis evaluated the usefulness of various conventional and novel sperm quality tests in assessing frozen-thawed (FT) AI doses produced from semen of young, unproven and older, progeny-tested dairy bulls of the Estonian Holstein Friesian (EHF) and Swedish Red and White (SRB) breeds and their relationship with field fertility after AI. In addition, the influence of sperm preparation methods prior to quality measurements was investigated. Semen AI doses were evaluated immediately post-thaw (PT) and after cleansing through washing/resuspension (W) and swim-up (SU). Special attention was given to age-related changes in semen from 1- v. 4-, and 3- v. 5- and 7-year-old bulls. Spermatozoa were evaluated for their morphology (microscopy on wet and dried, stained smears), motility (assessed subjectively and by computer-assisted sperm analysis [CASA]) and membrane integrity (using SYBR-14/propidium iodide [PI] fluorophores and microscopy). Mitochondrial activity (with MitoTracker Deep Red), membrane fluidity (using Merocyanine 540 lipid dye/Yo-Pro-1/H332) and deoxyribonucleic acid (DNA) integrity (using acridine orange [AO] staining) were assayed using flow cytometry (FC). Use of SU provided spermatozoa with significantly better motility, morphology, membrane integrity, mitochondrial activity and chromatin stability compared with either PT or W treatment. Although the SU selection step increased the proportion of spermatozoa with a stable plasma membrane, it also initiated membrane destabilization. Age differences in sperm quality (motility, membrane integrity and mitochondrial function) were seen PT (at 4 years of age for SRB and at 3–7 years for EHF bulls) and were accentuated when using SU, but not W, as pre-selection procedure. No changes in chromatin stability were, however, registered for either breed. Only few FT sperm quality attributes (e.g. average path velocity (VAP), proportion of cells with tail abnormalities, and non-linear motility) showed significant correlation to fertility after AI when evaluated PT or after W. More attributes (e.g. CASA total sperm motility, concentration of motile and linearly motile spermatozoa, VAP, as well as the percentage of spermatozoa with unstable plasmalemma) had a significant relationship with non-return rate (NRR) when spermatozoa were examined after SU. In conclusion, SU was superior to W in harvesting intact spermatozoa with attributes essential for fertilization, distinguishing bulls and revealing age-dependent changes. Also, the use of CASA and FC of fluorophore-loaded spermatozoa increased the objectivity of the tests assayed. Combining SU and CASA, methods considered to be easily applicable at the semen-producing enterprises, indicated that the overall semen quality of proven bulls was predictable from measurements done at an early age. While sperm membrane stability and mitochondrial activity were seen as suitable markers for monitoring semen quality, measurements of chromatin intactness could not yield additional information.

Key words: frozen-thawed (FT) bull semen, swim-up (SU), computer-assisted sperm analysis (CASA), flow cytometry (FC), in vivo fertility.

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Papers I-V

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

I. Hallap, T., Hååard, M., Jaakma, Ü., Larsson, B., Rodriguez-Martinez, H. 2004. Does cleansing of frozen-thawed bull semen before assessment provide samples that relate better to potential fertility? *Theriogenology* 62, 702–713.

II. Hallap, T., Hååard, M., Jaakma, Ü., Larsson, B., Rodriguez-Martinez, H. 2004. Variations in quality of frozen-thawed semen from Swedish Red and White AI sires at one and four years of age. *International Journal of Andrology* 27, 166–171.

III. Hallap, T., Jaakma, Ü., Rodriguez-Martinez, H. 2005. Changes in semen quality in Estonian Holstein AI bulls at 3, 5 and 7 years of age. *Reproduction in Domestic Animals* (accepted for publication).

IV. Hallap, T., Nagy, Sz., Hååard, M., Jaakma, Ü., Johannisson, A., Rodriguez-Martinez, H. 2005. Sperm chromatin stability in frozen-thawed semen is maintained over age in AI bulls. *Theriogenology* 63, 1752–1763.

V. Hallap, T., Nagy, Sz., Jaakma, Ü., Johannisson, A., Rodriguez-Martinez, H. 2005. Mitochondrial activity of frozen-thawed spermatozoa assessed by MitoTracker Deep Red. *Theriogenology* 63, 2311–2322.

VI. Hallap, T., Nagy, Sz., Jaakma, Ü., Johannisson, A., Rodriguez-Martinez, H. 2005. Usefulness of a triple fluorochrome combination Merocyanine 540/Yo-Pro-1/Hoechst 33342 in assessing membrane stability of viable frozen-thawed spermatozoa from Estonian Holstein AI bulls. *Theriogenology* (in press).

Abbreviations

AI	artificial insemination
AO	acridine orange
ATP	adenosine triphosphate
BSA	bovine serum albumin
CASA	computer-assisted sperm analysis
COMPα$_t$	cells outside the main population
CTC	chlortetracycline
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetic acid
EHF	Estonian Holstein Friesian breed
FACS	fluorescence-activated cell sorter
FC (FCM)	flow cytometry
Fert-TALP	modified Tyrode's albumin, lactate and pyruvate solution for *in vitro* fertilization
FSC	forward scatter
FT	frozen-thawed
GAG	glycosaminoglycan
GLM	general linear model
IVF	*in vitro* fertilization
MMP	mitochondrial membrane potential
NRR	non-return rate
PBS	phosphate-buffered saline
PI	propidium iodide
PT	post-thaw
ROS	reactive oxygen species
SCSA	Sperm Chromatin Structure Assay
SD	standard deviation
SM-CMA	Strömberg-Mika computer-assisted motility analyser
SP-TALP	modified Tyrode's albumin, lactate and pyruvate solution
SRB	Swedish Red and White breed
SSC	side scatter
SU	swim-up
TNE	TRIS, NaCl and EDTA
TRIS	Tris(Hydroxymethyl) Aminomethane
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight linear velocity
W	washing/resuspension

Introduction

The aim of the breeding industry is to identify genetically superior bulls and maximize the number of offspring conceived with their semen through artificial insemination (AI), thus increasing the dissemination of their genes. For obvious reasons, the fertility of these bulls is of utmost importance in effectively reaching this goal. In most cases the laboratory evaluation of semen from bulls, before and after freezing for AI, is entirely based on subjective scoring of sperm motility under a light microscope and measurement of sperm concentration. Occasionally morphology is also determined, mostly when certain pathologies are suspected. Although use of these assessment procedures and of a single protocol for freezing has led to formation of very homogeneous sire populations regarding sperm quality, the sires still give varying results with regard to field fertility. These varying results are due to the innate differences in fertility between males but also, and perhaps to a large extent, to the fact that the methods used are not sufficiently discriminative. The development of objective and rapid laboratory tests for semen assessment would, if they prove a significant relation to fertility, help to select highly fertile bulls at an early age and reduce the costs involved with extensive insemination trials for every sire. So far, semen from young bulls has been routinely collected as early as possible (usually around 1 year of age) and tested for volume, concentration and motility in the laboratory and then for fertility in herds that are enrolled in a milk-recording programme. The percentage of cows not returning to oestrus during 56–90 days post-AI (the so-called "non-return to oestrus rate", or "non-return rate [NRR]"), as well as pedigree information and first lactation production results, is recorded for each daughter and used to select genetically superior bulls that will return to produce semen for further AIs and thus become parents of the next generation of cows. A small proportion of these bulls are used to sire the next bulls to be progeny-tested. By the time a sire is proven, he is 4–5 years old and qualitative and/or quantitative changes may have occurred in his semen so that the fertility level assessed from the recorded NRRs when the bull was young may no longer be valid. Obviously, there is a need to determine whether changes in sperm quality occur with increasing age, within the frame of the functional lifetime of an AI bull, and whether there is any predictive value of early semen measurements.

   Among possible ways to improve the objectivity of laboratory semen evaluation methods is the assessment of multiple sperm attributes, either simultaneously or using separate tests on the largest sperm number possible, at one single time. Combinations of several sperm quality parameters, assessed via batteries of tests, could explain more variation in fertility between the bulls than can any single sperm quality trait (Wood *et al.* 1986; Truelson *et al.* 1996; Zhang *et al.* 1998; Januskauskas *et al.* 2000). The objectivity of motility measurements has also been improved by incorporation of computer-assisted sperm analysis (CASA), which measures a number of variables of sperm motility and could be applied to routine assessment of bull semen, although variation between instruments and measurements still exists (Budworth *et al.* 1988; Holt *et al.* 1997). However,

relationships between CASA measurements and field fertility have been reported for several species (Budworth *et al.* 1988; Holt *et al.* 1997). Still, lack of a direct relationship between single sperm quality parameters and fertility, on the one hand, and rapid development of technology, on the other, have led to a search for new markers of male fertility. One example of this search is the use of fluorescent markers in combination with flow cytometry (FC). This technique allows the simultaneous assessment of several parameters, such as semen viability, acrosome integrity and deoxyribonucleic acid (DNA) structure on numerous spermatozoa (>1 000 cells can be screened per second) loaded with specific fluorophore probes (Graham, 2001).

Many investigators have demonstrated relationships between sperm morphology and viability (Wiltbank & Parish 1986; Bredbacka *et al.* 1997). As the microscopic evaluation of sperm morphology is considered relatively subjective and non-repeatable, FC could help to reveal differences in AI bull populations. For instance, incubation of spermatozoa in an acid solution induces the *in situ* denaturation of DNA. Spermatozoa can then be stained with the metachromatic dye acridine orange (AO) and examined with FC, the basis for the Sperm Chromatin Structure Assay (SCSA) (Evenson *et al.* 1980). An increased susceptibility to denaturation denotes heterogeneity in the chromatin structure and has been related to disturbed spermatogenesis, abnormal morphology and constrained embryonic development (Hamamah *et al.* 1997; Larson *et al.* 2000; Lewis & Aitken, 2005) and consequently to potentially reduced fertility (Evenson *et al.* 1980; Ballachey *et al.* 1987; Sailer *et al.* 1995; Evenson & Jost 2000; Lewis & Aitken, 2005). This would explain an association between infertility cases and abnormal chromatin structure even where all the classic quality parameters are within acceptable limits (Evenson & Jost 2000). In such a case chromatin stability assessment may provide additional information about semen quality. Consequently testing spermatozoa for their susceptibility to DNA denaturation *in situ* would increase our ability to discriminate between bulls with different fertilizing ability compared with conventional methods.

As already mentioned, the most common criterion of sperm quality in bull stations is still motility, an indirect measure of metabolic activity and therefore, of sperm viability. Although the evaluation technique is simple, it is very dependent on the experience of the operator (Graham *et al.* 1980), yielding variable correlations when post-thaw (PT) motility and fertility are compared (Söderquist *et al.* 1991; Rodriguez-Martinez, 2003). As spermatozoa show flagellar movement only in the presence of adenosine triphosphate (ATP)-derived energy, direct measurements of mitochondrial function may be useful as an alternative, more objective measure of sperm quality (Evenson *et al.* 1982). There are approximately 100 mitochondria in the midpiece of the spermatozoon. Fluorescent dyes, such as Rhodamine 123 (R123), MitoTracker Green (MTG), JC-1, MitoTracker Orange (CMTMRos), MitoTracker Red (CMXRos), MitoTracker Red 580 and MitoTracker Deep Red 633 (Ericsson *et al.* 1993; Garner *et al.* 1997; Gravance *et al.* 2000; Cossarizza, 2005), can be used to visualize these organelles. The working mechanism for most of these probes is that they diffuse into living cells and accumulate into mitochondria provided that an internal 100–200 mV-negative gradient occurs across the mitochondrial membrane potential (MMP).

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Despite numerous published protocols there are several problems connected with most of these fluorophores, including low sensitivity, presence of several energy-independent binding sites in mitochondria, loss of staining in cases where MMP is diminishing, and interaction with semen extender (Troiano *et al.* 1998; Garner & Thomas 1999; Cossarizza, 2005). Owing to the incompleteness of information regarding the staining/binding mechanism of MitoTracker probes (Gregory, 2002) a novel probe, the MitoTracker Deep Red, recently suggested by Nagy *et al.* (2003) to test mitochondrial activity in spermatozoa, ought to be tested and the results compared with results achieved with parallel motility analyses (i.e. microscopic analysis and CASA).

It is widely known that spermatozoa bind to the oocyte only after capacitation in the female genital tract (Chang, 1951; Austin, 1952). During this process, the sperm plasma membrane becomes dynamically reorganized (for more details on the lipid order–disorder transition and protein relocation, see Harrison *et al.* [1996] and Gadella & Harrison [2000]). Since capacitation destabilizes the sperm plasma membrane, it can be assumed that if destabilization occurs prematurely, leading to acrosome exocytosis or cell death, fertilization is impaired (Gadella & Harrison 2000; Januskauskas *et al.* 2000). It is also known that the cooling of spermatozoa causes changes in the plasma membrane, which resemble the changes seen during physiological capacitation (Cormier *et al.* 1997). Whether these changes are really a sign of capacitation or only a destabilization of the membrane is still under debate (Green & Watson 2001). Recent evidence suggests that changes in the scrambling of plasma membrane phospholipids of cryopreserved boar spermatozoa differ from those in fresh semen undergoing bicarbonate-induced capacitation (Guthrie & Welch 2005). The hydrophobic dye Merocyanine 540 (M540) can be employed to monitor the level of scrambling of the phospholipids of the plasma membrane lipid bilayer, increasing the intensity of its fluorescence when the membrane lipid disorder increases (Harrison *et al.* 1996; Gadella & Harrison 2000). In addition, M540 staining can be combined with the membrane-impermeable DNA-binding probe Yo-Pro-1 to simultaneously analyse sperm cell viability. Any non-sperm event such as presence of DNA-free biological debris in semen extenders could be excluded based on a lack of a Hoechst 33342 (H33342) signal. Knowledge of the amount of viable spermatozoa with a stable plasma membrane in the inseminate would give very valuable information about its quality (Thundathil *et al.* 1999). It would also be of interest to disclose whether these cells, which have preserved their membrane stability after freezing and thawing, would capacitate, and how many would capacitate, when challenged with effectors of capacitation (e.g. heparin).

One more factor to take into consideration in fertility prediction is the population of spermatozoa we evaluate. During sperm transport in the female genital tract and following sperm-oocyte interaction there is an *in vivo* selection of spermatozoa with preserved attributes for fertilization. It could therefore be hypothesized that *in vitro* selection and evaluation of a sperm subpopulation within the inseminate, with attributes which *in vivo* would potentially allow the sperm to reach and fertilize the oocyte, would give a more realistic picture of the fertilizing ability of the examined sample. An additional advantage of such pre-selection by cleansing is the elimination of extenders and cryoprotectants which

may act as a source of variation during the measurements. In *in vitro* fertilization (IVF) protocols, cleansing of semen samples is done either by washing (repeated centrifugation/resuspension) of the sample (W) or by swim-up (SU) (Rodriguez-Martinez *et al.* 1997b). Centrifugation, an easily performed procedure, results in a final pellet with large numbers of spermatozoa including dead, moribund and abnormal cells. Use of the migrating ability of spermatozoa in an SU procedure provides samples with highly motile spermatozoa but yields only around 10–20%, for neat semen, or <10% of the original sample when frozen-thawed (FT) semen is used (Rodriguez-Martinez *et al.* 1997b). It has been reported that the total concentration as well as the concentration of motile spermatozoa after SU is related to bull fertility after AI (Zhang *et al.* 1998). It should therefore be determined whether assessment of sperm characteristics in a cleansed subpopulation of spermatozoa from an FT semen sample yields superior results in terms of correlating to *in vivo* fertility compared with measuring the sample immediately after thawing.

Aims of the study

The overall aim of the study was to determine the usefulness of several sperm evaluation methods for AI dairy bull sires and their relationship with field fertility after AI. Specific aims were to –

> ➢ estimate the influence of cleansing methods on FT bull sperm attributes, such as sperm morphology, sperm motility and membrane integrity;

> ➢ determine bull age-dependent changes in the quality of FT spermatozoa for AI; and

> ➢ test novel methods using fluorescent probes combined with FC, to evaluate –

>> ✓ mitochondrial activity, applying the fluorescent probe MitoTracker Deep Red
>> ✓ chromatin stability, using the SCSA assay
>> ✓ plasma membrane stability and fluidity, using a combination of the fluorophores M540/Yo-Pro-1/H342.

Materials and Methods

Reagents and media

Unless otherwise stated, all media components were purchased from Molecular Probes, Inc. (Eugene, OR, USA), Becton Dickinson Immunocytometry Systems, (San José, CA, USA) and Sigma (Sigma-Aldrich Sweden AB, Stockholm, Sweden). The basic culture medium used for SU was Tyrode's albumin, lactate and pyruvate solution for fertilization (Fert-TALP) except in **Paper IV** where Tyrode's albumin, lactate and pyruvate solution for sperm treatment (SP-TALP) was used (Parrish *et al.* 1988). The latter was also used for sperm cleansing by centrifugation/resuspension (W).

Animals

Frozen AI semen samples prepared from ejaculates collected from six Swedish Red and White (SRB) dairy bulls housed at the Svensk Avel ek. för. bull stud in Skara, Sweden (**Papers I, II and IV**), and six Estonian Holstein Friesian (EHF) bulls kept at the AI station of the Animal Breeders' Association of Estonia in Kehtna, Estonia (**Papers III–VI**), were used in the experiments. The bulls were part of the national breeding programmes and all collections and freezing of semen were performed at the AI stations under commercial conditions. From the SRB bulls, semen was collected at 11–13 months of age (hereafter referred to as "1 year") and again, at 52–58 months (hereafter referred to as "4 years"). During the 3–4-year waiting period (during which progeny testing of the bulls was underway) the bulls were kept in groups of eight to twelve animals at the bull station. The age of the EHF bulls at the time of first collection for these experiments was 32–44 months (hereafter called "3 years"). Thereafter, semen was collected when the bulls were 63–66 months ("5 years") and again, at 78–90 months ("7 years") of age. In the case of the EHF bulls, semen was collected for commercial purposes before and in between these samplings. Before being allowed to enter the progeny testing programme, a young bull must have produced semen with a total sperm number of ≥ 4 x 10^9 and an initial sperm motility of $\geq 60\%$, and with $>85\%$ spermatozoa of the total sperm number depicting normal morphology including $<5\%$ of morphologically immature spermatozoa (i.e. having proximal cytoplasmic droplets).

Semen processing

In all cases, semen was collected once weekly using an artificial vagina. Two consecutive ejaculates were pooled (hereafter referred to as a "batch"), extended with a commercial extender (Triladyl®, Minitüb, Altdorf, Germany), packed in 0.25 mL plastic straws, each containing either ~15 x 10^6 (SRB bulls) or ~30 x 10^6 spermatozoa (EHF bulls), and frozen using programmable biological freezers. The frozen straws were stored in liquid nitrogen until tested. Following preservation, a PT motility $\geq 50\%$ was set up as threshold.

Semen preparation for laboratory tests

The semen analyses were performed after the following preparations: immediately after thawing (PT, control), after thawing and SU separation (**Papers I and VI**) or after thawing and washing by centrifugation/resuspension (W) (**Papers I and II**). The straws were thawed by immersion in water at +35ºC for 12 seconds. The semen from two straws of the same batch was pooled and used for SU and W.

Cleansing by swim-up

For SU, 200 μL of semen were placed at the bottom of a siliconized soda-glass tube and covered with 400 μL of Fert-TALP (Parrish *et al.* 1988) supplemented with fatty acid-free bovine serum albumin (BSA) (6 mg/mL), sodium pyruvate (0.25 μM), heparin (5 μg/mL), D-penicillamine (20 μM), hypotaurine (10 μM) and epinephrine (1 μM). After 60 minutes of incubation (39ºC, 5% CO_2) the upper 300 μL of the medium were collected for sperm quality measurements.

Cleansing by centrifugation/resuspension

After thawing and pooling as described above, 150 μL of semen were extended with 350 μL SP-TALP (Parrish *et al.* 1988). After centrifugation at 400 g for 10 minutes (20–25ºC) the upper 450 μL of the suspension were carefully removed, 350 μL of SP-TALP were added and the spermatozoa resuspended. After a second centrifugation the final sperm pellet was extended with 150 μL of SP-TALP.

Semen evaluation after preparation for laboratory tests

Sperm motility and concentration (*Papers I–III, V and VI*)

Samples of 5 μL were placed in a Makler Counting Chamber (Sefi-Medical Instruments, Haifa, Israel) and analysed with a CASA instrument (SM-CMA, MTM Medical Technologies, Montreaux, Switzerland). At least 100 spermatozoa/sample were tracked and assessed at +38ºC to estimate the percentage of motile spermatozoa and the proportions of these showing linear, non-linear or circular motility. The kinetic characteristics of motility recorded were straight linear velocity (VSL), average path velocity (VAP) and curvilinear velocity (VCL). The percentages of linearly motile spermatozoa were manually calculated from the total population of spermatozoa present in the fields. Subjective sperm motility was estimated from the same sample (5 μL) under a phase contrast microscope (x 400) equipped with a warm stage (+38ºC). The mean value from the observation of four fields was recorded. Sperm concentration was assessed in two separate counting chambers of a Bürker haemocytometer, as described by Bane (1952), and expressed as spermatozoa x 10^6/mL. The same operator performed all analyses.

Sperm morphology (**Papers I–IV and VI**)

Sperm morphology was evaluated in wet preparations of semen fixed in buffered formalin (Bane, 1952) or in air-dried smears stained with carbol-fuchsine (Williams, 1920). In the wet preparations 200 cells were counted under a phase contrast microscope (x 1 000) and numbers of cells with abnormal acrosomes or midpieces, as well as cells with coiled tails, were recorded. Head abnormalities were monitored in the stained smears by counting 200 spermatozoa under a light microscope (x 1 000). Heads that were pear-shaped, narrow at the base, abnormal in contour, loose, undeveloped, narrow or variable in size were registered. For each region of the spermatozoa, the number of morphological abnormalities was expressed as a percentage of the total cells evaluated.

Sperm membrane integrity (**Papers I–III and VI**)

A combination of the fluorophores SYBR-14 and propidium iodide (PI) (Sperm Viability Kit L-7011, Molecular Probes Inc., Eugene, OR, USA) was used, as described by Januskauskas *et al.* (1999). Semen samples (100 μL) were extended in 400 μL TRIS-citrate with addition of 2.7 μL PI and 10 μL SYBR-14, which resulted in final dye concentrations of 24 μM and 100 nM, respectively. After incubation at 37ºC for 15−20 minutes, 2 x 100 spermatozoa were assessed (x 600) on a warm stage (+37ºC) under epifluorescence, using a microscope (Laborlux-11, Leitz, Jena, Germany) equipped with a ParaLens® objective lens set (Becton Dickinson, Leiden, The Netherlands). The nuclei of spermatozoa with intact plasma membranes stained green with SYBR-14, while those with damaged membranes stained red with PI. The results were expressed as the percentage of spermatozoa with intact membranes.

Sperm chromatin stability (**Paper IV**)

The susceptibility of sperm DNA to undergo acid-induced denaturation *in situ* was measured by FC using the ability of AO to metachromatically shift from green (double-stranded DNA) to red (single-stranded DNA) fluorescence (Evenson *et al.* 1980). Denaturation was expressed as function α_t, which shows the ratio of red (denaturized, single-stranded DNA) to red+green (total sperm DNA) fluorescence intensity. The α_t was calculated for each spermatozoon within a sample and the results were expressed as the percentage of cells with high α_t values (excess of single-stranded DNA), called "cells outside the main population (% COMPα_t)". The thawed semen samples were extended to 1–2 x 10^6/mL in TNE buffer (0.01 M TRIS, 0.15 M NaCl and 1 mM ethylenediamine tetra-acetic acid [EDTA], pH 7.4). After 1 minute, 200 μL of the extended sperm suspension was mixed with 400 μL of acid-detergent solution (0.15 M NaCl, 0.08 N HCl, 0.1% Triton-X 100, pH 1.2). Exactly 30 seconds later, samples were stained with 1.2 mL of AO staining solution (0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, 0.1 M citric acid, 6 mg/mL AO, pH 6.0). Samples were transferred to the flow cytometer, and analyses started 3 minutes after the acidic treatment. The same staining procedure was used after SU. Measurements were done on a FACSStar Plus flow cytometer (Becton

Dickinson Immunochemistry Systems, San José, CA, USA) equipped with standard optics. Acridine orange was excited with an Ar ion laser (Innova 90; Coherent, Santa Clara, CA, USA) at 488 nm and running at 200 mW. In association with double-stranded DNA, AO fluoresced green (530 ± 30 nm, as detected with the FL1 detector), but in the presence of single-stranded DNA the resulting fluorescence was red (>630 nm, as detected with the FL3 detector). The fluorescence stability of the FC was monitored daily using standard beads (Fluoresbrite plain YG 1.0 μM; Polysciences, Inc., Warrington, PA, USA). Equivalent instrument settings were used for all samples. From each sample a total of 10 000 events were measured at a flow rate of ~200 cells/sec. Scattergram analysis of raw data, with each point representing the coordinate of red and green fluorescence intensity values for every individual spermatozoon, was carried out using CellQuest, version 3.1, software (Becton Dickinson Immunochemistry Systems, San José, CA, USA). Events accumulated in the lower left-hand corner were viewed as sample debris and were excluded from the analysis. The FC results were stored as list mode files and further analyses of the parameters were done using FCSExpress software, version 2 (DeNovo Software, Thornhill, Ontario, Canada).

*Sperm mitochondrial activity (**Paper V**)*

A 100 μM SYBR-14 stock solution was prepared in dimethyl sulphoxide (DMSO) and a 100 nM working solution was prepared in CellWash (CellWash optimized phosphate-buffered saline [PBS], cat. No. 349524; Becton Dickinson, San José, CA, USA) just before sample preparation. Similarly, a 1 mM MitoTracker Deep Red stock solution was prepared in DMSO, followed by preparation of 20 μM working solution in CellWash (Becton Dickinson, San José, CA, USA) just before sample preparation. The staining protocol was as follows: sperm samples were extended with CellWash to a final sperm concentration of approximately 1 x 10^6/mL in 5 mL Falcon tubes (Becton Dickinson Immunochemistry Systems, San José, CA, USA). Five nM of SYBR-14 working solution and 100 nM of MitoTracker Deep Red working solution were added to the samples, mixed and incubated at 38°C in the dark for 10 minutes and remixed before analysis. The FC measurements were done on an LSR flow cytometer (Becton Dickinson, San José, CA, USA). The SYBR-14 dye was excited by a 20 mW Ar ion 488 nm laser while MitoTracker Deep Red was excited by a 17 mW HeNe 633 nm laser. The SYBR-14 fluorescence (cells with intact plasma membrane) was detected on detector FL 1 (530/28 nm) while MitoTracker Deep Red fluorescence was detected on detector FL 3 (670 LP). Forward and side scatter (FSC and SSC) values were recorded on a linear scale while fluorescent values were recorded on a logarithmic scale. Compensations were set according to Roederer (2000). Acquisitions were done using the CellQuest, version 3.1 software (Becton Dickinson Immunochemistry Systems, San José, CA, USA). Non-sperm events were gated out based on SYBR-14 fluorescence (DNA content). The FC was used at low flow rate (6–24 μL/min). Acquisitions were stopped after recording 10 000 SYBR-14-positive events and the data stored in list mode for further analysis.

*Sperm plasma membrane stability (**Paper VI**)*

The following working solutions were prepared: M540 (M 24571; Molecular Probes, Inc., Eugene, OR, USA) 1 mM in DMSO; Yo-Pro-1 (Y 3603; Molecular Probes, Eugene, OR, USA) 25 μM in DMSO; H33342 5 mg/mL in distilled water. Suspensions of FT spermatozoa before (e.g. PT) and after SU were extended to approximately 1 x 10^6/mL in CellWash (Becton Dickinson, San José, CA, USA) in 5 mL Falcon tubes containing 1 μL Yo-Pro-1, 2.6 μL M540 and 2 μL H33342 working solutions. Stained samples were incubated at 38°C in the dark for 10 minutes and remixed before analysis. Measurements were done on an LSR flow cytometer (Becton Dickinson, San José, CA, USA). The M540 and Yo-Pro-1 dyes were excited by a 20 mW Ar ion 488 nm laser while H33342 was excited by a helium–cadmium (HeCD) ultraviolet (UV) 325 nm 8 mW laser. Yo-Pro-1 fluorescence was detected on detector FL1 (530/28 nm), M540 fluorescence was detected on detector FL3 (670 LP) and H33342 fluorescence was detected on detectors FL4 (510/20 nm) and FL5 (380 LP). Forward and side scatter values were recorded on a linear scale while fluorescent values were recorded on a logarithmic scale. Compensations were set according to Roederer (2000). Acquisitions were done using the CellQuest, version 3.1 software (Becton Dickinson Immunochemistry Systems, San José, CA, USA). The non-sperm events were gated out based on H33342 fluorescence (DNA content). The FC was used at a low flow rate of 6−24 μL/min. Acquisitions were stopped after recording 10 000 H33342-positive events. Acquisition data were stored in list mode until further analysis. On FL1/FL3 (Yo-Pro-1/M540) dot plots, regions were set to differentiate viable and stable (Yo-Pro-1-negative and M540-negative) plasma membranes, viable and unstable (Yo-Pro-1-negative and M540-positive) plasma membranes and dead (Yo-Pro-1-positive) events.

Fertility

Inseminations were performed *as praxis* within 1 year of semen freezing on heifers and cows of different parity during all seasons of the year. One to four batches from each bull were included in the experiments. The number of AIs per batch ranged from 45 to 150 for EHF and from 162 to 332 for SRB bulls. Results of the AIs were available only for the 3-year-old EHF and the 1-year-old SRB bulls. In Sweden 56 d-NRR were recorded for each batch and corrected for season, area and parity, while in Estonia the NRRs were collected at day 60 and not corrected.

Statistical analyses

The statistical analyses were performed using the Statistical Analysis Systems (SAS) package (SAS®; SAS Institute Inc., Cary, NC, USA). To assess the influence of treatment, age and bull a three-factor analysis of variance was implemented using the SAS MIXED procedure and the equation $y_{ijkl} = \mu + B_i + T_j + A_k + e_{ijkl}$. In the statistical model bull was considered a random effect. Similarly, two-factor analyses of variance were used to measure the influence of treatment and bull among age groups. To compare certain bulls among age groups and treatments, multiple comparisons based on least square means were performed using the general linear model (GLM) procedure considering bull as fixed effect. A Pearson's correlation test was used to establish simple correlations between measured quality variables and field fertility. Differences in NRRs between batches were calculated using the chi-squared test. All influences and differences were considered statistically significant if $p<0.05$.

Results

Influence of preparation procedures (washing/resuspension and swim-up) on frozen-thawed sperm quality

In SRB bulls SU provided spermatozoa with significantly better motility (subjectively or CASA-assessed), acrosome, midpiece and tail morphology, membrane integrity ($p<0.05$, **Paper II**) and chromatin stability ($p<0.001$, **Paper IV**) compared with either PT or W-treated samples. All measured kinetic parameters were higher after SU as well ($p<0.05$, **Paper II**). Washing/resuspension treatment resulted in increased proportions of acrosome abnormalities and proximal droplets while midpiece abnormalities decreased, compared with PT samples ($p<0.05$, **Paper II**). In EHF bulls the following parameters improved after SU selection (**Paper VI**): motility (both subjectively and CASA-assessed), VAP, membrane integrity, mitochondrial activity (**Paper V**) and chromatin stability (**Paper IV**) ($p<0.001$). There was also an increase in spermatozoa with stable ($p<0.05$) and unstable plasma membranes ($p<0.01$) owing to a substantial concomitant reduction in the number of dead cells ($p<0.01$). At the same time the proportions of spermatozoa with tail abnormalities and linear motility decreased ($p<0.001$ and $p<0.01$, respectively) (**Paper VI**).

Sire age-dependent changes in sperm quality

In SRB bulls (**Paper II**), when semen was analysed PT, objective motility (p<0.01), concentration of motile spermatozoa and membrane integrity improved at 4 years of age in comparison to 1 year (p<0.05). After SU there was an improvement in membrane integrity and concentration of motile spermatozoa as well (p<0.05), but linear motility decreased (p<0.05) together with VSL (p<0.001), with a parallel rise in VCL (p<0.05). No significant age differences were recorded after W treatment. Neither was there any age-related change in chromatin stability (**Paper IV**). In the EHF bulls (**Papers III, V and VI**) when analysed PT there was an improvement in membrane integrity (p<0.05) but a decrease in plasma membrane stability (p<0.05) in the 7-year-old v. the 3-year-old group (**Paper VI**). Also, an increase in midpiece abnormalities (p<0.01) and cytoplasmic droplets (p<0.05) was registered between the same age groups (**Paper III**). After SU preparation, subjectively assessed motility, mitochondrial activity (p<0.05) (**Papers III and V**) and membrane integrity improved also for the 7-year-old bulls (p<0.05) (**Papers III and VI**). Similarly to the SRB bulls, no change in chromatin stability was registered along ages (**Paper IV**). In addition, there was an increase in non-linear motility (p<0.05) for 5- v. 3-year-old bulls and a decrease in acrosome and tail abnormalities for 7- v. 3-year-old bulls (p<0.05) (**Paper III**).

Sperm chromatin stability (*Paper IV*)

The COMPα_t values ranged from 0.5–3.6% (PT) to 0.2–1.7% (SU) for SRB bulls and from 0.4–1.8% (PT) to 0.2–1.5% (SU) for EHF bulls.

Sperm mitochondrial activity (*Paper V*)

The proportion of spermatozoa with high mitochondrial activity (i.e. bright MitoTracker Deep Red fluorescence) PT was 37.5–59.7%, 44.4–49.7% and 45.8–49.8% for 3-, 5- and 7-year-old bulls, respectively. The corresponding values after SU were 33.3–78.6%, 78.2–78.8% and 72.3–83.9%. There were no differences between the age groups after thawing, but following SU the semen from 7-year-old bulls showed a significant improvement compared with that from 3-year-old bulls (p<0.05). This tendency was seen also in semen from the 3–5-year group (p=0.06). After SU treatment significantly more spermatozoa showed high red fluorescence compared with PT results, viz. p<0.05 (3-year-old bulls), p<0.001 (5- and 7-year-old bulls) and p<0.001 (when all age groups were combined). Sperm mitochondrial activity also differed significantly among some 3-year-old bulls after SU (p<0.05).

Sperm plasma membrane stability (*Paper VI*)

In the EHF bulls the proportions of stable, unstable and dead spermatozoa PT were 53.1±9.7, 1.1±0.9% and 45.7±9.9%, respectively. After SU treatment the proportions of spermatozoa with stable plasma membrane increased (66.3±20.1%; $p < 0.05$) but so also did instability (5.9±6.6%; $p < 0.01$) owing to a substantial concomitant decrease in dead cells (27.8±19.4%; $p < 0.01$) compared with PT. The age of bulls did not influence the degree of membrane destabilization, except in the 3- v. 7-year-old bull samples, where the proportion of spermatozoa with destabilized plasmalemma increased PT ($p < 0.05$).

Relations among sperm quality parameters (*Papers IV–VI*)

The chromatin stability assessment showed very little relationship with the conventional light microscopic morphology evaluation in the two bull populations separately and combined. In the SRB bulls the percentage of spermatozoa with denatured chromatin (COMPα_t) PT was related to the percentage of undeveloped spermatozoa after SU ($p < 0.05$). In addition, there were tendencies of a relation between COMPα_t PT and the percentage of spermatozoa with abnormal head shape after SU ($p = 0.059$) in the SRB bulls. Also, a tendency was detected of a relation between COMPα_t immediately PT and the percentage of spermatozoa with abnormal head shape ($p = 0.086$) and with a narrow-based head shape ($p = 0.081$) PT in the EHF bulls. The percentages of COMPα_t PT correlated with those after SU ($p = 0.01$) only in SRB bulls. The percentages of spermatozoa with high MMP PT correlated well with the percentages after SU ($p < 0.01$) in EHF bulls. In relation to conventional sperm quality measurements, the proportion of spermatozoa with high MMP correlated with sperm motility both PT and after SU ($p < 0.01$). Also, a positive correlation between number of motile spermatozoa and MMP after SU was recorded ($p < 0.01$). Regarding plasma membrane stability in EHF sires, the proportion of spermatozoa with a stable membrane was related to many more conventional sperm quality parameters than was that of spermatozoa with membrane instability. Post-thaw, for instance, a relation was registered with membrane integrity ($p < 0.01$), motility and normal head morphology ($p < 0.05$). After SU there was a correlation between sperm concentration and number of spermatozoa with stable membranes ($p < 0.01$). In general, the proportion of spermatozoa with stable plasma membrane PT correlated to the respective results after SU ($p < 0.01$).

Relations between sperm quality parameters and fertility

Of all parameters measured in the SRB bulls (**Paper II**) PT and after W or SU the following showed significant correlation to *in vivo* fertility: VAP and proportion of cells with tail abnormalities assayed PT (r=0.479, p=0.044, and r=0.498, p=0.035, respectively), total sperm motility (CASA) after SU (r=0.503, p=0.034) and non-linear motility after W (r=–0.691, p=0.002). In EHF bulls (**Papers III and VI**) only parameters measured after SU, such as the proportion of total motile spermatozoa (r=0.804, p=0.005), the concentration of motile spermatozoa (r=0.741, p=0.01) and of linearly motile spermatozoa assessed by CASA (r=0.762, p=0.01), VAP (r=0.886, p=0.0006) and the percentage of spermatozoa with an unstable plasmalemma (r=0.629, p=0.015), had a significant relationship with NRR.

General discussion

Evaluation of fertility is essential to the AI industry. Although this can either be done by performing test inseminations in the field or through IVF in the laboratory (Larsson & Rodriguez-Martinez 2000), the use of AI for assessing bull fertility is the most accurate method. However, it is time-consuming and expensive compared with IVF, which, albeit more rapid and less expensive, is still not considered a reliable routine laboratory procedure because of technical complexities. In the laboratory certain characteristics of spermatozoa essential for reaching, binding and penetrating the oocyte vestments and, ultimately, for fertilizing the oocyte can be tested to assess normal attributes of sperm function and estimate potential fertility. Among these attributes are concentration, motility, morphology, membrane and acrosome integrity and the ability to interact with the oocyte and its vestments. However, considering the complexity of the fertilization process, measurements of any of these single sperm quality attributes can not reflect the real fertilizing ability of a semen sample. They could, however, be useful in eliminating samples of very poor quality (Graham *et al.* 1980; Stålhammar *et al.* 1994; Hirano *et al.* 2001). As the range in fertility among sires in commercial bull studs is usually narrow owing to pre-selection of the sires (Amann, 1989), the tests used to predict bull fertility need to be highly sensitive and several quality markers should be assessed simultaneously and should be related to the fertility in the field or in the laboratory. Such reasoning constituted the core of the present thesis.

One possible way to improve the objectivity of semen evaluation tests is to eliminate most of the extender and cryoprotectants from the manufactured sample since they could be an additional source of variation. This removal could be done through gradient centrifugation (Percoll, Avery & Greve 1995), through W or by SU (Rodriguez-Martinez *et al.* 1997a, Rodriguez-Martinez *et al.* 1997b). Out of these three, W is unequivocally the easiest method. Information in the literature about the influence of centrifugation on spermatozoa is controversial. Several reports have shown that it damages sperm quality and constrains pronucleus formation (Risopatron *et al.* 1996; Palomo *et al.* 1999) as well as increasing the formation of undesirable reactive oxygen species (ROS) (Aitken *et al.* 1998). At the same time the literature indicates that centrifugation has no detrimental effect on sperm fertilizing ability (Avery & Greve 1995). In any case, centrifugation results in a final pellet with large numbers of spermatozoa including dead, moribund and abnormal cells. During the SU procedure the spermatozoa are both surface-cleansed and selected for functionality, thus imitating processes occurring in the female genital tract during sperm transport towards the oocyte (Rodriguez-Martinez *et al.* 1997b).

Use of the migrating ability of spermatozoa in a SU procedure provides samples with highly motile spermatozoa but yields <10% of the original sample when FT semen is used, or up to 20% for neat semen (**Paper I**, Rodriguez-Martinez *et al.* 1997a, Rodriguez-Martinez *et al.* 1997b). Despite the low harvest recorded, both the total concentration and the concentration of motile spermatozoa after SU are reported to be associated with bull fertility after AI (Zhang *et al.* 1998). Such an

association with fertility after AI was registered for EHF bulls but not for SRB bulls, probably owing to the wider range in NRRs for EHF bulls (23.8 percentage unit) compared with SRB (8.5 percentage unit). Under these circumstances, the results confirm the value of exploring this parameter as a simple and reliable method to assess the potential fertility of a semen sample or bull. Because of the simplicity of the procedure it is recommended that bull semen-processing enterprises explore the outcome of SU on a larger bull population to eventually adopt this preparation method if its general value is proved.

Capacitation, a prerequisite for fertilization (Rodriguez-Martinez, 2001), occurs during sperm transport *in vivo* and can be induced *in vitro* by incubation in a heparin-containing medium such as Fert-TALP (Parrish *et al.* 1988; Rodriguez-Martinez, 2003). From the two cleansing methods tested in **Paper I**, SU provided spermatozoa with significantly better motility, acrosome/tail morphology and membrane integrity than seen immediately PT or after W. Membrane integrity values were significantly lower after W than PT or after SU, illustrating the detrimental influence of centrifugal manipulation mentioned above. Swim-up also allowed for a better differentiation of bulls, based on motility results, than did W or PT analysis.

In **Papers V and VI** the ability of SU to select for motility (both subjectively and CASA-assessed) was proved again. The common tendency was towards a decrease in linearity, with a simultaneous increase in VAP after SU compared with PT, which could be explained by the presence of a hyperactive-like motility pattern, a change in motility that accompanies capacitation (Suarez & Ho 2003). Hyperactive spermatozoa are characterized by less linear and progressive, but more vigorous movement (Yanagimachi, 1994). As the capacitating ability of heparin, a compound in the SU medium used here, is well known (Parrish *et al.* 1988), such a change in motility pattern is justified. However, both capacitation and hyperactivation can occur independently of each other, particularly *in vitro* (Ho *et al.* 2002). The selective abilities of the SU pre-treatment were seen also in the sperm chromatin stability study (**Paper IV**), showing significantly fewer spermatozoa with denatured chromatin post-SU than PT; in **Paper V** where the MMP was higher after SU in every age group of EHF bulls; and in **Paper VI**, where the proportion of spermatozoa with both stable and unstable membranes increased after SU selection, owing to a substantial concomitant reduction in the number of dead cells. The increase in instability after exposure to the capacitating agent heparin during SU may be related to the different nature of "cryocapacitation" and real capacitation (Guthrie & Welch 2005). Spermatozoa PT can be dead, acrosome reacted or intact (e.g. those that have restored their stability), while those that are intact can still undergo capacitation when exposed to capacitating conditions or agents, such as *in vivo*, or to heparin *in vitro*, and therefore be recorded with M540 after SU. The present results indicate that this could have been the case with SU through a heparin-containing medium. Use of SU through a heparin-free medium should therefore be tested in the future.

To ensure that the cost for extensive insemination trials necessary for approval of every young bull in the AI industry is justified, knowledge of whether the semen quality of 1-year-old bulls changes when the animals reach maturity at 3–4

years of age or further on during their reproductive age is of importance since it is the semen of the older bulls that is commercially exploited. It has been shown that early collections from young Holstein bulls are often substandard and that improvements occur from puberty until 2 years of age (Almquist & Amann 1976). Age-related changes in semen quality were recorded PT and SU but not after W in SRB bulls (**Paper II**), with both CASA-assessed motility (PT) and membrane integrity (PT, SU) increasing with age. These findings are not in agreement with those reported by Januskauskas *et al.* (1999) who did not find differences in PT motility between young and mature bulls of the SRB breed. These different results can be explained by the fact that two different groups of animals (young and old) were used in that study. However, when looking at motility in a sperm subpopulation harvested after SU, there was no difference between ages. From the above results it could be hypothesized that, as SU mimics the selection inside the female genital tract, the number of cells capable of reaching the oocyte is not dependent on the age of the sire. Yet when the age-related differences in spermatozoa of 3- to 7-year-old EHF bulls were measured, an increase in subjectively assessed motility was registered again after SU (**Papers III and V**), providing evidence that motility could still be related to sire age.

The improvement registered in the proportion of spermatozoa with intact membranes (PT, SU) was also confirmed in EHF bulls (**Papers III and VI**). Similar results were reported by Januskauskas *et al.* (1999) for PT samples assessed by microscopy. However, these authors did not report age-related changes for viability assessed with SYBR-14/PI when FC was used, as also reported by Garner *et al.* (1996). In the morphological evaluation no sire age-related changes were recorded in the group of 1- and 4-year-old SRB bulls. Karabinus *et al.* (1990) had the same results, in contrast to Söderquist *et al.* (1996), when examining FT semen. When age-related changes were monitored for longer periods in EHF sires (**Paper III**) the proportion of midpiece abnormalities and proximal cytoplasmic droplets increased in 7- v. 3-year-old bulls PT. At the same time the older bulls had fewer acrosome and tail abnormalities after SU. With the exception of acrosomes the overall measured values did, however, not exceed 1%, which gives them little biological significance. As mentioned, the highest values measured for sperm abnormalities were for acrosomes, in most cases linked to defective semen handling and only related to fertility when present at high levels (Rodriguez-Martinez *et al.* 1997a).

Somewhat contradictory results have also been shown during analyses of fresh bull semen. Foote *et al.* (1977) showed improvements in morphology by 3 and 4 years while in Padrik & Jaakma (2002) normal morphology is reported to have decreased using bulls from 1–3 to 4–5 and 6–7 years of age. Done at regular intervals, morphology examinations of fresh semen are valuable for monitoring semen quality and, indirectly, for monitoring testicular and epididymal function. Assessment of FT semen seems, therefore, redundant in this respect, but valuable in assessing freezing and thawing-related abnormalities such as acrosome ruptures. The lack of serious morphological abnormalities in the semen of these evidently normal sires, and of abnormal sperm head shapes in particular, was not accompanied by any biologically significant age-related changes in chromatin stability.

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Mitochondrial activity showed no differences between the age groups after thawing but, following SU, the semen from 7-year-old EHF bulls showed a significant improvement over the semen examined when the sires were 3 years old. This tendency was seen also in semen from the 3- to 5-year-old group being in agreement with motility results from the same bull population (**Papers III and V**). As motility and mitochondrial activity are interrelated and the evaluation of the latter included the objectivity of FC as a measuring instrument, it is recommended that such measurement be considered in the future.

The proportion of spermatozoa showing destabilization of the membrane (high M540 fluorescence) did not differ between the age groups of EHF sires, except in the 3-year-old v. the 7-year-old group, where the proportion of spermatozoa with unstable membranes increased PT (**Paper VI**). Since the levels of instability were unquestionably very low (<2%), the change probably does not influence either the freezability or the resulting functionality of spermatozoa from 7-year-old sires.

In conclusion, older bulls showed obvious improvement in membrane integrity and motility when analysed PT or after SU under the conditions of the present experiments. However, as none of these changing parameters showed positive relations between age groups, and as they did not correlate to NRR, we can conclude that in our study, the semen quality at 1 year of age was predictive of that at 4-years old and, similarly, that the semen quality at 3 years of age was predictive of that at 7 years of age. This result is in agreement with Karabinus *et al.* (1990) who suggested that the quality of a randomly selected semen sample from a young bull is generally indicative of the quality of a random sample of his semen as a mature bull, using conventional tests.

As microscopic evaluations of sperm attributes are considered relatively subjective and therefore as not always repeatable, FC, allowing simultaneous evaluation of thousands of cells, appears to be more sensitive and was therefore chosen in an attempt to reveal any difference in the selected AI bull populations. With this intention, in **Paper IV** both sperm chromatin stability and morphology were investigated in SRB and EHF AI bulls.

It has been stated by Sailer *et al.* (1995) that abnormal chromatin structure may lead to problems in sperm nuclear material packaging during spermatogenesis, which can be related to morphologically abnormal spermatozoa. The reason for DNA fragmentation in ejaculated spermatozoa could be reactive oxygen species (ROS), either produced during sperm deterioration or originating from leukocytes (Evenson, 1999). With chromatin stability measurements, it is possible to detect sperm deviations of importance for early embryonic development sooner than with light microscopy (e.g. at 3 v. 11 days following an insult caused by thermal stress, as described in Karabinus *et al.* [1997]). This would explain an association between infertility cases and abnormal chromatin structure while all the classic quality parameters would still be within acceptable limits (Evenson & Jost 2000). If this is the case, chromatin stability assessment may provide additional information about semen quality. The percentage of spermatozoa with denatured DNA for 1-year-old bulls found in our study (1.7±0.7%) was indeed very low compared with that reported by other authors using bulls at the same age (e.g.

2.6±1.6% in Januskauskas *et al.* [2001]) and 15.1±2.4% in Ballachey *et al.* [1987]). As the sires used here had passed through selection in relation to normal sperm structure and function, and had been subjected to fertility recording, low values of chromatin instability were not unexpected. However, Bochenek *et al.* (2001) report that verified ejaculates at AI centres could contain as many as 23.8% spermatozoa with denatured chromatin. In human studies COMPα$_t$ thresholds of 0–15%, 16–29% and ≥30% have been related to high, moderate and low fertility potential, respectively (Larson *et al.* 2000; Evenson & Jost 2000). Sperm morphology measurements in the present studies showed extremely low abnormality values, a major difference from the situation in humans. The highest frequencies were for abnormal acrosomes and for tail abnormalities. The rest of the abnormalities did not exceed 5%, a figure considered the limit for young AI dairy bulls. More sire age-dependent changes were present in the EHF population, where bulls were monitored 3 years longer than were bulls in the SRB group. There were few relationships between sperm chromatin (in)stability figures and sperm morphology for both sire groups, either separately or in combination. Although the breeds did not share a common variable that changed through the years, measurements after SU always showed an improvement in sperm head morphology for the mature bulls.

If chromatin stability assessments could offer more information about sperm quality than morphology, we would expect it to show a change over the years as well. Karabinus *et al.* (1990) found an improvement in chromatin stability for mature bulls and concluded that FC measurements are more sensitive than are conventional sperm quality measurements in disclosing these changes. However, the mean values for COMPα$_t$ in the study by Karabinus *et al.* (1990) were noticeably (>10%) higher than in the present study. We have no basis for questioning their results, but it seems important to clarify the differences in chromatin stability between the sire groups used. To summarize, the measurement of sperm chromatin stability in our study could not yield more knowledge than could the conventional sperm quality measurements, either PT or after SU, perhaps due to the sire population used.

In an attempt to find a method for evaluating sperm quality that is more objective than the current subjective motility evaluations, we measured the MMP by use of the cationic lipophilic dye MitoTracker Deep Red 633. Since the energy necessary for sperm motility is produced and consumed in the mitochondria, the measurements of their function were obviously compared with microscopic and computer-assisted sperm motility analyses. On average, the results of FC measurements of mitochondrial function were 10–15% lower than the recordings of motility, measured either subjectively or by CASA (SM-CMA). Such a difference between subjective motility evaluations and FC evaluations of mitochondrial activity is in line with results reported in several other publications (Garner *et al.* 1997; Gravance *et al.* 2000; Wu *et al.* 2003), where it was explained by human error, leading to an overestimation of the proportion of motile cells. However, since we can expect CASA to be more precise than the visual motility evaluation, we should not have the same discrepancy between mitochondrial activity and motility results. The reason for this discrepancy needs to be clarified. In the work by Garner *et al.* (1997) the SD for subjective motility was >15% and

for FC measurements, <4%. We did not notice any smaller variability in FC results in the present work than that reported in the literature. The FC measurements provided data similar to data from conventional semen analysis, which proves that MitoTracker Deep Red 633 is suitable for evaluation of mitochondrial function as a marker for sperm viability. Once again, being linked to a higher objectivity by its ability to assess thousands of cells, FC appears preferable to subjective microscopic or more "objective" CASA analyses.

Paper VI was aimed to test the usefulness of a triple fluorochrome (M540/Yo-Pro-1/H33342) staining in determining early changes in the plasma membrane of FT bull semen. For this purpose, the FC results were compared with results of other, more conventional measurements of sperm quality. It is known that the process of cryopreservation induces changes in the sperm plasma membrane, which resemble a physiological, heparin-induced capacitation (Watson, 1995; Cormier *et al.* 1997) but apparently with different regulatory mechanisms (Cormier & Bailey 2003).

During capacitation the sperm plasma membrane is highly polarized and dynamically organized. The fluorescent probe M540 detects plasma membrane changes interpreted as increases in lipid disorder (Gadella & Harrison 2000). An increased degree of disorder (destabilization) in the plasma membrane of PT spermatozoa reduces the lifespan of the cells (Watson, 1995), thus constraining fertilization *in vivo* (Thundathil *et al.* 1999; Januskauskas *et al.* 2000) due to a decrease in the number of viable spermatozoa at the fertilization site. Therefore the amount of viable, uncapacitated spermatozoa in the inseminate would give valuable information about its quality. The results showed that the proportion of spermatozoa with a stable plasma membrane tested by the triple combination of fluorophores and FC correlated to motility, normal head morphology and membrane integrity, thus proving the reliability of this new staining technique. Similar relations between sperm capacitation-like changes and other tests have been reported in the work by Januskauskas *et al.* (2005).

In the EHF bull population the proportion of live spermatozoa with stable membranes PT was similar to or even higher than that reported in other studies (Thundathil *et al.* 1999, Januskauskas *et al.* 2005). To further challenge this population of spermatozoa, we used SU through a heparin-containing medium, Fert-TALP. This modified Tyrode's medium was used to mimic the electrolyte composition of oviductal fluid (present at the physical site of capacitation). In the cow oviduct, heparin and heparin-related glycosaminoglycans (GAGs) are believed to act as effectors of capacitation *in vivo* (Parrish *et al.* 1988). As expected, the number of spermatozoa with high M540 fluorescence (indicating a higher degree of lipid disorder in the plasma membrane, as seen during the initial steps of capacitation) rose after SU compared with PT, within a higher ($p<0.05$) population of spermatozoa with stable membranes. This indicates that SU provides a high degree of sperm selection. Moreover, the finding that PT spermatozoa had a lower degree of instability than after exposure to the capacitating agent heparin may be explained by the different nature of "cryocapacitation" and real capacitation (Guthrie & Welch 2005). Although the issue of cryopreservation-induced capacitation-like changes (the so-called "cryocapacitation" described in

Pommer *et al.* [2003]) is still debated, more and more evidence is now available suggesting that the destabilization that occurs during cryopreservation is not necessarily a process of capacitation, although it resembles it (Green & Watson 2001; Guthrie & Welch 2005). Spermatozoa PT can be dead, acrosome reacted or intact (e.g. those that have restored their stability). Those that are intact can still undergo capacitation when exposed to capacitating conditions, such as found *in vivo*, or to agents, such as heparin *in vitro*, and thus be recorded with M540 after SU.

The above-mentioned rise in membrane instability recorded by M540 appears lower than that reported in the study of Thundathil *et al.* (1999), in which the chlortetracycline (CTC) assay was used (up to 36%). It should be noted that the methods monitor different events in the lengthy process of capacitation. The scrambling of phospholipids detectable with M540 is induced by bicarbonate and takes place early, while CTC monitors late events during capacitation and acrosome exocytosis triggered by a secondary Ca^{2+} displacement (Gadella & Harrison 2000; Rathi *et al.* 2001; Rodriguez-Martinez,. 2001). The presence of nearly 30% dead cells after SU could be explained by the time needed for staining and FC measurement (15−20 minutes in total) since they were both performed without CO_2 supplementation. Final measurements were also done at room temperature (with waiting samples held in a pre-warmed styrofoam box). Cell deterioration and spontaneous acrosome exocytoses could be expected under these conditions (Gadella & Harrison 2000). In conclusion, a triple fluorophore M540/Yo-Pro-1/H33342 in combination with FC is a suitable method for screening the degree of membrane stability among viable FT bull spermatozoa.

Relations among sperm quality parameters were investigated in **Papers IV–VI** to estimate the usefulness of newly developed sperm quality tests by comparing them with the conventional ones. The available data about correlations between conventionally measured parameters and chromatin stability vary greatly. Evenson *et al.* (1991) and Larson *et al.* (1999) found only moderate correlations between sperm morphology and sperm chromatin stability while Ostermeier *et al.* (2001) and Sailer *et al.* (1996) found similar relationships between sperm morphometry and sperm chromatin stability. Karabinus *et al.* (1990), however, found that frequencies of sperm head abnormalities and COMPα$_t$ values were strongly related (p<0.001). In **Paper IV** the relations between chromatin stability and morphology were very low in all cases, probably due to the small variation within the measurements. For the evaluation of MMP (**Paper V**), the proportion of spermatozoa with high MMP was related to sperm motility (p<0.01) both PT and after SU. In **Paper VI** plasma membrane stability correlated significantly to several routinely measured sperm quality variables, such as motility, normal head morphology and membrane integrity. Therefore, based on two AI bull populations used, the chromatin stability assessment did not supplement the routinely used sperm quality tests (morphology analysis by microscopy) as did the evaluation of mitochondrial activity and plasma membrane stability.

Besides the correlation among sperm quality parameters, the ultimate precision of a laboratory test is estimated for its relationship with fertility estimates (Amann, 1989). The fertility of AI bulls is in turn measured by NRRs. The range in 56-day

NRRs of the six SRB donor bulls used in **Papers I and IV** was 8.5 percentage points (625–910 inseminations per bull), with no significant differences among batches or bulls. Nevertheless, to make a reliable estimation of bull fertility, more than one batch (ejaculate) should be analysed (Amann 1989). In this study three semen batches per bull were assessed. In the EHF bulls (two to four batches per bull) the 60-day NRRs ranged from 52.2% to 76.0% (141–392 inseminations per bull), with significant differences among batches within two bulls (p<0.05) (**Papers III–VI**).

It is difficult to obtain relationships between sperm quality parameters and field fertility. One of the constraints is the pre-selection of sires, which means that both fertility values and quality measurements drop into a relatively narrow range, without any extreme values. The number of animals/inseminations is also usually limited, particularly if the same individuals are monitored for several years. These constraints could be the reason for the weak statistical relationships between fertility and quality parameters detected in this study. However, statistically significant relationships were registered (as presented in **Papers I and III–VI**) between measured sperm quality parameters and fertility both PT and after all preparation methods (W and SU). After SU more variables showed a significant relation to fertility than was the case after PT or W. Most variables derived from CASA analyses (motility characteristics) which correlated not only to AI results but also, between the age groups.

General conclusions

The results of the present study indicate that –

- ➢ Sperm cleansing after thawing by an SU procedure is superior as pre-treatment for analyses to W since it –

  - ✓ selects spermatozoa with higher motility, VAP, membrane integrity and chromatin stability than found in W or FT samples,
  - ✓ reveals changes between different ages of the sires, and
  - ✓ helps distinguish relationships between sperm quality and fertility among bulls in a homogeneous sire population.

- ➢ The overall semen quality at 1 year of age was predictive of that at 4 years and, similarly, the semen quality at 3 years of age was predictive of that in 7-year-old bulls.

- ➢ Measurement of sperm chromatin stability could not yield more information than obtained with conventional sperm quality measurements because of the very low correlation to conventional sperm quality parameters and the lack of relation to field fertility.

- ➢ MitoTracker Deep Red 633 (M 22426) was found to be a reliable marker for FT bovine semen viability as the proportion of spermatozoa with high mitochondrial activity correlated with sperm motility both PT and after SU.

- ➢ Exposure to a heparin-containing medium during SU further destabilizes the lipid bilayer of the FT spermatozoa, as detected by a triple combination of the fluorophores M540/Yo-Pro-1/H33342.

- ➢ Most sperm variables with significant correlation to *in vivo* fertility resulted from computerized analysis of motility (CASA). They were total motility, concentration of motile spermatozoa, linearly and non-linearly motile spermatozoa and VAP.

- ➢ Evaluation of selected, highly motile spermatozoa, as measured after SU by use of CASA, may be preferable to studies of bulk semen samples during quality trials, mostly due to the method's higher relation to fertility.

- ➢ Because of the simplicity of the procedure, it is recommended that bull semen-processing enterprises explore the outcome of SU in a larger bull population to eventually adopt this method if proved to be of general value.

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