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Semen quality and fertility after artificial insemination in dairy cattle and pigs

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Abstract

The economic importance of a high breeding efficiency in sows and dairy cows emphasizes the benefit of accurate prediction of fertility of boar and bull semen. The artificial insemination (AI) studs need an objective and rapid, but inexpensive, method to evaluate ejaculates. We developed a new quick and easy fluorescence method for frozen-thawed bull semen that uses an automatized fluorometer and the fluorophore stain propidium iodide that stains only cells with damaged membranes. The fluorescence of the semen sample and the totally killed subsample were measured simultaneously and viability was calculated. For fertility evaluation, the non-return rates (NR%) obtained from 92,120 inseminations with the analyzed batches were recorded. The correlation between the total number of viable spermatozoa in the insemination dose and field fertility was low but significant, suggesting that plasma membrane integrity evaluation can serve as a cost-effective quality control method for frozen-thawed semen at bull AI stations.

In the second study we describe a sudden, though long-lasting, drop in bull semen quality at an AI station. During five consecutive months, the number of rejected ejaculates and discarded frozen semen batches due to poor motility increased, and the number of all forms of abnormal spermatozoa increased. However, for the accepted ejaculates, a 60-day NR% was normal. The previous summer had been rainy, and the hay used in the AI station was visibly moldy. Immunoassay and gas chromatography-mass spectrometry detected *Fusarium* mycotoxins HT-2 and T-2, but no zearalenone in the hay. Occurrence of mycotoxins such as T-2 and HT-2 in the moldy hay coincided with, and may have been responsible for, the impaired semen quality in AI-bulls.

In the third study we describe the deteriorating effects of Bovine Respiratory Syncytial Virus (BRSV) epizootics on sperm quality in young AI bulls. The disease caused by BRSV is often mild in animals older than 6 months and is therefore neglected. However, it seems to be able to cause testicular fibrosis and disturbances in sperm morphology, thus requiring attention at AI stations with valuable breeding animals.

In the remaining two studies we concentrated on boars and studied the effect of altering insemination dose, sperm morphology and insemination technique (traditional vs. intrauterine insemination). The insemination dose for boars should exceed 3×10^9 spermatozoa under commercial circumstances to avoid decrease in prolificacy at farms. Morphological examination of spermatozoa requires some experience, but it is quite an easy and inexpensive method to screen-out overtly poor-quality ejaculates. Therefore, we suggest routine morphological examination of all young boars entering the AI station and regularly thereafter. The intrauterine insemination was easy and fast to perform in sows, but it did not result in marked increase in NR% or litter size when inseminating with 3×10^9 spermatozoa / dose.

This thesis focuses on different aspects affecting male reproduction. In the research projects we studied the deleterious effects of trichothecenes in feed and BRSV infection on sperm quality, developed a new method for sperm viability determination as well as studied the importance of semen quality and insemination dose for reproductive success.

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List of original publications

This thesis is based on the following original publications referred to in the text by their Roman numerals:

I Alm K, Taponen J, Dahlbom M, Tuunainen E, Koskinen E and Andersson M. A novel automated fluorometric assay to evaluate sperm viability and fertility in dairy bulls. Theriogenology 2001; 56(4): 677-684.

II Alm K, Dahlbom M, Säynäjärvi M, Andersson MA, Salkinoja-Salonen MS and Andersson MC. Impaired semen quality of AI bulls fed with mouldy hay: A case report. Theriogenology 2002; 58(8): 1497-1502.

III Alm K, Koskinen E, Vahtiala S and Andersson M. Acute BRSV infection in young AI bulls – effect on sperm quality. Reproduction in Domestic Animals. DOI: 10.1111/j.1439-0531.2008.01116.x

IV Alm K, Peltoniemi OA, Koskinen E and Andersson M. Porcine field fertility with two different insemination doses and the effect of sperm morphology. Reproduction in Domestic Animals 2006; 41(3): 210-213.

V Peltoniemi OAT, Alm K and Andersson M. Deep uterine insemination with standard AI dose in a sow pool system. Reproduction in Domestic Animals. DOI: 10.1111/j.1439-0531.2008.01094.x

Abbreviations

AI	artificial insemination
BRSV	Bovine Respiratory Syncytial virus
CASA	Computer Assisted Sperm Analysis
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FABA	Finnish Animal Breeding Association
LS	Least-Squares
MTT	Maa- ja elintarviketalouden tutkimuskeskus (Agrifood Research Finland)
NR%	non-return rate %
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SD	standard deviation
SEM	standard error of mean
SPF	specific pathogen free

1 Introduction

Artificial insemination (AI) is an assisted reproductive technology that is very important in intensive breeding and production of cattle and swine. AI is commonly used on a global basis; in Finland some 90% of cows and 80% of sows are artificially inseminated. In cattle, the AI is usually performed using cryopreserved semen as bull spermatozoa maintain their viability very well during the freezing-thawing process. On the other hand, the success rate for fertilization of sows with frozen-thawed semen is poor, so swine AI is usually performed with doses stored in a liquid state at +15-20 °C for max 3-5 days. To achieve acceptable results with AI, one needs to be successful in every procedural step from semen collection, dilution and storage to inseminating at the optimal time regarding estrus and ovulation.

Assessment of *in vivo* fertilizing capacity of semen represents a challenge because it is influenced not only by semen-related factors but also by female fertility and by many other factors that may or may not be determinable (Amann and Hammerstedt 2002). Due to the complexity of the fertilization process, several sperm attributes are required for successful fertilization, such as the ability to undergo capacitation, hyperactivation, the acrosome reaction, binding to the *zona pellucida*, and oocyte penetration. Despite the limitations as predictors of fertility, evaluation of sperm morphology and progressive motility is the most common method used to assess viability of fresh and frozen-thawed semen at AI stations.

The importance of using low sperm numbers for AI to initially determine relative fertility *in vivo* has been confirmed in a number of studies (Tardif et al. 1999; Amann and Hammerstedt 2002; Watson and Behan 2002; Ardon et al. 2003). This approach is likely to avoid the potential compensatory effect when using high sperm numbers per AI dose. This compensation can, however, be only partial, and some sperm defects are clearly uncompensable (Saacke et al. 1994; Saacke et al. 1998; Walters et al. 2005b). In practice, the possibility of using reduced sperm numbers for insemination in the field is limited due to potentially lowered fertility, which can cause marked economic losses to farmers. Another limiting factor in clinical trials is the wellbeing of the animals. It is especially relevant when studying factors harmful for the animal itself, as is the case with mycotoxins. Despite the limitations of large-scale field trials, they remain the only way to study the efficacy of different treatments under commercial conditions that laboratory assessment cannot adequately duplicate.

Farm animals in Finland are dependent on stored feed and bedding during the 7 to 8 month indoor feeding season. The microbiological quality of hay varies according to weather conditions during harvest and storage. In humid conditions, both bacteria and fungi may colonize hay and produce various toxins. These microbial impurities in feed and bedding enter farm animals orally and through inhalation.

The fungal genera most frequently reported to produce mycotoxins with adverse effects on animal health are *Fusarium* (T-2 and HT-2 toxins, zearalenone), *Aspergillus* (aflatoxins, ochratoxin), *Stachybotrys* (satratoxin), and *Penicillium* (ochratoxin) (Hintikka 1984; Karppanen et al. 1985; Ueno 1985; Gabal et al. 1986). Clinical mycotoxicosis is rare in Finland, but low doses of mycotoxins in feed and bedding can cause subclinical

symptoms, such as decreased fertility (Haase et al. 1983). Hanhela et al. (1995) studied the occurrence of microfungi on Finnish dairy farms and found *Fusarium* spp. to be common in grain and straw. However, neither the occurrence of mycotoxins in Finnish hay nor their subclinical manifestations in farm animals are well studied.

Many contagious animal diseases cause reproductive problems worldwide, but Finland has been almost free of many of these, including Bovine Respiratory Syncytial Virus (BRSV). In winter 2000, however, a major outbreak of BRSV occurred, spreading to the quarantine section of a rearing station for young dairy bulls intended for use in AI. BRSV is a pneumovirus and an important component of the calf pneumonia complex (Larsen 2000; Viuff et al. 2002). The most severe disease is observed in calves less than 6 months old (van der Poel et al. 1994; Grell et al. 2005), but it is also occasionally isolated from adult cattle with acute respiratory disease (van der Poel et al. 1993; van der Poel et al. 1994; Ellis et al. 1996; Elvander 1996). BRSV is widely distributed in most countries (Baker et al. 1986; Uttenthal et al. 1996; Baker et al. 1997). A BRSV outbreak was recently associated also with fibrotic lesions in bull testes (Barth et al. 2008).

The pig is considered to be, at least partly, an intrauterine ejaculator (Senger 2003). Therefore, deposition of the semen in the uterus may enhance reproductive success compared with using the caudal portion of the cervix as the primary site of semen deposition. Usually intrauterine insemination has been practiced in order to allow for a reduction in the number of sperm per dose (Martinez et al. 2002; Watson and Behan 2002). There is little information available to indicate whether intrauterine insemination with a standard AI dose is beneficial.

High quality semen is crucial for successful AI. Quality is, however, influenced by many factors that may or may not be determinable. This thesis concentrates on semen quality and on some factors affecting it in dairy bulls and boars, with emphasis on field fertility. The investigations were based on laboratory assessments and large field trials, as well as on historical datasets from Finnish AI companies and the breeding organization (FABA). This study resulted in the five original papers presented at the end of this thesis.

2 Review of the literature

2.1 Artificial insemination

High reproductive performance is a key factor for optimal economic success in cattle and swine production (Gröhn and Rajala-Schultz 2000). AI is the oldest and currently most common assisted reproductive technology and an important tool in animal production (Vishwanath 2003). Originally AI was introduced as a means of preventing spread of venereal diseases. Today AI represents a much more cost-effective means of disseminating superior genes (Roca et al. 2006). AI has been most widely used for breeding dairy cattle; 253 million frozen AI doses and 11.7 million liquid doses are produced worldwide every year (Thibier and Wagner 2002). In Finland, all bull semen used for AI is cryopreserved, allowing long storage times and easy distribution, and inseminations are generally done by trained inseminators.

The extent of AI use in the pig industry varies greatly among countries. AI accounts for 80% of litters in Finland (Gadea 2003). Over 19 million inseminations of gilts/sows are performed worldwide each year, almost all of them (99%) with liquid semen stored at +15-20 °C for up to 3-5 days (cooled semen) (Johnson et al. 2000; Roca et al. 2006), but this figure is expected to rise.

The AI industry has to deal with a time lag between semen collection and insemination and subsequent fertilization. Storage of semen doses for a certain time is necessary for their distribution and to optimize efficient use of semen for AI. It is of practical and economic importance that the semen storage time does not negatively influence fertility. However, the natural ageing process cannot be prevented in liquid diluted boar semen, not even during the first days of storage (Waberski et al. 1994b; Johnson et al. 2000). Spermatozoa progressively lose viability when stored in a semen extender at ambient temperature. A suppression of spermatozoal metabolic activity, to reduce energy consumption and by-product formation, is needed to ensure sperm longevity (Althouse et al. 1998). Maintaining extended semen at a temperature between 15 °C and 20 °C has been reported as optimal for storage of liquid boar semen (Paulenz et al. 2000). This practice is widely used and was followed also in the boar experiments in this study.

As a generalization, some 40-50% of the sperm population in mammals does not survive cryopreservation even with optimized protocols (Watson 2000). When comparisons are made on the basis of similar numbers of motile (assumed viable) spermatozoa, results are still generally poorer than with fresh semen, indicating that even the viable subpopulation after cryopreservation is compromised. Boar spermatozoa are very sensitive to cold shock, possibly because of the low cholesterol/phospholipid ratio of their membranes (Johnson et al. 2000). Therefore, the success of fertilization with frozen-thawed semen differs significantly between bull and boar (Holt 2000a; Holt 2000b; Johnson et al. 2000; Vishwanath and Shannon 2000). Another important observation related to cryopreserved boar semen is the timing of ovulation in the pig, which can occur over an extended period of estrus such that spermatozoa may be required to survive up to 40 h in the oviduct. Waberski et al. (1994a) noted that fertility with cryopreserved semen

can be high providing that the insemination is carried out within 4 h before ovulation. Outside this period, fertility with cryopreserved spermatozoa declined substantially, but fresh semen maintained its fertility for a much longer period. Cryopreserved spermatozoa do not survive as long as fresh spermatozoa in the female genital tract. These factors explain the use of liquid (fresh) semen in pigs and cryopreserved semen in cattle.

2.2 Semen evaluation

When choosing a male for breeding, especially for AI, it is imperative to assess its potential fertility by undertaking clinical and laboratory examinations. The *in vitro* semen evaluation, complementary to the clinical examination, is of high diagnostic value for assessing testicular and epididymal function, and/or the genital tract of the male, allowing elimination of clear-cut cases of infertility, or potential sub-fertility (Martin Rillo et al. 1996; Rodriguez-Martinez 2003; Saacke 2008). Likewise, the degree of normality of the semen before being processed for AI can be analyzed. The semen analysis routinely includes an immediate assessment of volume, appearance (i.e. color, contamination, etc.), sperm concentration and motility, as well as later determination of sperm morphology and the presence of foreign cells. Once screened for normality, ejaculates preserved for AI are assessed for sperm concentration and sperm motility. These are the parameters most often used to determine sperm viability in post-thaw semen samples as well as to estimate breeding potential of a sire under field conditions (Januskauskas et al. 1996; Martin Rillo et al. 1996; Correa et al. 1997; Rodriguez-Martinez 2003). Unfortunately, neither a simple semen analysis nor the routine evaluation post-thaw enable the determination *a priori* of the potential fertility level that the analyzed semen will reach, particularly after AI. The usefulness of these parameters to measure fertility of a semen sample accurately is controversial (Januskauskas et al. 1996; Correa et al. 1997) and correlations between sperm motility and fertility have revealed large ranges of variation (Kjaestad et al. 1993; Stalhammar et al. 1994b; Tardif et al. 1999; Januskauskas et al. 2003). Correlations between sperm morphology and fertility have also been found vary widely, and have most often been statistically non-significant when the semen of AI quality grade has been assessed (Rodriguez-Martinez 2003). Researchers have also used additional laboratory assays to predict accurately the fertilizing potential of a semen sample. Individual laboratory assays, which evaluate a single parameter, are not effective predictors of fertility. However, a combination of several assays may provide a better prediction of fertility (Ericsson et al. 1993; Rodriguez-Martinez 2003; Gil et al. 2005). The testing of a large number of parameters should lead to a higher accuracy because fertilization is a multi-factorial process (Amann 1989). However, most of these analyses are expensive and time-consuming and cannot be applied under field and/or commercial conditions. In conclusion, sperm analysis conducted under commercial conditions leads to the detection of ejaculates of very poor quality (associated with poor fertility). However, the pre-selection of the samples, the high number of sperm per dose and the high quality of the semen used in the AI programs reduces the variability, giving a low probability of detecting fertility differences associated with seminal parameters (Gadea et al. 2004).

2.2.1 Sperm concentration

Accurate and precise determination of sperm concentration in an ejaculate is important for AI stations in order to produce uniform insemination doses containing an adequate number of sperm. A certain safety margin is often used by AI stations to ensure that all insemination doses contain a minimal number of sperm. This also implies that some insemination doses contain an excessive number of sperm and that males of high genetic value are not used efficiently. This safety margin also affects the average revenue per ejaculate for the AI station.

The hemocytometer has often been referred to as the "gold standard" for assessing sperm numbers (Christensen et al. 2005; Kuster 2005; Prathalingam et al. 2006). The equipment is slow, however, and multiple measurements of each sample are needed to obtain a precise result (Evenson et al. 1993; Prathalingam et al. 2006). The use of a spectrophotometer is probably the most frequent method used by AI stations for assessment of sperm concentration (Woelders 1991; Evenson et al. 1993). For satisfactory results, periodic calibration of hemocytometers is necessary. The detection spectrum is limited for these instruments, and accurate quantification of sperm numbers in dilute or concentrated samples is problematic (Fenton et al. 1990; Christensen et al. 2005). Spectrophotometers overestimate sperm numbers in dilute semen samples and underestimate sperm numbers in concentrated sperm samples. For individual raw ejaculates of boar semen, differences in the amount of gel particles or debris (cytoplasmic droplets, bacteria) can result in an inaccurate determination of the sperm concentration (Woelders 1991). Electronic particle counters allow rapid determination of sperm concentration but tend to include any debris in the size range of sperm (Evenson et al. 1993). Fluorometric measurements of the amount of DNA, using DNA-specific fluorochromes have been investigated (Fenton et al. 1990; Hansen et al. 2002), but this method requires stoichiometric staining of all DNA and minimal unspecific fluorescence from the extender.

2.2.2 Motility

Most frequently, the semen quality of dairy bulls and boars in AI centers is evaluated using sperm concentration and motility in fresh semen and motility in post-thaw samples for bulls. While some authors established a correlation between motility and field fertility, others did not (Januskauskas et al. 1996; Correa et al. 1997; Holt et al. 1997; Christensen et al. 1999; Tardif et al. 1999).

Good progressive motility of spermatozoa is an indicator of both unimpaired metabolism and intactness of membranes (Johnson et al. 2000). Estimation of motility has fundamental importance in daily quality control of semen. The percentage of motile spermatozoa is used to calculate the required degree of dilution and to estimate the number of intact spermatozoa per insemination dose. Regular motility checks of boar semen after dilution and during the holding period furnish information on the capacity for preservation of the semen of each boar and its individual peculiarities. Motility is mostly assessed

visually with a light microscope. It is inexpensive and quick, but accuracy depends on the subjective estimation by individuals even though surprisingly consistent results can be obtained (Woelders 1991). Objective Computer Assisted Sperm Analysis (CASA) systems have become commercially available, but these systems are not frequently used in commercial AI-centers because of the high investment costs (Verstegen et al. 2002). Encouragingly small sampling errors and high correlations with fertility have been reported (Farrell et al. 1998), but the reported procedures have to be applied to an independent dataset to test their repeatability. The main problem in CASA systems is related to the standardization and optimization of the equipment and procedures (Rodriguez-Martinez et al. 1997; Verstegen et al. 2002). A simple visual estimation of sperm motility remains a useful tool for routine semen assessment for research purposes and in the AI industry.

As boar spermatozoa show a higher percentage of circular movement than those from other species, except stallions, it is recommended to estimate the different forms of motility, including proportions of progressive spermatozoa (Johnson et al. 2000). Estimates undertaken using phase contrast microscopy within 20-30 min of dilution cannot be integrated easily into the production processes. Stored boar semen should be examined regularly and motility values above 60% should be considered satisfactory (Johnson et al. 2000).

2.2.3 Morphology

Morphological abnormalities of sperm can have a detrimental impact upon fertilization and embryonic development (Walters et al. 2005a; Saacke 2008). Bulls and boars used for commercial AI are selected to a certain degree on the basis of a low incidence of morphologically abnormal spermatozoa, so that statistical calculations concerning their correlation with fertility are not very informative (Rodriguez-Martinez et al. 1997; Johnson et al. 2000), although some evidence for a relationship between sperm morphology and fertility in bulls has been presented (Söderquist et al. 1991; Al-Makhzoomi et al. 2008). A complete morphological examination is recommended when bulls and boars are introduced into the AI station and during subsequent regular routine examinations (Johnson et al. 2000; Al-Makhzoomi et al. 2008). Principles for determining sample size for morphological assessment of spermatozoa were extensively discussed by Kuster et al. (2004). The percentage of cytoplasmic droplets in boar ejaculates used for AI should not exceed 15%, especially when stored semen is used. In addition to the incidence of cytoplasmic droplets, the percentage of other morphological alterations should not exceed 20% (Johnson et al. 2000).

A number of classification systems exist for morphological abnormalities of sperm, including i) primary and secondary defects, which classify sperm abnormalities on the basis of their presumptive origin (Barth and Oko 1989); ii) major and minor defects, a revised system where sperm defects are classified in terms of their perceived adverse effects upon male fertility (Blom 1983); and iii) compensable and uncompensable semen traits according to a theoretical increase in numbers of functionally competent sperm that

will or will not solve the problem (Saacke et al. 1994, 2000; Saacke 2008). A compensable defect is one where the defective spermatozoa either do not reach the site of fertilization, or fails to initiate the fertilization process. Defects that lead to failed fertilization or early pregnancy loss are termed uncompensable.

2.2.4 Fluorescent methods / Assessment of the plasma membrane status

Plasma membrane status of spermatozoa is of outmost importance due to its role, not only as a cell boundary, but also for cell-to-cell interactions, e.g. between spermatozoa and the epithelium of the female genital tract and between the spermatozoon and the oocyte and its vestments. The relationship between the degree of sperm damage post-thaw and fertility is not always clear (Januskauskas et al. 1996; Garner et al. 1997b), but tends to increase when damage is extensive or when fertility values are widely spread (Januskauskas et al. 2001). Fluorescent viability staining in semen evaluation serves many purposes depending on the used combination of fluorochromes, such as determination of the sperm membrane integrity, acrosomal status, or function of mitochondria (Halangk and Bohnensack 1982; Harrison and Vickers 1990; Woelders 1991; Garner et al. 1997b; Januskauskas et al. 2000; Silva and Gadella 2006). Fluorophore-incubated spermatozoa have been mostly studied with fluorescence microscopy (where an operator is required for counting) (Althouse and Hopkins 1995). It is a simple and cheap method, but one that only allows assessment of a few hundred spermatozoa per sample. Sperm counting can be markedly increased using a fluorescence-activated cell-sorting instrument (FACS) (Garner and Johnson 1995) where thousands of spermatozoa can be examined in minutes, reaching correlations with *in vitro* (Maxwell et al. 1998) and *in vivo* fertility (Ericsson et al. 1993; Januskauskas et al. 2001). However, the costs of purchase and running this equipment are quite high. Alternatively, computerized fluorometry can be used to evaluate membrane integrity of large sperm numbers (Halangk and Bohnensack 1982) yielding significant, albeit variable, correlations with fertility (Januskauskas et al. 2001).

The usual approach in fluorescent viability staining is to use two fluorophore stains that react with the same cellular constituent – one stain that identifies only living sperm and another that stains only dead (or moribund) sperm, like membrane-permeant (SYBR-14) and impermeant stains (PI) together. Using a fluorometer, either rapid freezing and slow thawing or detergents are required to induce membrane damage as detected by PI in all sperm cells to create a totally killed subsample (Halangk and Bohnensack 1982; Juonala et al. 1999). Measuring simultaneously the fluorescence of the sample and the fluorescence of the totally killed subsample allows calculation of relative values.

Based on plasma membrane studies, the viability of post-thaw ejaculates varies within and between bulls (Ericsson et al. 1989; Ericsson et al. 1993), and a correlation between sperm viability after thawing and fertility has been demonstrated (Januskauskas et al. 2000), although the predictive value is limited. In pigs, plasma membrane integrity parameters in liquid semen stored for seven days correlated significantly with motility and fertility parameters (non-return rate and litter size) in multiparous sows (Juonala et al. 1999), but not in gilts with synchronized estrus (Tardif et al. 1999).

2.3 The number of spermatozoa / insemination dose

The number of sperm in the insemination dose is an important factor affecting the probability that a female will become pregnant after AI, and in litter-bearing animals also the litter size (Flowers 2002). To maximize pregnancy rate, the number of sperm in a dose is intentionally set high, but this management approach tends to obscure differences among males (or treatments) that might impact outcome of breeding when fewer sperm are used (Colenbrander and Kemp 1990; Woelders 1991; Amann and Hammerstedt 1993; Amann and Hammerstedt 2002; Amann 2005; Ruiz-Sanchez et al. 2006). Certain males achieve maximum fertility after AI with very few motile sperm (e.g. 1 million for cattle), whereas for other males 20-30x more motile sperm are required to maximize fertility (den Daas 1992; den Daas et al. 1998; Flowers 2002). At high sperm numbers per AI dose individual bulls differ in their maximal NR%. That is unrelated to the rate at which they approach this maximum (Saacke et al. 1994; den Daas 1998). *Vice versa*, subfertile bulls could not be restored to normal fertility by increasing numbers of sperm per inseminate (den Daas 1992; Saacke et al. 1994). Data for cattle are most comprehensive, but it would be erroneous to assume that this principle, which results from so called "compensable defects" of sperm (Saacke et al. 2000), is not operational in other species. Actually, it has been stated by several authors that insemination trials with reduced sperm numbers are needed to reveal subfertile males and/or to detect differences between males (Colenbrander and Kemp 1990; Woelders 1991; Tardif et al. 1999; Watson and Behan 2002; Ardon et al. 2003; Ruiz-Sanchez et al. 2006).

From the perspective of validating a diagnostic assay, the use of an excessive number of sperm when measuring fertility increases the probability that the compensable defects in sperm will be masked (Woelders 1991; Amann and Hammerstedt 2002). A compensable defect is one in which low fertility can be overcome, at least in part, by increasing the number of sperm in the AI dose (Saacke et al. 1994; den Daas et al. 1998; Saacke et al. 2000). Low fertility caused by an uncompensable defect persists regardless of the number of sperm per insemination. Hence, with a compensable defect of sperm, the "problem" causing low fertility results from the failure of sperm characteristics being expressed before sperm enter the oocyte. An uncompensable defect involves an attribute (or attributes) being expressed only after a spermatozoon enters an ovum (Amann and Hammerstedt 2002). When a spermatozoon with an uncompensable defect fertilizes an oocyte, it is unable to complete the fertilization process or sustain embryonal development, so pregnancy may not be detected.

There is an increasing interest among AI/breeding organizations to decrease the number of spermatozoa per straw to be used for AI, related to economic revenues and the expected increased use of sex-sorted semen in bulls. It is generally accepted that a total of 15×10^6 spermatozoa in a frozen 0.25 ml straw is enough to achieve an acceptable fertilization rate in cattle provided that post-thaw motility is equal to or above 50% (Shannon and Vishwanath 1995; Vishwanath and Shannon 2000; Ballester et al. 2007). Extension of semen to low sperm numbers per AI-dose has been related to a decrease in bull sperm viability *in vitro* with significant bull variation (Garner et al. 1997a; Ballester et al. 2007).

In AI of swine several dose regimens are applied, ranging from 1.5 x 10^9 to 6.0 x 10^9 spermatozoa per intra-cervical insemination dose (Colenbrander et al. 1993; Martin Rillo et al. 1996; Althouse 1997a; Althouse 1997b). A lower sperm dose is more profitable for AI centers and makes more effective use of superior boars. However, when decreasing the insemination dose, the effect of semen quality becomes more important and otherwise compensable morphological deficiencies can no longer be overcome (Althouse 1997a; Althouse 1997b; Althouse 1997c; Johnson 1997; Althouse et al. 1998; Saacke et al. 2000). This leads to decreased fertility rates and smaller litter sizes (Althouse 1997a).

2.4 Intrauterine (transcervical) insemination in sows

The pig is considered to be, at least partly, an intrauterine ejaculator (Senger 2003). Deposition of the semen in the uterus may enhance reproductive success compared with the standardized insemination procedure termed intra-cervical insemination that involves deposition of the semen dose in the posterior portion of the cervical canal by means of a catheter that engages with the posterior folds of the cervix, simulating the corkscrew tie of the boar's penis. The standard insemination is a simple, inexpensive and quick procedure, but requires a large number of spermatozoa per dose (generally more than 2.5 x 10^9 cells), of which approximately 30-40% flow back within 1 h of insemination (Roca et al. 2006). The intrauterine insemination technique was developed to overcome this event and improve the number of spermatozoa able to reach the uterotubal junction and sperm reservoir in the isthmus.

To perform intrauterine (transcervical) AI, a thin and semi-rigid insemination device is passed through a conventional catheter previously inserted between the cervical folds. As the specialized inner insemination device is 15-20 cm longer than a conventional catheter, it can extend over the remaining folds of the cervix and enter the uterine body (Watson and Behan 2002; Roca et al. 2006). Criticism for probable traumatic injury indicated by the presence of blood in the vulva after insemination, which has been reported in some animals, could be caused by incorrect manipulation of the device. The device should always be inserted carefully and not forced if some resistance is encountered. One important limitation is that the method is unsuitable for insemination of gilts because of difficulties in passing the device through the cervix (Roca et al. 2006).

Intrauterine insemination has been usually practiced in order to allow for a reduction of the number of sperm per dose (Martinez et al. 2002; Watson and Behan 2002; Roberts and Bilkei 2005), which is especially important with sex-sorted or frozen-thawed spermatozoa. A similar technique has been developed to allow transcervical embryo transfer (Martinez et al. 2004). In field trials comparing this technique using cooled semen and reduced sperm numbers (1 x 10^9 spermatozoa / dose) or conventional AI (3 x 10^9 spermatozoa / dose), farrowing rates have been similar (Watson and Behan 2002; Rozeboom et al. 2004; Roberts and Bilkei 2005). However, it has been accompanied with a reduction in the number of piglets born per litter (Rozeboom et al. 2004; Roberts and Bilkei 2005). When an even greater reduction of sperm dose has been attempted (0.5 x 10^9 sperm / dose), both farrowing rate and litter size have been poor (Rozeboom et al. 2004).

The two major reasons why the sperm numbers can be somewhat lowered using intrauterine insemination without a reduction in farrowing rates are the full passage of the semi-rigid insemination device past the cervical folds and a substantial reduction in semen backflow to less than 20% of inseminated spermatozoa (Mezalira et al. 2005). However, the reduction in the number of piglets born per litter indicates that 1×10^9 is not an adequate number of spermatozoa to achieve high fertility with intrauterine insemination under farm conditions, and a higher number of spermatozoa / dose is suggested (Roca et al. 2006).

2.5 Assessment of reproductive performance / field fertility

The most appropriate assessment of reproductive performance will vary depending on whether emphasis is placed on semen quality, differences among females or comparison of different AI strategies. In semen quality studies, reproductive success is often evaluated using the likelihood of conception after a particular AI (Amann and Hammerstedt 2002). Of the alternative outcomes after copulation or AI, non-pregnant or pregnant, pregnant is a better estimate of the normalcy of sperm function and non-pregnant of abnormal sperm function (Amann and Hammerstedt 2002). In this thesis we used a 60-day non-return rate to estrus (NR%) as the outcome variable for AI with bull semen, and farrowing rate and litter size for boar semen. It can be questioned whether NR% is an appropriate measurement for monitoring fertility, but the assessment of pregnancy rate, which would be more precise, requires that all animals in the herd are pregnancy tested, which is not practical or economical in large field studies where the farms are located throughout the country. For the calving rate, there is a 9 month delay for data which is impractical for study purposes. For all types of assessments of reproductive performance, there are some potential systematic errors, including culling and selling of animals, that one needs to be aware of when drawing conclusions. The many important aspects in determining differences in male fertility were critically reviewed by Amann and Hammerstedt (2002).

2.6 Seasonality

2.6.1 Cattle

Reproductive strategy in a seasonal breeder results in births during the spring when climate and food supplies are favorable for early postnatal development. Although cattle are not seasonal breeders in the strict sense of distinct seasons of reproductive activity and inactivity, there is evidence for seasonality in bovine reproduction (Dahl et al. 2000). Several studies demonstrated the effect of season on fertility in cattle (Gwazdauskas 1985; Stalhammar et al. 1994a; Söderquist et al. 1997; Oseni et al. 2003; Rensis and Scaramuzzi 2003), and long-term statistics from Finland show a similar trend for higher fertility of

cows during summer months with long but not too warm days, compared with winter (Harman et al. 1996), similarly as in Sweden (Stalhammar et al. 1994a). Extended photoperiods were also shown to hasten the onset of puberty in prepubertal heifers (Hansen et al. 1983), as well as milk yield in dairy cattle (Reksen et al. 1999).

2.6.2 Swine

Swine have been shown to be seasonal breeders with reduced NR% and litter size as well as increased early pregnancy loss during late summer - early autumn in a temperate climate like Finland (Peltoniemi et al. 1999; Tast et al. 2002). Claus et al. (1985) reported photoperiodic influences on sperm quality and libido in boars, and this was supported by the report of Ciereszko et al. (2000). It has been noted that a short-day length stimulates the pubertal maturation of spermatogenesis, while a long-day reduces the sensory scores for boar taint at slaughter in whole male pigs (Andersson et al. 1998), demonstrating the broad effects of changing light patterns mediated by changes in melatonin secretion (Tast et al. 2001).

2.7 Mycotoxins / Trichothecenes

Many types of mold are able to form toxic secondary metabolites termed mycotoxins. *Fusarium* molds are probably the most important mycotoxin producers that infect cereals in northern temperate regions (Hintikka 1984; Karppanen et al. 1985; Ueno 1985; Gabal et al. 1986; Eskola et al. 2001). *Fusarium* molds are known to produce different mycotoxins, including trichothecenes such as deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), nivalenol (NIV), T-2 toxin (T-2), HT-2 toxin (HT-2), and also other toxins such as zearalenone (ZEN) in grains (Eskola et al. 2001). Some molds are able to produce more than one mycotoxin and some mycotoxins are produced by more than one mold species, and thus several mycotoxins are often simultaneously found in a single commodity. Despite the fact that in natural contaminations of grain and grain products with mycotoxins, different combinations of mycotoxin mixtures are often detected, research has largely focused on the toxic effects of single trichothecenes (Koshinsky and Khachatourians 1992).

Cereals are the main source of trichothecenes and ZEN for consumers in the Nordic countries (Eskola et al. 2001). In Finnish cereals trichothecenes are more often found than ochratoxin A (OA) or ZEN (Hietaniemi and Kumpulainen 1991; Eskola et al. 2001). Mycotoxin contaminations in foods and animal feeds are usually heterogeneous (Eskola et al. 2001), which causes difficulties in sampling. The method of sampling is very important: a sufficient number of equal sample portions should be taken at random points throughout the lot. Gas chromatography (GC) with electron-capture (ECD) or mass spectrometric (MS) detection is the most frequently used technique for trichothecene analysis today (Eskola et al. 2001).

Exposure to mycotoxins has been associated with health problems in both humans and animals. The trichothecene mycotoxins are known to be potent inhibitors of protein synthesis, specifically in eukaryotes (Ueno 1985). The target organelle of trichothecenes is the 60S subunit of mammalian ribosomes. In mammalian cells, the trichothecenes inhibit DNA and RNA synthesis (Ueno 1985) injuring organs with rapidly dividing cell populations such as germinal epithelium in testes (Ueno et al. 1984). Furthermore, T-2 is a potent inducer of apoptosis (Ihara et al. 1997). Symptoms and toxic effects on animals vary according to species, amount of toxin, and route of administration. The acute and chronic toxicities of trichothecenes are characterized by the depletion of lymphoid tissues. This feature indicates that they impair the immune system and modify immune responses. In cattle, T-2 toxin induces immunosuppression (Hussein and Brasel 2001) by decreasing serum concentrations of IgM, IgG, and IgA, neutrophil functions and lymphocyte blastogenesis, and the response of lymphocytes to phytohemagglutinin. This toxin was also shown to induce necrosis of lymphoid tissues. Other significant effects of trichothecenes are dermal toxicity, feed refusal, vomiting, diarrhea, intestinal hemorrhage, neurological disorders, heart lesions, alimentary toxic aleukia, hepatic or kidney damage, impairment of the hematopoietic system and alteration of the levels of biogenic amines such as dopamine and norepinephrine in the central nervous system (Ueno 1985; Gabal et al. 1986; Fekete et al. 1989; Glavits et al. 1989; Pestka and Bondy 1990; Szilagyi et al. 1994). Bovine infertility and abortion in the final trimester of gestation have resulted from consumption of feed contaminated with T-2 toxin (Hussein and Brasel 2001). In interstitial cells from testes of adult gerbils incubated with T-2, testosterone synthesis and secretion were dose-dependently inhibited (Fenske and Fink-Gremmels 1990). Similarly, degeneration and necrosis of spermatogenic cells in the semiferous tubules of guinea pigs have been described (DeNicola et al. 1978). Another characteristic of trichothecene toxicity is the high susceptibility of animals, when exposed by the inhalation route (Ueno et al. 1984).

Generally, ruminants are less susceptible to trichothecene mycotoxicosis than monogastric animals (Hussein and Brasel 2001). Rumen microorganisms, mostly protozoa, have been shown to deacetylate T-2 toxin to HT-2 toxin, which is less cytotoxic (Kiessling et al. 1984; Ueno 1985; Swanson et al. 1987; Hussein and Brasel 2001). The detoxifying capacity of the rumen microflora is saturable and varies with changes in the diet, or as a consequence of metabolic diseases, such as rumen acidosis (Fink-Gremmels 2008). The metabolism of ingested material by the ruminal microbes may be considered as a first line of defense against toxic materials present in the diet. On the other hand, ruminants may be at a disadvantage if substances become toxic as a result of the action of ruminal microbes.

2.8 Bovine Respiratory Syncytial Virus (BRSV)

Bovine Respiratory Syncytial Virus (BRSV) is a pneumovirus belonging to the family Paramyxoviridae, subfamily Pneumovirinae, like its close relative Human Respiratory Syncytial Virus (HRSV) (Easton et al. 2004; Valarcher and Taylor 2007). BRSV infection

is an important part of the calf pneumonia complex (Larsen 2000; Viuff et al. 2002) causing up to 100% morbidity and 5-20% mortality in calves less than 6 months of age (Baker et al. 1986; van der Poel et al. 1994; Baker et al. 1997; Larsen 2000; Grell et al. 2005; Valarcher and Taylor 2007). The virus is occasionally isolated from adult cattle with acute respiratory disease (Ellis et al. 1996; Elvander 1996), and can infect sheep (Evermann et al. 1985). BRSV is widely distributed in most countries (Baker et al. 1986; van der Poel et al. 1993; van der Poel et al. 1994; Uttenthal et al. 1996; Baker et al. 1997). Respiratory disease caused by BRSV is mainly observed in autumn-winter in temperate climates (van der Poel et al. 1993), and is often diagnosed in combination with other viruses, bacteria or mycoplasmas (Kimman et al. 1989; Ellis et al. 1996; Tegtmeier et al. 1999).

The lability of BRSV in the environment has been underlined (Larsen 2000), but cell culture stocks of this virus have been reported to remain infective at 5 ºC for 300 days (Hägglund et al. 2006). Investigations are lacking on the time of quarantine required to avoid transmission to sensitive animals. The infection dose might be much smaller than the amount of virus that is required for isolation *in vitro* (Hägglund et al. 2006). The mode of transmission during the cause of natural infection has not been defined, but direct contact is probably required (Larsen 2000).

It is not known exactly how long antibodies against BRSV remain in sera in adult cattle, but months or even years can pass without re-infection (Elvander 1996). In calves, maternal antibodies to BRSV have been documented to remain up to a maximum of 7 months of age (Baker et al. 1986; Fulton et al. 2004; Grell et al. 2005). On farms where there are respiratory disease problems, including in Finland, BRSV seropositivity is frequently recorded (Härtel et al. 2004). The overall incidence of BRSV in Finnish herds is unknown, but the outbreak in winter 2000 and minor outbreaks in the following winters suggest that the virus is currently widespread.

Incubation time for BRSV infection is estimated to be 2-8 days. Symptoms include cough, nasal discharge, and fever. Major BRSV induced lesions occur in the airways and lungs of calves (Kimman et al. 1989; Philippou et al. 2000), but viral RNA has also been demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) outside the respiratory tract in lymphatic tissues and kidneys (van der Poel et al. 1997). RNA of BRSV has been found up to 71 days after an experimental infection of calves (Valarcher et al. 2001) and in naturally infected cows in peripheral leucocytes and nasal mucosa by nested RT-PCR (nRT-PCR) in acute phase and again 4 weeks later (Valentova et al. 2003). HRSV has been reported to be present in peripheral blood circulating mononuclear leucocytes of infected patients (Domurat et al. 1985). Both HRSV and BRSV can spread in their hosts also outside the respiratory tract (Sharma and Woldehiwet 1996; van der Poel et al. 1997), but the viremic phase of infection is not routinely detected (Valentova et al. 2003).

BRSV is present and replicates mainly in bronchial and alveolar epithelial cells on the luminal side of the respiratory tract (Viuff et al. 1996; Viuff et al. 2002). The virus infects several ciliated and non-ciliated epithelial cell types (Baker et al. 1997) leading to an alteration of the ciliogenesis and partial or total loss of cilia (Philippou et al. 2000; Viuff et al. 2002). BRSV can replicate in a wide range of primary bovine cell cultures derived

from the testis, turbinate, trachea, aorta, spleen and lung (Larsen 2000), as well as bovine and ovine alveolar macrophages, lymphocytes and peripheral monocytes (Sharma and Woldehiwet 1996; Keles et al. 1998) and ovine testicular cells (Sharma and Woldehiwet 1996). After *in vitro* exposure to BRSV, the viability of lamb testis cells, but not of lymphocytes or monocytes, was significantly reduced as early as 24 h post exposure (Sharma and Woldehiwet 1996). In the same study, the lamb testis cells were the most permissive cells, with viral antigens present on 96 ± 2.2% of cells by 24 h post exposure and on all cells by 48 h post exposure.

BRSV infection in young (6-12 month old) bulls seems to increase testicular fibrosis (Barth et al. 2008) by some unknown mechanism. It might have some immunological background similar to the lung pathology caused by RS viruses (Viuff et al. 2002; Valarcher and Taylor 2007) where fibrosis is common (Kimman et al. 1989; Ellis et al. 1996). Barth et al. (2008) suggested that during the active process leading to fibrosis of testicular tissue spermatogenesis is adversely affected and this is later seen as poor sperm morphology.

3 Aims of the study

The quality of semen used in AI is crucial to the outcome. This thesis concentrates on semen quality and on factors affecting it, with emphasis on field fertility. It is based on laboratory assessments and large field trials as well as on historical datasets from Finnish AI companies and the breeding organization (FABA).

The specific aims of the research were to:

1) develop a new, easy-to-use and cost-effective method for sperm viability testing (I)

2) investigate potentially harmful factors for semen quality and subsequent fertility in cattle (II and III)

3) study the effect of sperm morphology in boars (IV)

4) compare different AI doses in swine (IV)

5) study the effect of two insemination methods on pig reproduction (V)

4 Materials and methods

An overview of materials and methods is presented in this section. More detailed descriptions are available in the original publications (I-V). Clinical trials were carried out in commercial environments with standardized housing and management practices.

4.1 Animals and management

Young bulls arrived in quarantine at 5 to 6 months of age, from farms where they had been born, and remained in quarantine for at least 30 days. Bulls were moved from quarantine to the rearing station at about 7 months of age and to one of two AI stations for semen collection for progeny inseminations at the age of 1 year.

The bulls (I-III) were of Finnish Ayrshire and Holstein-Friesian breeds and located at two bull stations. They were all between 12 and 36 months of age. The bulls were housed indoors and fed according to Finnish standards (in Finnish: MTT).

The boars were of Finnish Landrace and Yorkshire breeds (IV), and Duroc X Hampshire crossbred as well as pure Duroc and Hampshire (V). They were located at two boar stations. The age of the boars ranged from 9 to 40 months. The boars were housed indoors and fed according to Finnish standards (in Finnish: MTT).

The cows (I-III) were in commercial farms belonging to the national health control system. The sows in study IV were in commercial farms belonging to the health control system and in study V in a sow pool consisting of 440 sows housed in five herds, one of which was the nucleus herd for breeding and pregnancy. Three weeks prior to term, each group of 40 sows was transported to one of the four satellite herds for parturition and lactation. The satellite herds were located 30-80 km from the nucleus herd. The sow pool was a SPF system.

4.2 Semen collection

Bull semen (I-III) was collected using the standard method, via an artificial vagina (2 ejaculates per bull per collection day). Semen from young bulls (13 to 18 months of age) was collected once a week, and from older bulls (over 18 months) twice a week, resulting in two or four ejaculates per week.

Boar semen (IV, V) was collected using a gloved hand technique no more than twice per week and the entire ejaculate was collected.

4.3 Semen quality assessment

Only grossly normal looking ejaculates were accepted for further evaluation and processing. The volume and density of the ejaculates were measured using a photometer (Novaspec II, Pharmacia LKB Biotechnology, Uppsala, Sweden) immediately after collection. Initial subjective progressive motility was estimated in all experiments and after freezing-thawing (I-III) by experienced technicians at the AI station. Motility was assessed with a phase-contrast microscope equipped with a heated stage, at 200x magnification. A drop of 10 µl of diluted semen was placed on a preheated (37 °C) glass slide and covered with a cover slip (22 x 22 mm).

The bull ejaculates were accepted for use in AI on the basis of their initial sperm concentration > 500 x 10^6 / ml, visual subjective sperm motility ≥ 60% and visual subjective post-thaw motility ≥ 40%. A batch consisted of two ejaculates (if both were acceptable, otherwise only one ejaculate) collected within a 15 minute interval.

Fresh boar ejaculates with total volume > 1 dl, progressive motility ≥ 60%, total number of spermatozoa ≥ 20 x 10^9 and total morphological abnormalities less than 20% of the sample were accepted. The average number of spermatozoa / AI dose was regularly controlled also in a hemocytometer (Bürker counting chamber, Fortuna, Germany).

4.3.1 Morphology

Morphology of semen samples was evaluated in studies II-IV. The smears were air-dried and sent to Saari laboratory, where they were fixed and stained using the Giemsa method according to Watson (1975); 100 spermatozoa / smear were examined, and classification of sperm morphology was performed as described by Blom (1983). Spermatozoa were divided into four classes: major sperm defects (e.g. pyriform head), proximal droplets, minor sperm defects (e.g. bent tail) and normal spermatozoa. Only the most serious sperm defect of each spermatozoon, based on its effect on fertility, was recorded. If there were less than 70% normal spermatozoa in the smear, an additional 300 spermatozoa were examined.

4.3.2 Sperm membrane integrity

Plasma membrane integrity of bull spermatozoa was studied (I) with fluorophore stain propidium iodide (PI) (Molecular Probes, Eugene, OR), which stains only dead or moribund cells. Simultaneous measurement of the fluorescence of the sample to be analyzed and the fluorescence of the totally killed subsample (rapidly frozen and slowly thawed) allowed calculation of relative values and the percentage of viable spermatozoa in the sample.

Two frozen semen straws from the same batch were pooled and dispensed into two vials after thawing. One of the vials from each batch was rapidly refrozen and slowly thawed to cause 100% disruption of the plasma membranes. The rapidly refrozen

subsamples were then analyzed in the same manner and in the same black well plate as the non-treated samples.

Equal aliquots of Beltsville Thawing Solution (BTS) diluted sample and PI solution were dispensed into the well plate (Black Cliniplate, ThermoLabsystems, Helsinki, Finland) in three replicates. Blanks containing diluted extender (1:1 BTS and Triladyl) and PI were dispensed into the microtiter plate in four replicates. The plate was shaken gently for 2 min and incubated in the fluorometer for 8 min before analysis. Eleven samples and their blanks were then analyzed simultaneously.

Fluorescence was measured using an automatic computerized fluorometer (Fluoroscan Ascent, Labsystems, Helsinki, Finland). Both excitation and detection of fluorescence were carried out from the top of the well (reflectance fluorescence). The interference filter in the excitation path and that of the emission filter had their maximum transmission at 544 nm and 590 nm, respectively. The analysis was done twice for each batch.

Percentage of fluorescence was calculated from the ratio of fluorescence intensities in the sample and in the rapidly refrozen subsample, in relation to background fluorescence (blank) (Garner et al. 1997b), the blank being a combination of diluent and PI without spermatozoa.

Viability of the frozen-thawed batch was calculated as follows:

(1 - [fluorescence of the sample - blank] / [fluorescence of the rapidly frozen subsample - blank]) x 100%.

The maximal fluorescence output value of the rapidly refrozen subsample was calibrated against the total sperm count of the AI doses by the use of a hemocytometer in advance (Juonala et al. 1999). Thereby, the fluorometric measurements simultaneously provided total sperm count in the straws and viability of the spermatozoa.

For comparison of the fluorometer with fluorescence microscopy, samples were prepared and stained as described. In addition to analyses of the microtiter well plate with the fluorometer, the samples underwent microscopic evaluation with a fluorescence microscope (Olympus BH2 with epifluorescence optics, Olympus Optical Co. Ltd., Tokyo, Japan) equipped with phase-contrast optics. Using only a little light, it was easy to visualize viable, unstained spermatozoa simultaneously with the fluorescent ones. From each sample 200 spermatozoa were evaluated. Only spermatozoa with completely unstained heads were considered viable.

4.4 Inseminations

In field trials, the cows (I-III) were inseminated in natural heat according to standard practice by trained inseminators. Similarly, the sows were inseminated in naturally occurring standing heat (IV-V). The sows were inseminated by farm owners (IV) or by technicians employed on the farm (IV-V). All experiments lasted at least a year to avoid bias caused by seasonal variation in fertility.

Frozen bull semen (I-III) was distributed throughout the country regardless of the AI station at which it was produced, and semen was used for inseminations only within the breed. The number of spermatozoa per straw was about 18 x 10^6.

In study IV, the age and breed-matched boars were randomly divided into two groups, with inseminations of 2 x 10^9 and 3 x 10^9 spermatozoa / dose, respectively. A sow was inseminated an average of 1.5 times / estrus.

In study V, the sows were transported to the nucleus herd on the day of weaning and housed in groups of 40 sows. After arrival at the nucleus herd, sows were checked for estrus symptoms twice a day. When in standing heat, sows were randomly allocated into either a uterine insemination group or a standard AI group and bred accordingly. In both treatment groups insemination was repeated once using semen from the same boar batch if the sow was still receptive 24 hours later. The sows were excluded from the study if not in estrus by day 6. The same technician performed both inseminations and the two technicians rotated intrauterine and traditional AI. In intrauterine AI, semen was deposited in the body of the uterus, whereas the caudal part of the cervix was used as the deposition site in the traditional AI. The catheters used were Verona (Minitüb, Tiefenbach, Germany) for the intrauterine AI and Goldenpig (IMV Technologies, L'Aigle Cedex, France) for the traditional AI. In both treatments 3 x 10^9 spermatozoa were included in each heterospermic dose.

4.5 Fertility parameters

Fertility was determined in clinical trials as the 60 day non-return rate (NR%) in dairy cows (I-III), and as the farrowing rate and litter size (total number of the piglets born / litter) in gilts and sows (IV-V). Fertility data in all experiments were obtained from the Agricultural Data-Processing Centre Ltd., Vantaa, Finland.

As in Norway (Refsdal 2007), a year in Finland can be divided into four seasons based on the fertility of the cows: a good season in summer to autumn (June to October), two intermediate seasons, one in spring (April to May) and another in late autumn (November to December), and a poor one in winter (January to March) (personal communication, Finnish Animal Breeding Association FABA). In sows seasonal infertility occurs in Finland during late summer-early autumn (August-October) (Peltoniemi et al. 1999). These seasonal variations in fertility are taken into account in statistical models in the experiments.

4.6 Mycotoxin investigations

Feeding of the macroscopically suspicious hay to bulls (II) began on July 7. On October 17, the moldy hay was discarded and feeding with new hay of better quality was started.

The surface of the contaminated hay was sampled onto Scotch Tape® and inspected with bright field light microscopy at x 1000 magnification.

A composite sample of hay from many hay bales was dissolved in acetonitrile and screened for the presence of T-2 toxin or zearalenone with a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) (Toxiklon, Agricultural Biotechnology Center, Gödöllö, Hungary) as described by Barna-Vetro et al. (1994). Testing was done for T-2 and zearalenone in 3 and 2 replicates, respectively. The measurement ranges of the tests were 100 to 2000 ng / g for T-2 and 25 to 400 ng / g for zearalenone. The T-2 ELISA test cross-reacts with HT-2 toxin (Barna-Vetro et al. 1994).

One sample of hay was also analyzed for *Fusarium* mycotoxins at the Finnish Food Safety Authority (Evira, Helsinki, Finland) using gas chromatography-mass spectrometry (GC-MS). The detection limit for all toxins tested was 20 ng / g.

4.7 BRSV studies

At the beginning of 2000, a severe BRSV epizootic occurred in Finland, with even adult dairy cattle dying of pneumonia (Veijalainen et al. 2000). In February 2000, the first outbreak of upper respiratory disease manifested as high fever, cough and nasal discharge in the quarantine area of the bull rearing station (III). Seven subsequent bull groups in quarantine were included in the study; four of these became infected, while the remaining three groups showed no signs of respiratory disease.

All young bulls from the above-mentioned seven subsequent quarantine groups that had passed the standard breeding soundness evaluation [i.e. physical examination, libido and semen quality (volume, motility, morphology)] at the AI stations were included in the study. When the animals were at the age of 14 ± 1 months, blood samples were taken for BRSV antibody testing. In addition, 68 older bulls already at the AI stations were randomly selected and sampled for BRSV antibodies. None of the old bulls showed any signs of respiratory disease. All antibody testing was done at the Finnish Food Safety Authority (Evira), Department of Virology (Helsinki, Finland), using a SVANOVIR RSV ELISA (SVANOVA Biotech AB, Uppsala, Sweden) method (Veijalainen et al. 2000). Eleven bulls (12.2%) did not pass the breeding soundness evaluation, mainly because of poor semen quality (motility, morphology). One of these eleven bulls was azoospermic and had moderate adherences between the testis and *tunica vaginalis*.

4.8 Statistical analyses

The bull station, the season, and the breed served in statistical analyses (I) to explain variation in the number of sperm and in the percentage of viable sperm, as well as in the number of viable sperm in insemination doses. These factors, as well as the insemination number and parity, served to explain variation in NR%. When significant residual correlation existed between sperm parameters and non-return rates, those sperm parameters were included in the model as regressions to reveal their effect on NR%. For

statistical analyses, the Least-Squares (LS) procedure was used (Harvey 1960) and means were expressed as LS means and standard error of means.

Statistical analyses (II) were carried out with the Statistix statistical software package, version 1.0 (Analytical Software, Tallahassee, FL). The Mann–Whitney *U*-test was used to compare morphology between young bulls in October 1998 and young bulls from previous years, and the Wilcoxon signed rank test for morphology in young bulls during different months.

The serological status for BRSV, bull station, insemination number (first vs. later inseminations), parity (heifers vs. cows) and season (month of insemination) as well as station by BRSV status and season by BRSV status interactions served as variables in statistical analyses (III) to explain variation in NR%. The LS procedure was used, and results were expressed as LS means and standard error of means. The T-test was applied to compare morphology between BRSV seropositive and negative young bulls at the age of 14 months.

Table 1 Summary of trials.

Study	Inseminations (n)	Species	Breed	Parameter(s) of interest	Statistical model	Aim
I	92 120	cattle	Ay, Fr	viability, NR%	LS	novel assay
II	-	cattle	Ay	motility, morphology	*U*-test, signed rank test	trichothecenes
III	128 299	cattle	Ay	morphology, NR%	LS, t-test	BRSV infection
IV	45 562	swine	L, Y	NR%, litter size, morphology	logistic & linear regression	AI dose and sperm morphology
V	326	swine	hybrids (L; Y)	farrowing rate, litter size	logistic & linear regression	transcervical AI

Ay=Ayrshire, FR=Holstein-Friesian
L=Landrace, Y=Yorkshire
LS=Least-Squares

Multiple regression models were used (IV) to assess the effect of the dose on fertility parameters. In the case of non-return rate, a logistic regression model was built, including dose, breed and semen characteristics as explanatory variables and the non-return rate as the outcome variable (R = 0.65). To study the effect of AI dose on fertility parameters, two separate linear regression models were used for first parity sows and older sows. In

both cases, the model included dose, breed and semen characteristics as the explanatory variables and the non-return rate as the outcome variable (R = 0.45 for the first parity model and R = 0.41 for older parities). No interactions for the variables were noted. Furthermore, the analyses indicated no collinearity problem between the variables. For the analyses, SPSS (SPSS Inc., Chicago, USA) version 11.0 was used.

Sample size (V) was calculated based on the following assumptions. Expected difference in the means of the groups was set at 0.8 piglets (11.8 vs. 11.0 live born piglets / litter was assumed for the two treatment groups). Level of confidence was set at 95%, power of the study at 0.8 and expected standard deviation at 2.9 piglets / litter. Using these assumptions, an independent sample size of 165 sows per group was required in order to detect a significant difference between the treatment groups.

Using farrowing rate (farrowed or not) and live-born litter size (normal distribution checked for and found, repeat breeders included) as the outcome variables, a logistic and linear regression approach, respectively, was chosen to study the effect of the following factors: treatment, AI operator, breed, satellite herd preceding weaning, parity, weaning-to-estrus interval and length of lactation. For an effect to be included in the model, a conservative 0.2 level of significance was applied. All analyses were carried out using SPSS 13.0 for Windows (Lead Technologies, inc., U.S.).

5 Results

5.1 Fluorometric studies (I)

The fluorometric analyses were done in three replicates and twice for each batch. The method was precise and accurate when compared to fluorescence microscopy. In a comparison test the percentages (±SEM) of viable bull spermatozoa detected by the fluorometer (67.5 ± 1.48) and by the microscope (67.8 ± 1.34) were almost identical.

The raw means of the sperm numbers and the overall NR% are given in Table 2.

Table 2 Summary statistics for semen and fertility parameters of 436 bull semen batches.

	Mean ± SD	Min	Max
Sperm viability (%)	64.8 ± 7.9	33.8	81.7
Total number of sperm/dose (x 10^6)	18.2 ± 2.9	10.1	26.2
Number of viable sperm/dose (x 10^6)	11.8 ± 2.2	5.71	19.0
NR%	71.1 ± 10.5	27.3	97.3

As for seasons, the NR% differed significantly, reaching its lowest value in winter (70.0 ± 0.47) and highest in summer (75.3 ± 0.54) ($P < 0.001$).

A significant residual correlation existed between NR% and number of viable sperm in an insemination dose ($R = 0.051$, $P = 0.016$). The correlation between NR% and viability of spermatozoa ($R = 0.046$, $P = 0.027$) was also significant, albeit not significant between NR% and total number of spermatozoa per dose ($R = 0.03$, $P = 0.147$).

An increase of one million viable spermatozoa in an insemination dose increased NR% by 0.2%, $P = 0.016$. An increase of 1% in the rate of viable spermatozoa increased NR% by 0.05%, $P = 0.027$.

5.2 Effect of trichothecenes in the hay (II)

Many species of fungi had contaminated the hay, *Fusarium* spp. being some of the most frequent. In the immunoassay, T-2 toxin was detected in concentrations of 220, 300, and 600 ng / g. This was confirmed with monoclonal ELISA tests. One composite sample of hay, when analyzed using gas chromatography–mass spectrometry (GC-MS), showed elevated levels of T-2 toxin (47 ng / g) and very high amounts of HT-2 toxin (570 ng / g). No zearalenone toxin was detected with any of the methods used.

Feeding of the moldy hay lasted from July 7 to October 17. The decrease in sperm quality, as detected by low progressive motility and poor morphology persisted several months after withdrawal of the moldy hay. The percentage of rejected ejaculates was significantly increased over that of the previous year (Figure 1).

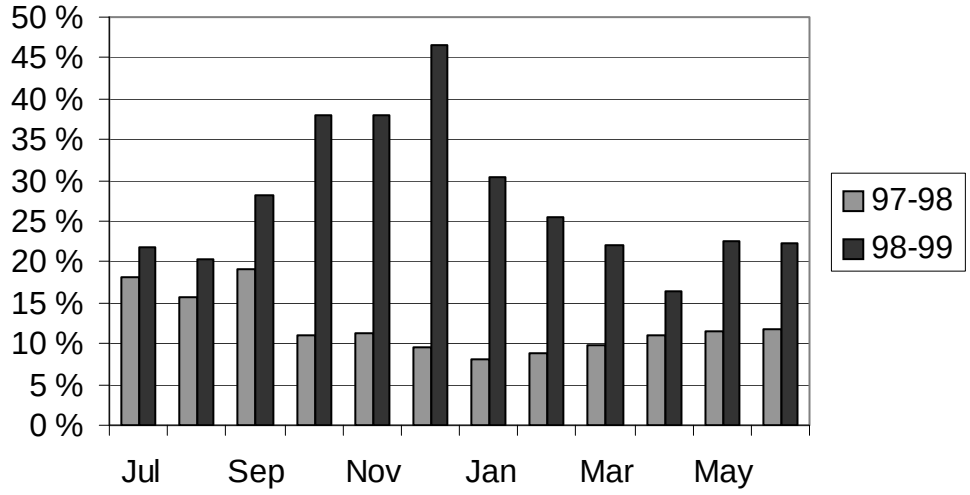

Figure 1 The average percentage of rejected bull ejaculates from July 1997 to June 1999.

Analyses of sperm morphology revealed that the ejaculates from October to December 1998 were inferior to those in breed- and age-matched bulls at the same AI station during previous years (Table 3). All forms of abnormal spermatozoa increased in number.

The number of spermatozoa per straw was about 18 x 10^6. Total number of spermatozoa in the accepted ejaculates remained unaffected, as did the fertility of accepted semen batches evaluated by the 60-day non-return rate.

Table 3 Sperm morphology in young bulls in October and December 1998 (n = 29) and in previous years (n = 103) at the same AI station.

	previous years	October 1998	December 1998
Normal spermatozoa (%)	84.0 ± 9.1	71.0 ± 13.6	77.3 ± 14.4
Major sperm defects (%)	8.3 ± 4.3	19.5 ± 11.0	13.8 ± 9.6

Data presented as mean ±SD. All values within a row differ significantly ($P < 0.01$).

5.3 Effect of acute BRSV infection (III)

At the AI station, 54 out of the 79 young bulls had positive BRSV antibody titers at the age of 14 months. In general, either all or 0-2 of the young bulls in a quarantine group became infected based on seropositivity to BRSV. Of the randomly sampled 68 older bulls at the AI stations, 59 had negative BRSV antibody titers, and only 9 old bulls were seropositive.

The NR% differed significantly ($P = 0.014$) between BRSV seronegative and seropositive young bulls, being 76.9% and 75.2%, respectively. Bulls at Station 1 showed better fertility; overall NR% was 76.7% vs. NR% 75.4% for bulls at Station 2. However, the negative influence of BRSV infection on NR% was similar at both AI stations. Other factors in the statistical ANOVA model having an effect on NR% were insemination number (first vs. later inseminations), parity (heifers vs. cows) and season (month of insemination) as well as interactions of station by BRSV and insemination month by BRSV.

Sperm morphology in BRSV-negative bulls was significantly better than in seropositive animals, the proportions of normal spermatozoa being 81.2% and 74.1%, respectively ($P = 0.035$). All classes of morphological abnormality were slightly elevated, although not significantly.

5.4 Comparison of two insemination doses and effect of morphology (IV)

The non-return rate in the 96 boars using the normal insemination dose of 3×10^9 spermatozoa (group B) was 84.0% compared with 75.8% in the 50 boars using a dose of 2×10^9 spermatozoa (group A) ($P < 0.001$). The corresponding size for primiparous litters was 10.7 versus 10.1 piglets ($P < 0.001$), and for multiparous litters 12.1 versus 11.7 piglets ($P < 0.001$). The results are summarized in Table 4.

When comparing those boars having ≥ 70% morphologically normal spermatozoa, the non-return rate in the 84 boars used with the normal insemination dose of 3×10^9 spermatozoa was 84.3% compared with 77.7% in the 46 boars using a dose of 2×10^9 spermatozoa ($P < 0.001$). The corresponding litter size for primiparous litters was 10.7 versus 10.2 piglets ($P < 0.001$), and for multiparous litters 12.2 versus 11.8 piglets ($P < 0.001$).

With the smaller insemination dose (group A), sperm morphology (percentage of normal spermatozoa) and all three fertility parameters were strongly correlated in the whole dataset ($P < 0.01$). For group B (normal insemination dose), the percentage of normal spermatozoa correlated with NR% ($R = 0.223$, $P < 0.05$), but there was no correlation with litter size.

When only the boars with more than 70% normal spermatozoa were included there were no correlations between the percentage of normal spermatozoa and any of the three fertility parameters, regardless of insemination dose. However, the negative correlation

between the percentage of major sperm defects and litter size of multiparous farrowings remained significant, although the poorest boars by sperm morphology were excluded.

Table 4 Summary of boar semen and fertility parameters, presented as means ± SD.

	Group A (2×10^9)	Group B (3×10^9)	P
Non-return rate %	75.8 ± 8.8	84.0 ± 3.7	< 0.001
Litter size of primiparous farrowings	10.1 ± 1.0	10.7 ± 0.6	< 0.001
Litter size of multiparous farrowings	11.7 ± 0.7	12.1 ± 0.6	< 0.001
Normal spermatozoa %	85.6 ± 15.3	85.2 ± 13.1	
Major sperm defects %	9.9 ± 14.7	5.5 ± 6.1	
Minor sperm defects	4.4 ± 4.5	9.4 ± 10.9	

5.5 Intrauterine (transcervical) insemination (V)

The intrauterine AI catheter was successfully passed through the cervix in 157 / 160 of the females (98.1%) at least at the second attempt (9.5%). Inserting the inner catheter took an extra minute. However, infusion of the AI dose was more rapid in the intrauterine AI so that for the whole insemination procedure, the intrauterine AI lasted for only an average of 22 seconds longer than the traditional AI. No blood was detectable on the uterine AI catheter after performing the AI.

Overall, live-born litter size was 11.3 ± 2.9, repeat breeding rate 4.2% and farrowing rate 91.2%. In the intrauterine insemination group, 93.6% of inseminated sows farrowed, whereas farrowing rate for the conventional insemination group was 88.8% (P = 0.13). Intrauterine insemination with a standard AI dose did not result in a significant improvement in the live-born litter size (11.5 ± 2.8 and 11.1 ± 3.0, respectively, P = 0.13).

The preceding satellite herd had a highly significant effect on the live-born litter size (12.4 ± 2.6; 11.1 ± 2.9; 10.8 ± 2.9 and 10.9 ± 2.9 for the four satellite herds, P < 0.01).

6 Discussion

6.1 General discussion

AI is a reproductive technology that has made possible the effective use of best males, thus greatly improving the genetic quality of breeding herds. A prerequisite for the optimal use of this genetic material is to obtain acceptable fertility after AI. At high sperm numbers per AI dose bulls differ in their maximal NR%. That is unrelated to the rate at which they approach this maximum (Saacke et al. 1994; den Daas et al. 1998). Therefore, screening of the semen for normality and evaluation of fertility is essential both to farmers and the AI industry.

Prediction of fertility is the primary goal of ejaculate examination. The concept of the male fertility is as complex as that of the female. Thus it is easier to find males and/or ejaculates with inferior fertility than to predict the exact fertility of a given sample. The *in vitro* semen evaluation, complementary to the clinical examination, is of high diagnostic value for assessing testicular and epididymal function, and/or the genital tract of the male, allowing elimination of clear-cut cases of infertility, or even potential sub-fertility (Martin Rillo et al. 1996; Rodriguez-Martinez 2003; Saacke 2008). Unfortunately, neither a simple semen analysis nor the routine post-thaw evaluation will enable the determination *a priori* of the potential fertility level that the analyzed semen will reach, particularly after AI. The usefulness of these parameters to accurately measure fertility of a semen sample is controversial (Januskauskas et al. 1996; Correa et al. 1997).

For this thesis different aspects of semen quality and its evaluation were studied. Furthermore, the ultimate outcome of fertility, production of offspring, was the end-point in all five research projects included in this thesis.

6.2 Semen evaluation/fluorometric method

The fluorometric plasma membrane integrity assay (I) provided additional information to the routinely used sperm concentration and subjective motility evaluation in semen screening at AI stations. The new method was considered simple, objective, and rapid, and suitable for quality control. However, it was not effective in rejection of ejaculates in daily semen production because the regression between the percentage of viable spermatozoa and field fertility was negligible. The reasons might be the pre-selection of the semen which reduces the variability among ejaculates used, or the relatively high number of spermatozoa per insemination dose that may mask their functional weaknesses (Tardif et al. 1999), so-called compensable deficiencies (Saacke 2008).

The correlation between sperm viability after thawing and fertility was demonstrated earlier (Januskauskas et al. 2000). However, calculation of fluorescent cells in the microscope is time consuming, subjective and less accurate (Woelders 1991), and therefore not suitable for routine assays of high numbers of samples. Fluorometric plasma

membrane integrity measurements provide a rapid and easy alternative for semen quality control. However, after our study, the increased cost-effectiveness of a newly launched flow cytometer in semen evaluation has made it the method of choice in many sperm laboratories.

6.3 Trichothecenes

The decrease in semen quality caused by trichothecene mycotoxins (II) was manifested as impairment of initial and post-thaw progressive motility. The impairment began gradually 1.5 to 2 months after ingestion, and probably also inhalation, of moldy hay, and the semen remained of low quality for about five months after the end of feeding with moldy hay. The lowest semen quality (lowest proportion of ejaculates accepted for use in AI) was observed in December 1998, i.e., about two months after the cessation of this feeding. These findings indicate that the mycotoxins in the hay may have increased during storage, and that they exert their main effects at an early stage of spermatogenesis that takes approximately 62 days (Barth and Oko 1989).

Epoxytrichothecenes, like T-2 and HT-2 toxins, inhibit protein as well as DNA and RNA synthesis (Ueno 1985), injuring organs with rapidly dividing cell populations such as germinal epithelium in testis (Ueno et al. 1984). Furthermore, T-2 is a potent inducer of apoptosis (Ihara et al. 1997). T-2 inhibited testosterone synthesis and secretion (Fenske and Fink-Gremmels 1990) in gerbils and guinea pigs, and induced degeneration and necrosis of spermatogenic cells in the semiferous tubules (DeNicola et al. 1978).

Ruminants are generally less susceptible to trichothecene mycotoxicosis than monogastric animals. The metabolism of ingested material by the ruminal microbes may be considered as a first line of defense against toxic materials present in the diet. On the other hand, ruminants may be at a disadvantage if substances become toxic as a result of the action of ruminal microbes. Another characteristic of trichothecene toxicity is the high susceptibility of animals, when exposed via the inhalation route (Ueno et al. 1984), probably because of passing by the intestinal biodegradation/defense mechanisms.

The concentration of HT-2 toxin (570 ng / g) was markedly high in the hay as measured in both chemical and immunochemical assays. For comparison, Eskola et al. (2001) found HT-2 in the concentration range 10-20 ng / g in Finnish cereal samples. The daily amount of ingested mycotoxin certainly varied as a result of the probable uneven distribution of toxins in the hay.

Mycotoxins such as diacetoxyscirpenol, aflatoxin B1, zearalenone, and ochratoxin A have been blamed for deterioration of semen quality, for example, increased abnormalities in sperm morphology and decrease in motility (Haase et al. 1983; Conner et al. 1990; Sinha and Dharmshila 1994; von Lange et al. 1998; Solti et al. 1999; Tsakmakidis et al. 2006). Fenske and Fink-Gremmels (1990) reported that T-2 inhibits testosterone secretion *in vitro*. However, HT-2 and T-2 toxins have not earlier been implicated in impairment of semen quality *in vivo*. The effects of low trichothecene contents on testicular function and spermatozoa remain to be fully evaluated in an experimental design.

6.4 BRSV

BRSV is often underestimated in animals older than 6 months, but our results (III) clearly show the influence of BRSV epizootics on sperm quality and field fertility in young AI bulls. Despite the usually mild disease in older animals, it may cause economic losses to farmers and AI organizations due to sub-fertility and need for early culling of bulls.

The mode of transmission in natural infection has not been confirmed, but direct contact is probably necessary (Larsen 2000). Available data to date do not allow conclusions to be drawn on the length of shedding of the virus and investigations are lacking on the length of quarantine required to avoid transmission to susceptible animals. However, quarantine of young bulls proved to be effective; at the AI stations none of the mature bulls fell ill or showed any decline in semen quality, and almost all remained seronegative for BRSV. It is not known exactly how long antibodies against BRSV remain in sera of adult cattle, but it is months or even years without re-infection (Elvander 1996), confirming the disease-free status of older bulls.

BRSV infection in young (6-12 months old) bulls seems to increase testicular fibrosis (Barth et al. 2008) by an unknown mechanism that might have some immunological background, similar to the pathological changes in lungs caused by the BRS virus (Viuff et al. 2002; Valarcher and Taylor 2007) where fibrosis is common (Kimman et al. 1989; Ellis et al. 1996). Barth et al. (2008) suggested that during the active process leading to fibrosis of the testicular tissue, spermatogenesis is adversely affected, and this is later seen as poor sperm morphology. This is in accordance with our findings during and after BRSV epizootics. The old bulls instead, having had BRSV infection long ago but being still seropositive, had normal sperm morphology, as expected, since no ongoing harmful process or acute infection affected sperm production in testicles, contrary to the case of young BRSV infected bulls.

6.5 Seasonality

Although often suspected, it became clear from these studies (I-V) that both cows and sows still show a tendency to be seasonal breeders despite domestication and active breeding. There were seasons with lowered fertility, although not total infertility. This phenomenon was earlier described for sows in Finland (Peltoniemi et al. 1999; Tast et al. 2002), and for cows in Norway (Refsdal 2007), where a marked heat stress during the summer months is not a problem/confounding factor. The seasonal effect in reproduction has been shown to be caused by variation in day length and mediated by melatonin (Malpaux et al. 2001; Tast et al. 2001; Thiery et al. 2002).

6.6 The effect of sperm morphology and insemination dose

The importance of normal sperm morphology in bulls, goats, stallions and men is well known. These studies confirm the significance of sperm morphology also in AI bulls and boars; earlier studies with this pre-selected material have been somewhat controversial (Graham 2001; Rodriguez-Martinez 2006). The controversy might be explained by the quite high numbers of sperm that are often used for insemination, even though we were able to find the effect also with higher sperm numbers (IV) in accordance with Saacke (2008). However, sperm morphology is not sensitive enough to rank males within "normal" limits for fertility.

The effect of poor semen morphology is multiplied when the insemination dose decreases and otherwise compensable morphological deficiencies can no longer be overcome (Althouse 1997a; Althouse 1997b; Althouse 1997c; Johnson 1997; Althouse et al. 1998; Saacke et al. 2000). This leads to decreased fertility rates and litter sizes in pluriparous animals (Althouse 1997a). The recommended total sperm count in an insemination dose ranges from 1.5 or 2 x 10^9 to 6 x 10^9 for boars (Colenbrander et al. 1993; Martin Rillo et al. 1996; Althouse 1997a; Althouse 1997c). With the routinely used insemination dose of 3 x 10^9, the number of spermatozoa is not a limiting factor and field trials may not detect small differences (Woelders 1991), which agrees with our results (IV). Some characteristics of spermatozoa, including chromosomal aberrations, DNA damage or aberrant chromatin structure, cannot be compensated for by increasing the number of spermatozoa, but affect the pregnancy rate and litter size at all insemination doses (Woelders 1991; Evenson 1999; Saacke et al. 2000). Other characteristics (most classical parameters) of spermatozoa affect their ability to reach and fertilize oocytes, and the increasing number of spermatozoa in the insemination dose can therefore improve the chances of success. The morphological classification introduced by Blom (1983) is well suited for practical purposes to detect different sperm populations in a sample, not only in bulls, but also in horses (Jasko et al. 1990) and boars, allowing its use to determine an optimal insemination dose for a male.

Conducting a semen morphology examination once before entering boars into AI use, as suggested by Larsson et al. (1980), proved to be beneficial in predicting the suitability of a young boar for breeding (IV). In the case of adult boars, repeated examinations are recommended (Woelders 1991; Colenbrander et al. 1993; Martin Rillo et al. 1996; Althouse 1997a; Althouse 1997b), but the optimal examination frequency remains unclear. This applies to AI bulls as well (Al-Makhzoomi et al. 2008). Semen morphology has a limited positive predictive value for field fertility, especially in pre-selected (for motility, concentration) samples. It does, however, help to screen-out overtly poor-quality ejaculates (Althouse 1997b). It is quite easy and inexpensive to perform, not only in a reference laboratory, but also in AI stations, although it requires some experience.

6.7 Intrauterine insemination in sows

With the standard insemination dose of 3 x 10^9 spermatozoa, both litter size and farrowing rate in sows were unaffected by intrauterine insemination (V), so the method did not lead to a significant improvement of fertility using such a high dose. Watson and Behan (2002) reported a slight reduction in fertility using intrauterine AI with 1 x 10^9 spermatozoa / dose compared with 2 or 3 x 10^9 spermatozoa / dose. We did not compare the effect on fertility of smaller insemination doses, but at least when using 3 x 10^9 spermatozoa, no additional benefit ensued in comparison with traditional AI.

The duration of insertion of the uterine AI catheter was only slightly longer than with the traditional method, as reported earlier (Watson and Behan 2002). Simultaneously, we also observed that the time to dispose the AI dose was shorter with the intrauterine than with the traditional catheter, so that the whole AI procedure lasted only 22 seconds longer using the intrauterine catheter. In principle, insertion of the inner catheter through the cervix, in addition to the traditional AI catheter, may provide additional cervical stimulation that appears to induce further myometrial contractility (Langendijk et al. 2005). This may explain the faster infusion time of the uterine AI dose found in the present study.

The uterine AI seems to be a practical and feasible method of inseminating the sow and the time required to pass the cervix with the catheter appears not to be a major obstacle either. It might be most profitable when maximizing the use of the superior boars.

6.8 Summary

The quality of semen used for AI is crucial to its outcome. Assessment of *in vivo* fertilizing capacity of semen presents a challenge because it is influenced by many different sources a variation, which may or may not be determinable (Amann and Hammerstedt 2002). Despite the limitations of large-scale field trials, they are still the only way to study the efficacy of various treatments under commercial conditions that laboratory assessment cannot accurately substitute for.

This thesis focuses on different aspects affecting male reproduction. In the research projects we developed a new method for sperm viability determination, studied the deleterious effects of trichothecenes in feed and BRSV infection on sperm quality as well as studied the importance of semen quality and insemination dose for reproductive success.

7 Conclusions

I) We conclude that routine fluorometric studies can be cost-effective to the AI industry in semen quality control.

II) We recommend that only hay and grain of good quality be used for valuable breeding animals. In doubtful situations, screening with monoclonal ELISA-assays for mycotoxins should be performed.

III) The avoidance of infectious diseases is critical for AI studs, and even "milder" diseases cannot be neglected.

IV) Based on our findings, we suggest routine morphological examinations for boars and bulls intended for AI use before they are taken into the regular collection scheme, and regular examinations, e.g. once a year, thereafter.

V) The number of spermatozoa per insemination dose should be accurately determined and set low enough in field trials to detect differences between males. In standard use the total number of spermatozoa in boars should exceed 3×10^9 per insemination dose.

VI) Intrauterine insemination in sows is easy and fast to perform, but may not provide additional benefit with the traditional AI dose of 3×10^9.

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