Eesti Maaülikool
Estonian University of Life Sciences

FACTORS INFLUENCING THE QUALITY OF SEMEN FROM ESTONIAN HOLSTEIN AI BULLS, AND RELATIONSHIPS BETWEEN SEMEN QUALITY PARAMETERS AND *in vivo* FERTILITY

EESTI HOLSTEINI TÕUGU SUGUPULLIDE SPERMA KVALITEET, SEDA MÕJUTAVAD TEGURID NING SEOS *in vivo* VILJAKUSEGA

PEETER PADRIK

A thesis
for applying for the degree of Doctor of Philosophy
in Agricultural Sciences

Väitekiri
filosoofiadoktori kraadi taotlemiseks põllumajandusteaduste erialal

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Opponent: **Kasia Kupisiewicz, PhD**
VikingGenetics

Supervisors: **Professor Ülle Jaakma, PhD**
Estonian University of Life Sciences

Professor Emeritus Olev Saveli, D.Sc.
Estonian University of Life Sciences

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Vivere est cogitare
(Elada tähendab mõtelda) Cicero
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To my mother and
in memory of my father

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on five original publications referred to by Roman numerals in the text. The papers in the thesis are reproduced with the kind permission of the publishers.

I. **Padrik, P.**, Hallap, T., Bulitko, T., Jaakma, Ü. 2010. Sügavkülmutatud/sulatatud spermide kvaliteedinäitajate seos sesoonsuse ja sugupulli vanuse ning emasloomade tiinestumisega. *Agraarteadus* XXI (1-2), 38–46.

II. **Padrik, P.**, Hallap, T., Bulitko, T., Januskauskas, A., Kaart, T., Jaakma, Ü. 2010. The quality of frozen-thawed semen of young A.I. bulls and its relation to the grade of Holstein genes and fertility. *Veterinarija ir Zootechnika* T. 50(72), 59–65.

III. **Padrik, P.**, Hallap, T., Bulitko, T., Kaart, T., Jaakma, Ü. 2012. Relationships between the results of hypo-osmotic swelling tests, sperm motility and fertility in Estonian Holstein dairy bulls. *Czech Journal of Animal Sciences* 57(10), 490–497.

IV. **Padrik, P.**, Hallap, T., Bulitko, T., Januskauskas, A., Kaart, T., Jaakma, Ü. 2012. Conventional laboratory test and flow cytometry in the prognostic testing of bull semen fertility. *Veterinarija ir Zootechnika* T. 60(82), 52–58.

V. Kurykin, J., Hallap, T., Jalakas, M., **Padrik, P.**, Kaart, T., Johannisson, A., Jaakma, Ü. 2016. Effects of insemination-related factors on pregnancy rate using sexed semen in Holstein heifers. *Czech Journal of Animal Sciences* 61(12), 568–577.

The contribution of the authors to the articles

Paper	Original idea and structure of paper	Data collection, sample analysis	Data analysis	Preparation of manuscript
I	**PP**, ÜJ, TH	**PP**, TH	**PP**, ÜJ, TH	All
II	**PP**, ÜJ, TH	**PP**, AJ, TH	**PP**, ÜJ, TH, TK	All
III	**PP**, ÜJ, TH	**PP**, TH	**PP**, ÜJ, TH, TK	All
IV	**PP**, ÜJ, TH	**PP**, AJ, TH	**PP**, ÜJ, TH, TK	All
V	JK, TH, MJ, TK, ÜJ, AJo	**PP**, JK, TH, MJ, TK, ÜJ, AJo	JK, TH, MJ, TK, ÜJ	All

PP – Peeter Padrik, ÜJ – Ülle Jaakma, TH – Triin Hallap, TB – Tanel Bulitko, TK – Tanel Kaart, AJ – Aloyzas Januskauskas, JK – Jevgeni Kurykin, MJ – Mihkel Jalakas, AJo – Anders Johannisson, All – all the authors of the paper

ABBREVIATIONS

AI	artificial insemination
ALH	amplitude of lateral head displacement
AO	acridine orange
ATP	adenosine triphosphate
BCF	beat cross frequency
CASA	computer-assisted sperm analyser
DMSO	dimethyl sulphoxide
$COMP\alpha_t$	cells outside the main population
%DFI	DNA fragmentation index
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetic acid
EHF	Estonian Holstein breed
FACS	fluorescence-activated cell sorter
FC(FCM)	flow cytometry
FSC	forward scatter
FT	frozen-thawed
GMot	general motility
GAG	glycosaminoglycan
h^2	heritability coefficient
HOT-1	hypo-osmotic swelling test 1
HOT-2	modified hypo-osmotic swelling test 2
HOT-3	modified hypo-osmotic swelling test 3
ΔHOT-2	HOT-2 result
ΔHOT-3	HOT-3 result
LIN	linearity
LSM	live stable membrane
MMP	mitochondrial membrane potential
MTDR-H	high mitochondrial activity
MTDR-L	low mitochondrial activity
NRR	non-return rate
PMot	progressive motility
PNRR	predicted non-return rate
PT	post-thaw

SCSA	Sperm Chromatin Structure Assay
SD	standard deviation
SP-TALP	modified Tyrode´s albumin, lactate and pyruvate solution
SS	sexed semen
SSC	side scatter
SubMot	subjective motility
VAP	average path velocity
VCL	curve line velocity
TNE	TRIS, NaCl and EDTA
TRIS	tris(hydroxymethyl)aminomethane
VSL	straight line velocity
WOB	wobble (VAP/VCL)

Abbreviations in Estonian/*Lühendid*

CASA	spermide liikuvuse kompuuteranalüüs
DFI%	DNA fragmentatsiooniindeks
ESM	elusad stabiilse membraaniga spermid
FCA	voolutsütomeetriline analüüs
HOT	Hüpo-osmootne test
HOT-2	modifitseeritud hüpo-osmootne test 2
HOT-3	modifitseeritud hüpo-osmootne test 3
ΔHOT-2	modifitseeritud hüpoosmootse testi 2 tulemuse väljendus
ΔHOT-3	modifitseeritud hüpoosmootse testi 3 tulemuse väljendus
SMA	suure mitokondriaalse aktiivsusega spermid
LS	liikuvad spermid
OLS	otseliikuvad spermid
SKA	spermide kõrvalekaldeamplituud liikumistrajektoorist
SKL	spermide kiirus liikumisteekonnal
SKS	spermide kiirus sirglõigul,
SKT	spermide kiirus trajektooril
SOL	spermide otseliikuvus (SKS/SKL)
SubL	subjektiivselt hinnatud spermide liikuvus
VLS	võnkuva liikumisega spermid (SKT/SKL)
VMA	väikese mitokondriaalse aktiivsusega spermid

INTRODUCTION

At present, dairy farming is the most profitable sector of Estonia's cattle industry. The country has a remarkably long history of cattle raising and breeding. A thousand years before Roman historian Cornelius Tacitus (55-117 AD) called Estonians '*Aests*' which means 'fire holders' (Meri, 1976) in his book Germania, these people had the words like 'cow', 'milk' and 'butter' in their language. These words derive from the era of development of Estonians in about 1000 BC (Libe et al., 1932) which means they are much younger than livestock keeping in the region. Cattle were raised as early as in the late Stone Age about 3000 BC. With the expansion of Western spirituality (the establishment of *Academia Gustaviana*) and implementation of innovative agricultural equipment (the heavy reversible plough) in the 16th and 17th centuries (Talve, 2004), new cattle breeds started spreading in Estonia. Black-and-white cattle were first introduced to Estonia (Purtse manor) from the Netherlands in 1624. Pedigree records of these cattle were discovered in 1885. Herdbook keeping has significantly contributed to selective breeding. Several useful techniques like artificial insemination, cryopreservation of semen, and the use of sexed semen that enable more effective use of valuable genetic material for improving the milk yield in dairy cattle, have developed rapidly since the mid-twentieth century.

However, the success in dairy farming is not merely due to the long, rich history of cattle breeding in this region, but rather due to the efforts of animal breeders, researchers and enthusiastic farmers, favourable climatic conditions, and a strong market for dairy products.

Effective milk production depends not only on the availability of valuable genetic material, but also on several other factors which include optimal calving intervals as well as the reproductive performance and health of cows. The quality of bull semen is among the factors that critically affects the pregnancy rate of cows and heifers. The assessment of fresh and frozen-thawed (FT) semen quality is crucial to attaining a high reproductive efficiency in each individual herd and the entire cattle population.

Evaluation of FT semen quality in the artificial insemination (AI) industry is often based on subjective assessment performed by experienced

professionals, not on using appropriate methods that guarantee objective outcomes. Although many AI laboratories are nowadays equipped with a computer-assisted sperm analyser (CASA) enabling objective measurement of motility, combined measurement of multiple sperm attributes could explain more precisely the variation in fertility among bulls than the variables of motility alone (Christensen et al., 1999). The use of fluorescent markers in combination with flow cytometry allows simultaneous assessment of several sperm parameters in thousands of sperm cells and has therefore become a valuable tool in semen quality assessment (Gillan et al*.,* 2005; Hua et al*.,* 2006; Sellem et al., 2015). Thus the developments in measurement techniques and assays have provided AI industry with a variety of objective tests for semen quality. The question is, however, if the traditional simple and cheap methods should be replaced by more precise but expensive methods, or could the latter rather be incorporated into a combined semen quality assay to increase the predictive value of the assessment. The single tests have not been sufficiently discriminative (Christensen et al*.,* 1999) because each of them measures only a single attribute necessary for the fertilization. Therefore, it would be rational to combine different parameters into a prediction model (Rodriguez-Martinez, 2006; Ahmed et al., 2016).

However, simple and cheap functional semen quality tests are still valuable at batch level. One of such simple tests is the hypo-osmotic swelling test (HOT) for the evaluation of sperm membrane integrity. The traditional HOT, originally presented by Jeyendran et al. (1984), enables determination of the functional intactness of sperm membranes as spermatozoa 'swell' under hypo-osmotic conditions due to the influx of water, and the expansion of the membranes causes the tails to coil. Several authors (Rodriguez-Martinez, 1998; Neild et al*.,* 1999; Imam et al*.,* 2008; Zubair et al., 2015) have emphasized the suitability of HOT for assessing the quality of fresh and frozen-thawed semen in different farm animal species.

It has to be determined if HOT alone or in combination with other tests can be used for routine semen quality testing by the AI industry.

Multiple other functional parameters, such as motility, stability of the membranes, and mitochondrial activity can be of significant predictive value. However, when predicting fertility, the possible effects of the

season of semen collection and the age of bulls on the quality of semen have to be considered.

Lately, there has been a growing interest in using sexed semen. However, the use of sexed semen has often resulted in decreased pregnancy rates. This can be due to lower sperm numbers per dose or reduced viability of sperm cells caused by the comparatively invasive sorting procedure (DeJarnette et al*.,* 2008, 2009; Schenk et al*.,* 2009). As the use of sexed semen is increasing, the studies on the quality and fertilizing ability of sexed sperms are of great practical importance for the dairy cattle industry.

Thus, bull fertility and high quality of semen are of utmost importance. Development of models for the prediction of male fertility is a continuous process. A smart combination of advanced analytical methods and simple traditional tests would offer great benefits for the industry. Among the multiple factors affecting the measurements, the age of bulls and the season of semen collection should be carefully examined. Also, additional sperm treatments, for example sex sorting before freezing of semen straws, may affect sperm quality and fertilizing ability.

There are relatively few scientific publications that investigate and suggest specific steps or tests that can be applied to assess the quality of spermatozoa when producing insemination doses in the AI industry. Moreover, the research results are often contradictory. For the AI industry, it is important to study semen quality tests that are relatively easy and inexpensive to implement in producing bull sperm AI doses. At the same time, it is necessary to strike a balance between the accuracy of the fertility assessment and the level of complexity, investments, and time commitment required for these tests.

In the present PhD dissertation, the relationships between the age of bulls, seasonal variation of semen quality, grade of Holstein genes (proportion of Holstein genes in the bull`s pedigree) and different sperm quality parameters (**I**, **II**, **III**) were studied. The sperm membrane intactness in Estonian Holstein AI bulls was determined using three different modifications of HOT, and the relationships between sperm quality parameters were revealed by conducting conventional AI laboratory tests and flow-cytometric tests (**III**, **IV**).

The relationships between the sperm quality parameters and field fertility estimated as non-return rates (NRRs) of cows and heifers (**I–V**) were determined. Mathematical models were constructed to estimate the potential fertility of young and mature bulls (**II**, **III**, **IV**). The structural and functional characteristics of flow-cytometrically sexed semen in comparison with unsexed semen of the same bulls were investigated (**V**).

1. REVIEW OF THE LITERATURE

1.1. Characteristics of the Estonian Holstein breed

The first recorded evidence of black-and-white cattle in Estonia dates back to 1624 when one breeding bull and seven cows were brought to Purtse manor (Karelson, Jaama, 1981). The breed was named Estonian Black-and-White in 1951 (Kurm, 1981), and renamed Estonian Holstein (EHF) in 1998. While in the 1960s the Estonian Black-and-White made up 30% of the dairy cattle population (Saveli, 1979), the breed expanded rapidly since the late 1970s, and by 2018, Estonian Holstein comprised 82.5% of the Estonian dairy cattle population (EPJ, 2015-2019). The popularity of the breed is due to its high milk productivity. While in the 1960s the Estonian Black-and-White cattle outperformed the Estonian Red by 200-300 kg in terms of the yearly milk yield per cow, the difference increased to 800 kilograms by the 1980s (Kaasiku, 2000), and is currently 1,356 kg (EPJ, 2015-2019). As a result of using Dutch breeding bulls, Estonian Black-and-White cattle became more compact, their muscularity improved, and milk productivity increased, but some udder problems remained.

Extensive use of American and Canadian Holstein bulls in Europe started in the 1970s. In 1961-1999, a total of 218 Holstein and Black-and-White bulls were imported to Estonia. The use of these bulls had a greatest positive impact on milk productivity. Additionally, improvement in udder shape and moderate improvement in leg condition as well as the shape of hooves were observed (Meier, 1977). Over the last five years, more than 130 young and mature Holstein bulls have been imported to Estonia from the Netherlands and Germany (Animal Breeders' Association of Estonia, database).

EHF cattle are tall and calm in nature. Herd instinct is not very strong, but both cows and bulls maintain well-developed herd hierarchy.

Holstein cattle have a high feed efficiency, but adequate forage base must be created to realise the breed's production capacity (Cromie et al., 1998). The EHF breeders aim for shaping a black-and-white cow with a yearly milk yield of over 10,000 kg that contains 4.4% fat, and more than

3.4% protein (EHF breeding programme). In 2018, these indicators were 10,059 kg, 3.88%, and 3.37%, respectively (EPJ, 2015-2019).

1.2. Development of artificial insemination technology

Farm animal breeding has undergone a spectacular development over the past 150 years. From the first herd books to the application of biotechnological methods, effective use of valuable breeding material has been the key objective of breeding programmes. Today, AI is an indispensable tool for securing successful breeding.

Sperms were first described by Antonij van Leeuwenhoek in 1677 in Delft, the Netherlands. The first successful artificial insemination experiments were performed by Lazzarro Spallanzani in 1784 (Jaakma and Jalakas, 2018).

Freezing of bull semen is possible thanks to Christopher Polge and his co-workers who discovered glycerol as a cryoprotectant in 1949. They worked out a feasible method for the preservation of bull semen in the frozen state at -79°C (Polge and Rowson, 1952). The freezing technology was further developed by Japanese researchers who used an extender containing sugar, egg yolk, and glycerol to dilute the semen (Nagase and Niwa, 1964).

During the last 20 years, much attention has been paid to the studies of producing calves of desired gender. One of the most successful methods of differentiating sperms carrying X or Y chromosomes is sorting them according to the deoxyribonucleic acid (DNA) content. The size of the X chromosome differs from that of the Y chromosome by 2-7%, whereas separation of X- and Y-chromosome-bearing spermatozoa is most effective when the difference in DNA is at least 3.5% (Johnson, 2000). The first reports on using DNA as a marker when differentiating sperms on the basis of X and Y chromosomes and getting descendants of desirable gender are from 1983 (Johnson et al., 1989). Dividing sperms into fractions carrying X and Y chromosome can be done using laser-based flow cytometry. Semen manipulated by using this technique have been frozen successfully, with 30-45% of motile sperm post-thaw (Seidel et al., 1999; Schenk et al., 2000).

In Estonia, the first AI experiments were performed by Edgar Keevallik at Kuusiku in 1938-1940. Cows were inseminated right after collecting semen from the bulls. The semen was undiluted and usable for six hours (Jaakma and Jalakas, 2018).

The period between 1948 and 1956 may be called the period of formation of AI stations. The collected semen was undiluted or diluted with a glucose extender and had to be used in 24 hours.

Since 1956, bulls with high breeding value have been used at the stations, and semen freezing and thawing has been applied. Cryopreservation of semen allowed the sperm to be used over a long period of time. Semen quality could be evaluated just before insemination and assessed at every step of the freezing process to adjust the semen handling regimen (Lass, 1996).

Currently, there is only one AI station in Estonia that belongs to the Animal Breeders' Association of Estonia and is located at Keava, Rapla County. There are 170 breeding bulls at the AI station, most of them genotyped (have genomic breeding values). About 500,000 insemination doses (straws) are produced annually, of which around 250,000 are sold to the Estonian farmers, and few thousands exported to Russia and Turkey.

1.3. Evaluation of fresh and frozen-thawed semen

The evaluation of the quantitative parameters of fresh semen and the study of the qualitative characteristics of both fresh and FT sperm are of dual importance. First, in assessing the quality parameters that are most relevant to achieving pregnancy in female animals and monitoring the factors that affect semen quality, and second, in identifying the most appropriate quality assurance tests that are easy and relatively inexpensive to implement in the AI industry.

Sperm morphology. The formation of spermatozoa in the seminiferous tubules of the testes is called spermatogenesis. Mammalian spermatozoa do not differ significantly in terms of their structure. They are composed of a flat oval head covered by the acrosome, the neck, midpiece, and the tail (Figure 1). The total length of the sperm of domesticated mammals varies from 55 μm to 88 μm (Lengerken et al., 2012).

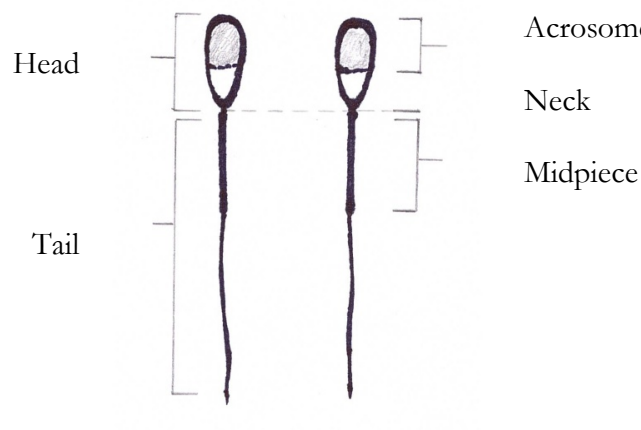


Figure 1. Morphologically normal bull spermatozoa.

Sperm morphology provides a good overview of the process of spermiogenesis in breeding bulls (Parviainen, 1993; Forsberg, 1996). Our previous studies have shown that the fresh ejaculate of a breeding bull should contain at least 85% of morphologically normal sperms to be successfully used in breeding (Figure 1, morphologically normal sperms; Figures 2 and 3 morphologically abnormal sperms; Padrik and Jaakma, 2002).

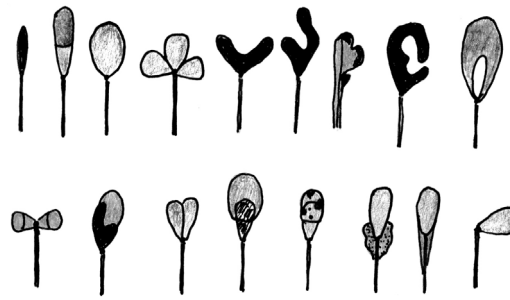


Figure 2. Morphologically abnormal heads in bull fresh semen (Padrik and Jaakma, 2002).

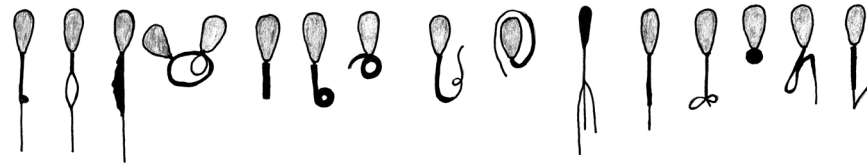


Figure 3. Morphologically abnormal midpieces and tails in bull fresh semen (Padrik and Jaakma, 2002).

A positive correlation has been found between sperm morphological quality and the fertility of bulls (Söderquist et al*.,* 1991; Barth, 1992; Correa et al*.,* 1997 a, b; Zhang et al*.,* 1998; Cranfield and Revell, 2000; Ahmed et al., 2016; Morrell et al., 2018). Söderquist et al. (1991) and Ahmed et al. (2016) noted that the more there are sperms with abnormal head and acrosome, the lower is the fertility. Correa et al. (1997 a, b) found a correlation between the incidence of abnormal head and acrosome and non-return rates (NRR) while no correlation was observed between sperm tail abnormality and NRRs. It is important to consider that when it comes to sperm head morphology, the evaluation criteria have not been harmonised and the tests performed at different laboratories differ significantly (Boersma et al*.,* 2000). Sperm morphology usually changes after FT. The major damage is to acrosomes and tails (Rodriques-Martinez, 1998).

The faster the percentage of normal sperms stabilises in the semen of young bulls, the greater is the count of normal sperms in a semen straw and the higher the conception rate (Shannon and Vishwanath, 1995). A decrease in normal sperm morphology occurs with bull aging (Söderquist et al*.,* 1991; Hallap et al*.,* 2005; Carreira et al., 2017).

There is a seasonal variation in sperm morphology (Slaweta, 1986; Söderquist et al*.,* 1996; Sharma et al., 2014). Söderquist et al. (1996) noted an increase in the incidence of abnormal sperms in the semen of Swedish Red and Swedish Holstein bulls in spring and summer. Such effects are due to changes in temperature that have an impact on spermatogenesis (Vogler et al*.,* 1991; Barth and Bowman, 1994; Janett et al*.,* 2003 a, b; Malama et al., 2017).

Sperm membrane intactness. The functional integrity of sperm membrane is one of the important characteristics of semen quality that gives a good overview of spermatogenesis as well as the damage that

occurs during freezing. Sperm membrane integrity can be evaluated by several methods, e.g., light or fluorescent microscopy combined with vital stains (Brito et al., 2003; Panemei et al., 2015), and flow cytometry (Hallap et al., 2004; Gliozzi et al., 2017). One of the simplest methods of evaluating the integrity of the plasmalemma of sperm cells is the hypo-osmotic swelling test (HOT). The traditional HOT, originally presented by Jeyendran et al. (1984), enables determination of the functional intactness of sperm membranes as viable spermatozoa 'swell' under hypo-osmotic conditions due to the influx of water, and the expansion of the membranes causes the tails to coil. Several authors (Rodrigues-Martinez, 1998; Hossain et al., 1999; Lagares et al., 2000; Imam et al., 2008; Sharma et al., 2014; Zubair et al., 2015; Ahmed et al., 2016) have emphasized the suitability of HOT for assessing the quality of fresh and FT semen in different farm animal species and humans.

In our earlier studies (Padrik et al., 2000) the percentage of sperm with functionally intact membranes ranged from 44.0 to 55.0% in the fresh semen from young EHF bulls. Similar data (40 to 65.5%) have been obtained by other authors (Mandal et al., 2003; Landing et al., 2010 a, b; Panmei et al., 2015).

A positive correlation has been found between the results of HOT or its modifications and the NRR of female animals (Revell and Mrode, 1994; Correa et al., 1997 a; Trojacanec et al., 2000), which potentially makes HOT one of the most appropriate and simple methods for semen quality evaluation for the AI industry. Several authors have found a positive correlation between plasmalemmal integrity and sperm motility characteristics (Neild et al., 1999; Mandal et al., 2003). Mandal et al. (2003) reported that ejaculates with less than 50% HOS-positive spermatozoa showed lower motility as compared with those with >50% HOS-positive spermatozoa in Murrah buffalo bulls. Stanger et al. (2010) observed that in humans, low HOT values of neat semen samples were significantly associated with increased deoxyribonucleic acid (DNA) damage identified by the sperm chromatin structure assay as well as the TUNEL assay. Zuge et al. (2008) observed a significant positive correlation between sperm cells with high mitochondrial activity (MTDR-H) and the percentage of cells with intact membrane assessed by HOT. Brito et al. (2003), and Tartaglione and Ritta (2004) found that the results of the post-thaw HOT could be used for predicting the fertility of bovine semen used for AI.

Sperm motility. In the AI industry, evaluation of the motility of FT sperm cells is often based on subjective evaluations by experienced professionals. Many authors have found a positive correlation between sperm motility and pregnancy rate subjectively evaluated by light microscopy (Saacke, 1983; Kjœstad et al., 1993; Januskauskas et al., 2003). Currently, many AI laboratories are equipped with a computer-assisted sperm analyser (CASA) for objective measurement of motility. The CASA system provides high repeatability, as the results do not depend on the evaluator. This enables better accuracy and correlation with NRR (Zhang et al., 1998; Verberckmoes et al., 2002; Januskauskas et al., 2003; Sellem et al., 2015 Ahmed et al., 2016; Morrell et al., 2017; Nogueira et al., 2018). A total of 19 different parameters characterising spermatozoa motility can be determined by CASA. The proportion of motile spermatozoa in fresh and FT semen is determined through the use of parameters, such as GMot, which expresses the proportion of motile spermatozoa in the general sperm population expressed as percentages, and PMot, which expresses the proportion of straight-moving spermatozoa in the general sperm population, also expressed as percentages.

It has been found, that in the fresh semen of young dairy and beef bulls, GMot ranged from 81.5 to 95.9%, and PMot from 81.5 to 92.0% (Landing et al., 2010 a, b), while in FT semen, GMot ranged from 64.3 to 78.9%, and PMot from 41.1 to 80.8% (Hallap et al., 2004; 2005).

Curve line velocity (VCL) that characterises the speed of sperm movement, and the amplitude of lateral sperm head displacement (ALH) are specific parameters providing important information about different sperm populations in the ejaculate, which is essential for further processing of bull semen. VCL ranges from 104.0 to 120.0 μm/s, and ALH from 2.55 to 3.43 μm in the fresh semen of dairy and beef bulls (Padrik, 2004; Landing et al., 2010 a, b). These studies also showed that in FT semen, VCL ranges from 76.2 to 157.0 μm/s and ALH from 2.0 μm to 3.3 μm.

There is a relationship between the motility of FT sperms and other sperm quality parameters. A positive correlation was demonstrated between sperm motility and mitochondrial activity as well as between the percentage of cells with stable membranes and the motility of fresh and FT sperms (Hallap et al., 2006; Hua et al., 2006). Moreover, a significant

relationship between the adenosine triphosphate content of FT semen and sperm motility has been described (Januskauskas et al*.,* 1996; Alam-Kristiansen et al., 2018).

Sperm mitochondrial activity. The approximately 100 mitochondria located in the midpiece of a sperm play an important part in determining whether or not a spermatozoon reaches the fertilisation site, since the sperm would not be able to move without the ATP generated largely by the mitochondria (Vishwanath et al*.,* 1986; Rajender et al*.,* 2010; Alam-Kristiansen et al., 2018).

Flow cytometric analysis is used to measure the mitochondrial membrane potential (MMP) of sperm cells. Different fluorescent dyes such as JC-1, Rhodamine 123 (R123), MitoTracker Green (MTG), MitoTracker Orange (CMTMRos), MitoTracker Red 580, and MitoTracker Deep Red 633 can be used to visualise the organelles (Ericsson et al*.,* 1993; Garner et al*.,* 1997; Gravance et al*.,* 2000; Cossarizza et al*.,* 2005; Carreira et al., 2017). The working mechanism 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 MMP. Thus, the more actively mitochondria synthesize ATP, the more likely these probes diffuse into living cells and make the mitochondria more fluorescent. This, in turn, could be measured by using flow cytometry that helps to specify high mitochondrial activity (MTDR-H) and low mitochondrial activity (MTDR-L). A positive correlation has been observed between MTDH-H and sperm motility in FT semen (Hallap et al*.,* 2005).

Sperm plasma membrane stability. The handling, dilution and deep-freezing of bull fresh semen may significantly damage the functional integrity of the sperm membranes, which may be caused by the processing methods or the particular traits of the bull. As described above, one of the important quality indicators of both fresh and FT bull semen is the percentage of sperms with functionally intact membranes determined by HOT. In addition, it is also important to study the changes in the sperm membrane such as the scrambling of phospholipids inside the plasma membrane (Harrison et al*.,* 1996). Membrane stability is an important quality parameter when evaluating sperm functionality (Hallap et al*.,* 2005). One way to estimate the sperm plasma membrane stability is to use the hydrophobic dye Merocyanine 540 (M540) and flow cytometry to determine the level of scrambling of the phospholipids in the plasma

membrane of the spermatozoa (Harrison et al., 1996). When staining with Merocyanine 540, the fluorescence increases in correlation to an increase in the level of scrambling phospholipids in the membrane (Harrison et al., 1996). Increase in phospholipid scrambling in the sperm membrane indicates that sperm membranes are not stable and have been damaged during processing. This, in turn, may decrease the viability, motility, and fertility of spermatozoa. A positive correlation has been found between the percentage of general motility (GMot; CASA-assessed), the percentage of subjective motility (SubMot), and the percentage of sperms with a stable membrane (LSM) (Hallap et al., 2005). Hallap et al. (2005; 2006) did not find a statistically significant correlation between LSM and MTDR-H sperms and female fertility. Thus, the reported data are often conflicting and depend on the population studied, the number of animals, semen batches, and variation.

Sperm chromatin stability. Sperm chromatin stability assay is used for assessing the status of DNA. It can be used to establish if the sperm DNA is denatured, or the sperm has an intact double DNA helix. Sailer et al. (1995) noted that abnormal chromatin structures may lead to problems in the nuclear material packaging of sperms, which in turn is related to morphologically abnormal spermatozoa. Thus, the chromatin stability of sperms may be influenced by factors disturbing the normal process of spermiogenesis (Karbinarius et al., 1997). Mahmoud et al. (2015) noted positive correlation between sperm abnormalities and DNA fragmentation ($r = 0.59$, $P < 0.05$). A correlation has been found between pregnancy rate in heifers and DNA fragmentation index (DFI) in FT semen (Januskauskas et al., 2001; Kasimanickam et al., 2006).

Prediction of bull fertiliy. Assessment of different sperm quality parameters allows more accurate prediction of male fertility (Rodriguez-Martinez, 2006; Rodderiguez-Martinez and Barth, 2007). There is evidence that the mathematical models for female pregnacy prognosis based on sperm quality parameters are quite accurate (Zhang et al., 1999; Januskauskas et al., 2000; Phillips et al., 2004; Ahmed et al., 2015; Giozzi et al., 2017; Kumaresan et al., 2017). The question remains – what are the most important sperm characteristics that must be included in the prediction models? Zhang et al. (1999) found that the motility of FT sperm fits well into this model, whereas the correlation between actual and predicted NRR was $r = 0.94$ ($P < 0.001$) and $R^2=0.71$. Januskauskas et al. (2000) included FT sperm motility and membrane functional

integrity in a predictive model the correlation of which with the female pregnancy rate was $r = 0.74$ ($P < 0.002$; $R^2 = 0.55$).

While the FT sperm quality parameters and the correlations between them and female fertility have been well studied, the correlation between fresh and FT sperm quality parameters has received much less attention. Finding the correlation between these quality parameters is important in two respects. First, to predict the freezing tolerance of sperms based on the quality parameters of fresh semen and second, to modify the technological process of freezing to minimise sperm damage. Defoin et al. (2008) found a strong positive correlation ($r = 0.75–0.82$; $P < 0.001$) between the percentage of progressive motility of sperm in fresh semen, and general and progressive motility of sperms in FT semen.

1.4. Factors affecting semen quality

Effect of age on semen quality. It has been well proven by AI practitioners that semen quality improves with age in young bulls. Improvement in semen volume and sperm quality parameters correlates with the body weight and the development and growth of testicles.

Coe et al. (1999) noted that an increase in the body weight and testicular weight in young bulls resulted in an increase in the percentage of morphologically normal sperms to at least 70%. Padrik and Jaakma (2002) found that while the percentage of abnormal sperms was about 20% in the first ejaculates collected from young EHF bulls, the percentage of abnormal sperms started to decrease after five ejaculates, and stabilized after 10–12 ejaculates. Söderquist et al. (1996) showed that with the increase in age from 14 to 36 months, the percentage of abnormal sperms decreased by 2.6%.

A correlation between the age and sperm motility in fresh and FT semen of breeding bulls has been demonstrated by Devkota et al. (2008). An increase in the concentration of sperms and the percentage of progressively motile sperms with an increase in age can also be explained by the growth of testicles in young bulls. Pant et al. (2003) described a positive correlation between the age, body weight, scrotal circumference, and testicular volume ($r = 0.56$; $r = 0.61$; $r = 0.71$) in bulls. Fontoura et al. (2016) observed a positive correlation between the age and scrotal circumference ($r = 0.72$). The age of bulls critically affects the ejaculate

volume as well as sperm concentration and motility (Brito et al., 2002; Pant et al., 2003; Fuerst-Waltl et al., 2006; Khezri et al., 2018).

Sperm quality parameters in the bulls aged over six years are much less studied. Hallap et al. (2005) determined that the sperm population with abnormal midpieces in the semen of seven-year-old bulls was significantly larger compared to a group of three-year-old bulls ($P < 0.05$) A decrease in the sperm morphological quality in the groups of 4–5- and 6–7-year-old bulls compared to the bulls aged 1–3 years has also been reported (Padrik and Jaakma, 2002). A decrease in sperm quality parameters in older bulls may be caused by a decline or fluctuations in the levels of hormones regulating spermatogenesis (Forsberg, 1996; Hafez and Hafez, 2000).

Seasonal effects on semen and sperm quality. Many authors (Mandal et al., 2003; Janett et al., 2003 a, b; Koonjaenak et al., 2007 a, b; Koivisto et al., 2009; Hidalgo et al., 2018) have described the seasonal effects on sperm quality. Fewer motile and morphologically normal sperms ($P < 0.05$) have been found in the semen collected in summer compared to other seasons (Söderquist et al., 1991; Janett et al., 2003 a, b). The percentage of sperm motility was ($P < 0.05$) higher in the ejaculates collected in winter than in those collected during the summer period (Westfalewicz et al., 2018).

Seasonal changes in bull semen quality are mainly due to changes in temperature that affect spermatogenesis (Vogler et al., 1991; Barth and Bowman, 1994; Ahirwar et al., 2018). Both CASA and flow cytometry analysis showed that in mature Holstein breeding bulls, the percentage of sperm with intact plasmalemma and acrosome as well as the percentage of sperm with high mitochondrial membrane potential were significantly higher ($P < 0.01$) in the ejaculates collected in winter compared to those collected in summer (Malama et al., 2017). According to Ax et al. (1984) and Cheng et al. (2016), the high temperature in summer causes an increase in the proportion of abnormal sperms and a decrease in sperm motility. To examine the effect of heat stress on spermatogenesis, Shojaei Saadi et al. (2013) conducted an experiment on scrotal insulation, during which bull testicles were covered for a short time. As a result of short-term scrotal insulation, the temperature in testicles increased by 1–2.3°C (Newton et al., 2010). It was found that a short-term rise in temperature during spermatogenesis significantly

increased the proportion of sperm with an abnormal head shape. One of the reasons may be the fluctuation of testosterone levels synthesised in Leydig cells, which resulted in a decreased production of actin, a cellular structure protein, during spermatogenesis. Shojaei Saadi et al. (2013) found that after scrotal insulation, the morphologically abnormal sperm population contained significantly less actin capping protein (CAPZB) than before scrotal insulation.

Effect of the grade of Holstein genes and gene defects on semen and sperm quality. There is not much information available about the relationships between the grade of Holstein genes and semen quality. In earlier studies (Padrik, 2001), it has been found that an increase in the grade of Holstein genes increases the percentage of sperms with abnormal morphology in fresh semen ($P < 0.001$). Padrik and Jaakma (2001) found that 100% Holstein bulls are more sensitive to seasonal changes in temperature. In the spring-summer period, the percentage of sperms with abnormal morphology in fresh semen increased significantly more in the bulls with the grade of Holstein genes >96.9%, compared with other bulls.

Complex Vertebral Malformation (CVM) was first described by Danish scientists in 2000 (*Nautra,* 2001). Kanae et al. (2005) found that CVM is an autosomal recessive disorder which is caused by a mutation in serotonine transporter gene (SLC35A3). Usually the calves born with CVM defect are stillborn, their spine's chest and/or neck are notably shorter, hind legs are shorter or deformed and heart failures may occur (Kanae et al*.,* 2005; Thomsen et al., 2006). In our earlier study, it was found that sperm concentration in fresh ejaculate and the percentage of progressively motile sperms in FT semen were significantly higher in the bulls that had no CVM gene in their pedigree (Padrik and Bulitko, 2004).

Heritability of semen quality. The heritability coefficient of sperm production and quality in different domestic animals – bulls (Gibson et al*.,* 1987; Graffer et al*.,* 1988; Smith et al*.,* 1989; Birkhead et al*.,* 2009), stallions (vanEldik et al*.,* 2006), boars (Wolf, 2010), rams (David et al*.,* 2007), and dogs (England et al*.,* 2010), is relatively moderate. Birkhead et al*.,* (2009) noted that the heritability coefficient of sperm and semen quality indicators depended on the breed. The heritability coefficient of sperm concentration in fresh semen of Holstein breeding bulls was $h^2 = 0.36$, while it ranged from $h^2 = 0.24–0.28$ in beef bulls. The heritability

coefficient of motile sperms in the fresh semen of Holstein bulls was h^2= 0.64, while it was significantly lower (h^2= 0.23) in beef bulls.

It can be concluded that there are different methods available for assessing semen quality in the fresh and FT semen of breeding bulls. The different factors referred to above that can influence the quality of semen and functional characteristics of sperms should be taken into consideration when producing semen doses for AI.

Despite the amount of data that has been published on different sperm quality tests and the relationships between specific sperm quality parameters and pregnancy rates, the results described are often contradictory. Relatively few articles have been published that suggest reliable combined assays for the assessment of semen quality by the AI industry. From the breeders' point of view, it is necessary to investigate and develop semen quality tests that are fast, easy, relatively inexpensive, and applicable in routine AI work. It is necessary to strike a balance between the accuracy of semen quality and bull fertility prediction, and the level of complexity, amount of investments, and time commitment required for these tests. The AI industry is looking for science-based, improved and effective technologies for the production of bull semen doses, where the semen quality control methods are of crucial importance.

2. AIMS OF THE STUDY

The overall aims of the study were to determine the usefulness of different semen quality evaluation methods for AI industry, and to develop prediction models for estimating the potential fertility of bull semen.

The specific aims of the study were:

1. to investigate the relationships between the age of bulls, season, grade of Holstein genes, and sperm quality parameters (**I**, **II**, **III**);

2. to assess sperm membrane intactness in EHF AI bulls using three different modifications of HOT, and to reveal the relationships between the HOT and sperm quality parameters estimated by conventional and flow-cytometric tests used at AI laboratories (**III**, **IV**);

3. to determine the relationships between the sperm quality parameters and field fertility estimated by NRR of cows and heifers (**I**–**V**);

4. to develop a better fertility prediction model for bull semen (**II**, **III**, **IV**);

5. to investigate the structural and functional characteristics of flow-cytometrically sexed semen in comparison with unsexed semen from the same bulls (**V**).

3. MATERIALS AND METHODS

3.1. Animals

The bulls selected for a breeding programme were involved in the study. Semen was collected and frozen under commercial conditions at the AI station of the Animal Breeders' Association of Estonia.

The fresh semen was collected from 91 EHF bulls aged between14 and 86 months (**III**). Semen was collected once weekly using an artificial vagina.

FT semen (49 ejaculates) from 10 mature bulls aged between 36 and 72 months and 7 young bulls aged between 14 and 22 months (**I–IV**) were examined and used for test inseminations.

3.2. Semen processing

Two consecutive ejaculates were pooled (hereafter referred to as a "batch"), extended with a commercial extender (Triladyl®, Minitüb, Tiefenbach, Germany), packed in 0.25 ml plastic straws each containing ~25×10^6 spermatozoa for mature bulls, and 30–40×10^6 spermatozoa for young bulls, and frozen using a biological freezer with manual temperature control (**I–IV**). The frozen straws were stored in liquid nitrogen until tested or used for insemination.

In FT semen, sperm were examined for membrane HOT, motility, plasma membrane stability, morphology, chromatin stability, and mitochondrial activity (**I–V**).

Sexed semen was purchased from Cogent Ltd. (Chester, UK) and Select Sires Inc. (Plain City, OH, USA) (**V**). Sexed semen from five bulls were frozen in 0.25 ml plastic straws and semen from three bulls in 0.5 ml plastic straws (**V**). Frozen flow-cytometrically sorted (sexed) and unsorted semen fractions from the Holstein bulls used in the study contained 2.1×10^6 X-chromosome-bearing sperm, and 15×10^6 unsorted sperm in one straw, respectively.

The semen straw was thawed in a water bath at +35°C for 20 s before emptying into a test tube (**I–IV**). For insemination, the straws with sorted sperm were thawed in a water bath at +37°C for 40 s, and unsorted semen at +35°C for 15 s (**V**).

3.3. Hypo-osmotic swelling test

A total of 683 ejaculates, collected over sixteen months in the AI station of the Animal Breeders` Association of Estonia at Kehtna, were examined for sperm membrane HOT and motility in fresh semen (**III**).

HOT-1. Three HOTs were performed, two of them modified. The traditional HOT (Jeyendran et al., 1984), labelled HOT-1, was performed by incubating 100 µl of fresh semen with 1 ml 150 mOsm kg^{-1} hypo-osmotic solution (7.35g sodium citrate and 13.51 g fructose per litre of distilled water) at +37ºC for 60 min.

The semen straw (0.25 ml) was thawed at +35°C in a water bath for 20 s before emptying into a test tube containing the hypo-osmotic solution. After incubating at +37ºC for 60 min, 0.2 ml of eosin (0.99%, Pioneer Research Chemicals, Ltd., England) was added to the test tube. The wet preparation was examined under a phase-contrast microscope (1000×, Olympus BX40, Olympus Corporation, Tokyo, Japan). The ratio of spermatozoa with swollen tails was expressed as a percentage of the total count (mean of three replicates). One hundred spermatozoa were assessed in each replicate (**I–V**).

HOT-2. In HOT-2 (Padrik, 1999), the proportion of FT spermatozoa with swollen tails was determined in 0.2% and 0.4% NaCl solutions (osmotic pressure 66 and 130 mOsm kg^{-1}, respectively). The semen straws were thawed in a water bath at +35°C for 20 s, and emptied into a test tube containing 1 ml of 0.2% and 0.4% NaCl solution. After incubating at room temperature (+20 to 22°C) for 2 min, 0.2 ml of eosin was added to each test tube. A wet preparation of each concentration was examined under a phase contrast microscope. The ratio of spermatozoa with swollen tails was expressed as a percentage of the total count (mean of three replicates). One hundred spermatozoa were assessed in each replicate.

ΔHOS-2 was calculated by subtracting the ratio of spermatozoa with intact membranes in the 0.2% NaCl solution from the similar value in the 0.4% NaCl solution (**III**, **IV**). The decrease in osmotic pressure from 130 to 66 mOsm kg^{-1} resulted in a significant difference in sperm behaviour in different semen batches (Figure 4).

HOT-3. In HOT-3 (Padrik and Jaakma, 2000), three straws of frozen semen were thawed at +35°C for 20 s, emptied into a test tube containing 3 ml of 2.9% sodium citrate solution (Tallinn Pharmacy Ltd., Estonia), mixed, and incubated for six hours at +37°C. A 100 µl fraction of the sperm suspension was pipetted into each of the two solutions – 1 ml of 0.2% and 1 ml of 0.4% NaCl. After 2 min incubation at room temperature (+20 to 22°C), 0.2 ml of eosin was added into each test tube and wet preparations were made.

One hundred spermatozoa were assessed in each preparation, and the ratio of the spermatozoa with swollen tails was expressed as a percentage of the total count (mean of three replicates).

ΔHOS-3 was calculated by subtracting the ratio of spermatozoa with intact membranes in the 0.2% NaCl solution from the similar value in the 0.4% NaCl solution (**III**, **IV**).

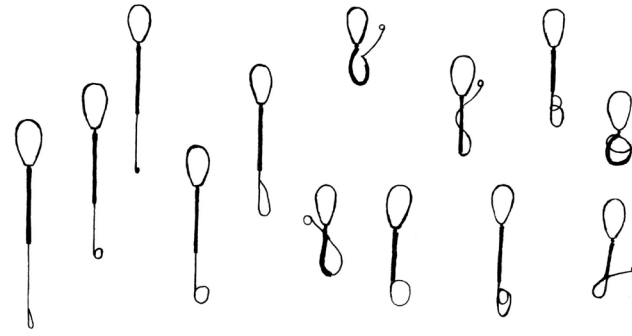


Figure 4. Pattern of tail swelling in spermatozoa depends on the osmotic pressure.

3.4. Sperm morphology

The morphology of sperms was evaluated in air-dried smears dyed with Spermac stain (Stain Enterprises Inc. Wellington, RSA) according to the manufacturer's instructions. A total of 100 cells were counted on the slides under a phase-contrast microscope (1000×, two replicates; Olympus BX40, Tokyo, Japan). The frequencies of abnormal head shapes, acrosomes, midpieces, coiled or missing tails, and cytoplasmic droplets were recorded. For each region of a sperm cell, the number of morphological abnormalities was expressed as the percentage of the total number of cells evaluated (**V**).

3.5. Sperm motility

Sperm motility characteristics were determined using a computer-assisted motility analyzer (Computer Assisted Cell Motion Analyzer, Sperm Vision, Minitüb GmbH&Co, Germany).

Samples of 5µl were placed in a Makler chamber (Makler Counting Chamber, Sefi-Medical Instruments, Israel) to track and assess ~400 fresh or post-thaw sperm cells at 200× magnification (Olympus BX40, Tokyo, Japan) (**I–IV**).

The samples of sexed and unsexed semen of 3µl were placed in 20µ-deep disposable four-chamber Leja slides (IMV, Maple Grove, MN USA), and about 400 cells were tracked and assessed at 200× magnification under a phase-contrast microscope (Olympus CX31, Tokyo, Japan) (**V**).

The following parameters were determined in sperm: GMot and PMot, VCL (µm/s), LIN (VSL/VCL), and amplitude of lateral head displacement (ALH, µm) (**I–V**); wobble (WOB = VAP/VCL), average path velocity (VAP, µm/s), beat cross frequency (BCF, Hz), linear motility and non-linear motility (**V**). The percentage of subjective sperm motility (SubMot, %) was estimated under a phase-contrast microscope (Olympus BX40, Tokyo, Japan) equipped with a warm stage (+37ºC) at 200× magnification. The percentage of subjectively motile sperm (SubMot) among the sperms in the general field of view was determined visually (**IV**).

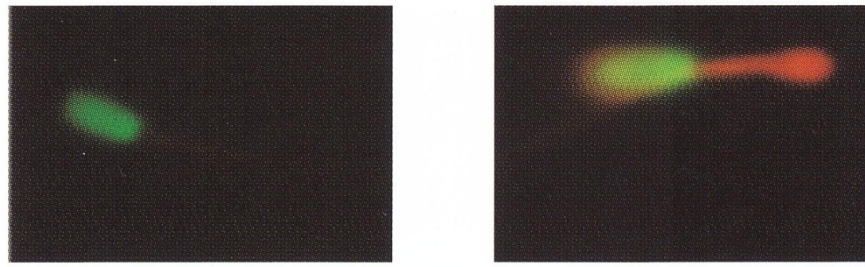
3.6. Sperm plasma membrane stability

The following working solutions were prepared: Merocyanine 540 (M-540; Molecular Probes, M24571, Leiden, The Netherlands) 1mM in dimethyl sulfoxide (DMSO); Yo-PRO 1 (Molecular Probes, Y3603) 25 μM in DMSO. Washed spermatozoa were stained with 25 nM Yo-PRO 1 and incubated at +38°C for 9 min in the dark as described by Harrison et al. (1996). Thereafter, 10 μL of a 40 μM solution of M-540 in SP-TALP was added to achieve a final M-540 concentration of 2.7 μM, and vortexed for 10 s before flow cytometry (FacsCalibur, Becton Dickinson, San Jose, USA).

Data collection was started 60 s after M-540 addition. Measurements were made with a flow cytometer equipped with standard optical lasers as excitation sources. The M-540 and Yo-PRO 1 dyes were excited by an Argon ion 488 nm laser running at 15 mW. Forward and side scatter values were recorded on a linear scale; fluorescent values were recorded on a logarithmic scale.

Obscuration bars were set for maximum sensitivity to obtain L-shaped forward light – scatter/sideways light scatter distribution of sperm cells. Fluorescence of Yo-PRO 1 was detected with the FL 1 detector (530/28 nm), while M-540 fluorescence was detected with the FL 2 detector (585/2 nm). From each sample, a total of 10,000 events were measured with the flow rate of approx. 200 cells/s. Data acquisitions were made using CellQuest Pro software (Becton Dickinson, San Jose, USA).

Dot plots for offline analyses were drawn by WinMDI, version 2.8. Events accumulated in the lower left corner correspond to sample debris and were excluded from the analysis by gating. On FL 1/FL 2 (Yo-PRO 1/M-540), dot plot regions were set to differentiate viable, stabile plasma membrane (live stabile plasma membrane – LSM, Yo-PRO 1 negative and M-540 negative; Figure 5A); viable, scrambled plasma membrane (live unstabile plasma membrane – LUSM, Yo-PRO 1 negative and M-540 positive; Figure 5B); and dead (Yo-PRO 1 positive) events (**I**, **II**, **IV**).



A-Sperm with live stable membrane (LSM)

B-Sperm with unstable membrane

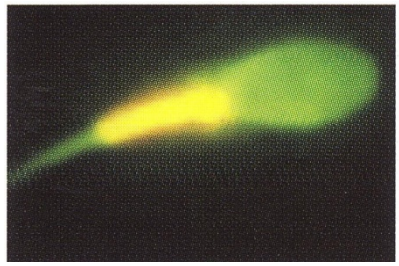
Figure 5. Spermatozoa with stable and unstable membranes (IMV-technologies, 2010).

3.7. Sperm mitochondrial activity

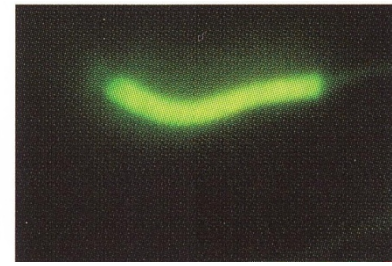
The staining protocol was identical to that described by Hallap et al. (2005). The measurements were made using a FacsCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The SYBR-14 dye was excited by a 15 mW Ar ion 488 nm laser while MitoTracker Deep Red was excited by a 17 mW HeNe 633 nm laser. The SYBR-14 fluorescence (cells with intact plasma membrane) was detected with the FL 1 detector (530/28 nm), while MitoTracker Deep Red fluorescence was detected with the FL 3 detector (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).

Data acquisitions were made using the CellQuest Pro software (Becton Dickinson, San Jose, CA, USA). Non-sperm events were gated out based on SYBR-14 fluorescence (DNA content). FC was used at a 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.

On SYBR-14 (FL 1/FL 2) dot plots, regions were drawn around the SYBR-14-positive cluster, and these events were classified as spermatozoa. In SYBR-14/ MitoTracker Deep Red dot plots sperm cells with low MTDR-L (Figure 6B) and high MTDR-H (Figure 6A) Deep Red fluorescence were specified (**I**, **II**, **IV**).



A-Sperm with high mitochondrial activity (MTDR-H**)**



B-Sperm with low mitochondrial activity

Figure 6. Spermatozoa with high and low mitochondrial activity (IMV-technologies, 2010).

3.8. Sperm chromatin stability

The susceptibility of DNA to acid-induced denaturation *in situ* was measured based on the metachromatic shift of acridine orange (AO) stain from green (double-stranded DNA) to red (single-stranded DNA) fluorescence (Evenson et al*.,* 1980). The susceptibility was expressed as a function of α_t, i.e., ratio of red (denatured, single-stranded DNA) to red + green (a total sperm DNA) fluorescence intensity. The α_t was calculated for each spermatozoon in a sample and the results were expressed as the percentage of cells with high α_t values (excess of single-stranded DNA), called DNA fragmentation index (%DFI). The %DFI corresponds to the previously used COMP α_t value. Thawed semen samples were diluted to 1-2 × 106/ml in TNE buffer (0.01M TRIS, 0.15M NaCl, 1mM EDTA, Ph 7.4). After 1 min, 200 µl of diluted semen was mixed with 400 µl of acid-detergent solution (0.15M NaCl, 0.08N HCL, 0.17% Triton-X100, Ph 1.2). After 30 s, the samples were stained with 1.2 ml of acridine orange staining solution (0.2M Na2HPO4, 1mM EDTA, 0.15M NaCl, 0.1M citric acid, 6µg/ml acidine orange pH 6.0) (Merck, Kebo Lab, Stokholm, Sweden). Samples were transferred to the flow-cytometer and analysis was started 3 min after the acidic treatment. Measurements were done on a FACSStar Plus flow-cytometer (Becton Dickinson Immunochemistry Systems, San Jose, USA) equipped with standard optics. Acridine orange was excited with Ar ion laser (Innova 90, Coherent, Santa Clara, CA, USA) at 488 nm, running at 200 mW. In association with double-stranded DNA, AO fluoresces in green (530±30 nm as detected with the FL1 detector) and after connection to single-stranded DNA the fluorescence is red (>660±20 nm, as detected with FL3 detector). Acquisitions were done using CellQuest 3.1 software

(Becton Dickinson, San Jose, USA) and stopped after recording 10,000 events. The resulting list-mode files were further processed using FCS Express version 2 (De Novo Software, Thornhill, Ontario, Canada), to calculate %DFI. (**V**).

3.9. Fertility

The fertility of bulls was evaluated based on the 60-day NRR after the insemination of heifers and cows with the tested semen batches.

The number of inseminations was as follows:

Publication **I**: FT semen (36 ejaculates) from 13 bulls were examined and used to inseminate 2,828 cows and heifers (an average of 79 inseminations per ejaculate and 218 inseminations per bull).

Publication **II**: Tested batches were used to inseminate a total of 1,338 cows and heifers (an average of 70 inseminations per ejaculate and 218 inseminations per bull).

Publication **III**: FT semen (49 ejaculates) from 10 mature and 7 young bulls was examined and used to inseminate 3,850 cows and heifers (an average of 78 inseminations per ejaculate and 226 inseminations per bull).

Publication **IV**: Doses from 45 FT semen batches were used to inseminate 3,475 cows and heifers (an average of 77 inseminations per batch and 231 inseminations per bull) by four AI technicians in four different herds. The 60-day NRRs were recorded for each semen batch and used for the analyses without correction for season, area, and parity. The fertility of an individual bull, calculated using NRRs for all semen batches, ranged from 37.5 to 71.5%. The fertility of semen batches ranged from 22.8 to 80.0%. Heifers and cows of different parity were inseminated routinely within one year during all seasons of the year (**I–IV**).

A total of 3,206 virgin heifers of the Holstein breed were used for inseminations to determine the relationships between the functional traits of sperm and the outcome of pregnancies after the use of sexed and unsexed semen of the same bull. The heifers from seven commercial dairy herds were 478.5 ± 0.71 (means ± standard deviations – SD)

days of age, and weighed 418.0 ± 40.4 kg. (**V**). Pregnancy diagnosis was performed by rectal palpation of the uterus 45–60 days after insemination.

3.10. Statistical analysis

The data on quantitative characteristics are expressed as means ± SD. The Pearson's correlation test was used to calculate correlations between different sperm parameters in fresh and FT semen, and between the measured sperm parameters and field fertility (60-day NRR). The pairwise t-test was used to assess the impact of treatments (**I–V**).

The differences were considered statistically significant if $P < 0.05$ (**I–V**).

The General Linear Models analyses for repeated measurements with SAS 9.1.3 procedure GLM (SAS System Version 9.1.3, Institute Inc., Cary, NC) were performed to compare the average sperm quality characteristics between the groups of bulls at batch level. The pairwise post-hoc comparisons were performed only after the group effect was statistically significant. The stepwise regression analyses were applied to find the optimal combination of sperm quality characteristics for predictive model of NRRs (**II**, **III**, **IV**).

The influence of season on sperm quality parameters was studied in 45 ejaculates from 15 bulls. Sperm quality parameters were assessed in spring (March, April, May), summer (June, July, August) autumn (September, October, November), and winter (December, January, February) (**I**).

To study the effect of age on sperm quality parameters, the bulls were divided into two age groups: mature bulls (Group I, aged 36 to 72 months, n = 10) and young bulls (Group II, aged 14 to 22 months, n = 7) (**III**), or three age groups (Group I, aged 1 to 2 years, n = 7; Group II, aged 3 to 4 years, n = 6; Group III, aged 5 to 7 years, n= 2) (**I**).

To study the effect of the grade of Holstein genes on sperm quality parameters, the bulls were divided into two groups: Group I – 87.5–93.8% grade of Holstein genes/ancestry (3 bulls, 7 batches), and Group II – 100% Holstein (4 bulls, 12 batches) (**II**).

4. RESULTS

4.1. Effect of age, season, and grade of Holstein genes on sperm quality parameters

Effect of age. Sperm motility parameters, plasma membrane intactness and stability, and mitochondrial activity were compared in 1–2-, 3–4-, and 5–7-year-old bulls. The results showed that the increase in age from 1–2 years to 5–7 years was accompanied by an increase in GMot, LSM and MTDR-H ($P < 0.05$), VCL ($P < 0.01$), and ALH; ($P < 0.001$) at batch level, and in ALH ($P < 0.05$) and VCL ($P < 0.01$) at bull level, but a decrease in LIN ($P < 0.05$) at bull level and ($P < 0.001$) at batch level (**I**).

The results of the second study (**III**) demonstrated that HOT-1 and HOT-3 results in FT semen did not differ between mature and young bulls neither at bull nor at batch level. However, the results of HOT-2 indicated an improvement in the quality of semen from mature bulls compared to that from young bulls at batch level ($P < 0.05$; **III**). The differences between the age groups were more pronounced when sperm motility parameters were assessed. An increase in age was accompanied by an increase in VCL ($P < 0.05$), and ALH ($P < 0.001$) at bull level, GMot and PMot ($P < 0.05$), VCL and ALH ($P < 0.001$) at batch level (**III**). An increase in age was accompanied by a decrease in LIN ($P < 0.01$) at batch level and ($P < 0.001$) at bull level (**III**).

Effect of season. Sperm motility parameters, plasma membrane intactness and stability, and mitochondrial activity were compared in frozen-thawed semen collected in spring, summer, autumn, and winter. The sperm motility parameters (VCL and ALH) of FT semen were significantly higher ($P < 0.05$) in the ejaculates collected in winter, autumn, and summer, compared to those collected in spring (**I**). Mitochondrial activity (MTDR-H) in FT semen was significantly higher in batches collected in winter and autumn ($P < 0.05$–0.001) compared to spring and summer. Similarly, sperm motility parameters GMot, PMot, and LSM tended to be lower in spring and summer compared to autumn and winter, however, the difference was statistically non-significant.

At bull level, it appeared again that most of the numerical values of quality parameters were higher in the winter and autumn period,

compared to those collected in spring and summer. However, only sperm mitochondrial activity (MTDR-H) of FT semen was significantly higher ($P < 0.001$) in winter and autumn, compared to spring (**I**).

Effect of the grade of Holstein genes. To investigate the effect of the grade of Holstein genes on semen quality, sperm motility parameters, plasma membrane intactness and stability, and mitochondrial activity were studied in FT semen of bulls with 87.5–93.8% and 100.0% of Holstein genes (**II**). An increase in the proportion of Holstein genes to 100% was accompanied by a decrease in GMot and MTDR-H ($P < 0.05$), PMot and LSM ($P < 0.01$) in FT semen at batch level (**II**). Similar tendencies were recorded for the HOT, PMot, GMot, VCL, MTDR-H and LSM at bull level, but the differences were not significant ($P > 0.05$; **II**).

4.2. Relationships between different sperm quality parameters

HOT-1 results in fresh semen and their relationship to motility characteristics. In fresh semen, the percentage of swollen spermatozoa was 57.9 ± 13.6% (range 7.0 to 85.0%) at batch level, and 57.6 ± 10.2% (range 31.0 to 77.4%) at bull level. A medium ($r = 0.32–0.33$; $P < 0.01$) correlation was found between the results of HOT-1, and GMot and PMot in fresh semen at bull level, but not at batch level. A medium to strong positive correlation was recorded between HOT-1 results in fresh semen, and GMot and PMot in FT semen at both batch ($r = 0.63–0.66$; $P < 0.001$) and bull ($r = 0.72–0.81$; $P < 0.001$) level (**III**).

HOT-tests in frozen-thawed semen and the relationships between different laboratory tests. The mean HOT-1 value in FT semen was 35.2 ± 6.8% (22.0 to 45.5%) at bull level, and 33.3 ± 9.7% (13 to 50%) at batch level. A significant positive correlation was detected between HOT-1 and PMot ($r = 0.55$; $P < 0.05$) at bull level, and between HOT-1 and PMot and GMot at batch level ($r = 0.47$, $r = 0.55$; $P < 0.01$, respectively; **III**).

The mean ΔHOT-2 was 7.1 ± 6.5% (-4.6 to 22.0%) at bull level, and 6.0 ± 8.9% (-15.0 to 25.0%) at batch level. The strongest correlation was found between ΔHOS-2 and GMot and PMot ($r = 0.66$, $r = 0.55$; $P < 0.05$, respectively) at bull level. A significant positive correlation was also

determined between GMot, PMot, VCL, ALH and ΔHOT-2 at batch level ($r = 0.42–0.64$; $P < 0.01$; **III**).

The mean ΔHOT-3 was -0.3 ± 3.7 (-6.5 to 7.3%) at bull level, and –0.7 ± 6.1% (-11.0 to 10.0%) at batch level. The ΔHOT-3 correlated with GMot and PMot at bull level ($r = 0.61$, $r = 0.52$; $P < 0.05$, respectively). Medium correlations were also determined between GMot, PMot VCL, ALH and ΔHOT-3 at batch level ($r = 0.36–0.55$; $P < 0.01$; **III**).

A positive correlation was found between HOT-1 and ΔHOT-2 at both bull ($r = 0.28$; $P > 0.05$) and batch level ($r = 0.49$; $P < 0.05$), and also between HOT-1 and ΔHOT-3 at bull ($r = 0.28$; $P > 0.05$) and batch level ($r = 0.38$; $P < 0.05$). ΔHOT-2 related to ΔHOS-3 at both bull ($r = 0.81$; $P < 0.001$) and batch level ($r = 0.72$; $P < 0.001$; **III**).

Relationships between the results of conventional laboratory tests of semen quality and flow cytometrically estimated mitochondrial activity and plasma membrane stability. Sperm motility parameters estimated either subjectively or by CASA, correlated to the percentage of LSM and MTDR-H (**IV**). Strong correlations were found between PMot and LSM at batch and bull levels ($r = 0.72$ and $r = 0.73$, respectively; $P < 0.001$), between PMot and MTDR-H at batch and bull levels ($r = 0.67$ and $r = 0.75$, respectively; $P < 0.001$), and between GMot and LSM and GMot and MTDR-H both batch ($r = 0.66$, $r = 0.59$; $P < 0.001$) and bull level ($r = 0.63$, $r = 0.66$; $P < 0.01$). Similarly, subjectively assessed SubMot correlated strongly with LSM and MTDR-H at both batch ($r = 0.67$; $r = 0.62$; $P < 0.001$) and bull level ($r = 0.71$, $r = 0.71$; $P < 0.01$). Also, a high positive correlation was found between LSM and MTDR-H at both batch and bull level ($r = 0.81$, $r = 0.86$; $P < 0.001$; **IV**). Medium correlations were recorded between HOT-2, LSM and MTDR-H, and HOT-3, LSM and MTDR-H at batch level ($r = 0.30–0.44$; $P < 0.05–0.01$).

4.3. Comparison of semen quality in sexed and unsexed semen

The quality of sexed and unsexed semen doses of the same batches from five bulls was studied. The total, progressive and linear motility of sperms were lower ($P < 0.01$, all values) and the proportions of non-linear and immotile sperm were greater ($P < 0.05$ and $P < 0.01$, respectively) in sexed semen compared to unsexed semen (**V**).

There were no differences between VAP (P = 0.22); VCL (P = 0.14); WOB (P = 0.74); VSL (P = 0.33); ALH (P = 0.29), and BCF (P = 0.81). The proportion of sperm with abnormal acrosome and membrane integrity was lower (P < 0.05 and P < 0.001, respectively) in sexed semen than in unsexed semen. The proportion of sperm with abnormal mid-pieces also tended to be lower (P = 0.06) in sexed semen. No difference in the chromatin stability was found between sorted and unsexed sperm (P > 0.05; **V**).

4.4. Relationships between single quality parameters of frozen-thawed spermatozoa and *in vivo* fertility

Relationships between HOT results and *in vivo* fertility. A medium positive correlation was found between HOT-1 results in FT semen and NRR at batch level (r = 0.37; P < 0.01), but not at bull level (**I**, **II**, **IV**). A medium to strong positive correlation was detected between ΔHOT-2 and NRR, at both batch (r = 0.63–0.65; P < 0.01–0.001) and at bull level (r = 0.63–0.73; P < 0.01–0.001), and between ΔHOS-3 and NRR at batch (r = 0.65–0.66; P < 0.01–0.001) and at bull level (r = 0.57–0.71; P < 0.05–0.01; **III**, **IV**).

Relationships between FT sperm motility parameters and *in vivo* fertility. A medium to strong positive correlation was found between SubMot and NRR at batch and bull level (r = 0.69 and 0.71, respectively; P < 0.01–0.001; **IV**). A medium to strong positive correlation was detected between GMot and NRR, at batch (r = 0.68–0.70; P < 0.01–0.001) and bull level (r = 0.71–0.85; P < 0.01–0.001; **I**–**IV**). A medium positive correlation was found between PMot and NRR at batch level (r = 0.64–0.69; P < 0.01–0.001) and a medium to strong correlation at bull level (r = 0.63–0.74; P < 0.01; **I**–**IV**). A medium positive correlation was also observed between VCL and NRR at batch level (r = 0.58–0.67; P < 0.05–0.001) and medium to strong positive correlation at bull level (r = 0.59–0.92; P < 0.05–0.001). A medium positive correlation was found between ALH in FT semen and NRR at batch level (r = 0.35–0.63; P < 0.05–0.001), and medium to strong positive correlation at bull level (r = 0.56–0.77; P < 0.05–0.001; **I**–**IV**).

Relationships between the results of flow cytometric analysis and *in vivo* fertility. A medium positive correlation was found between the

LSM results in FT semen and NRR at batch level ($r = 0.45–0.54$; $P < 0.05–0.001$) and at bull level ($r = 0.32–0.55$; $P < 0.001$; **I**, **II**, **IV**).

A medium positive correlation was also detected between MTDR-H and NRR at batch ($r = 0.49–0.57$; $P < 0.05–0.001$) and at bull ($r = 0.51–0.67$; $P < 0.05$) level (**I**, **II**, **IV**).

Relationships between the quality parameters of sexed sperm and *in vivo* fertility.

The total (general) motility of sexed sperm tended to correlate positively with pregnancy rate ($r = 0.82$, $P = 0.09$; **V**).

Effect of the grade of Holstein genes on *in vivo* fertility. The average NNRs were higher in the group of bulls with 87.5–93.8% Holstein genes – 52.3% (42.9 to 69.2%) than in the group with 100% Holstein genes – 43.9% (22.8 to 56.8%; **II**).

4.5. Relationships between predicted and observed non-return rates

Relationships between predicted (PNRR) and observed NRRs in mature and young bulls.

Based on the results of stepwise regression analyses, the predictive model for NRR (PNRRage; **III**) included five parameters: HOT-1, ΔHOT-3, GMot, PMot, and LIN.

$R^2 = 0.80$ in mature bulls at batch level:
PNRRage = 36.423 + 0.174 HOT-1 + 0.634 ΔHOT-3 – 0.388 GMot + 0.652 PMot + 16.32 LIN

$R^2 = 0.79$ in young bulls at batch level:
PNRRage = – 0.88 + 0.648 HOT-1 + 0.466 ΔHOT-3 + 1.291GMot – 0.887 PMot + 135.3 LIN

A strong positive correlation was found between PNRRs and 60-day NRRs (%) at batch level ($r = 0.89$; $P < 0.001$; **III**) in mature bulls. A medium positive correlation was found between PNRRs and 60-day NRRs (%) at batch level ($r = 0.64$; $P < 0.001$; **III**) in young bulls.

Relationships between predicted and observed NRRs when using only conventional AI laboratory tests or a combination of them along with flow cytometry.

The NRR prediction equation I (PNRR-I; **IV**) included five conventional quality parameters: HOT-2, SubMot, GMot, PMot, and ALH.

Bull level: $R^2 = 0.77$
PNRR-Ibull = 26.78 + 0.70 × ΔHOT-2 + 0.46 × SubMot +1.51 × PMot – 30 × GMot + 25.52 × ALH

Batch level: $R^2 = 0.62$
PNRR-Ibatch= 4.43 + 0.35 × ΔHOT-2 + 0.39 × SubMot + 0.61 × PMot – 58 × GMot + 12.06 × ALH

Other prediction equations – PNRR-II, were obtained by including five parameters from conventional laboratory tests and two flow cytometric analyses.

Bull level: $R^2 = 0.91$
PNRR-IIbull = -7.13 + 0.49 × ΔHOT-2 + 0.22 × SubMot + 0.77 × PMot - 1.65 × GMot + 33.23 × ALH - 0.21 × LSM + 0.58 × MTDR-H

Batch level: $R^2 = 0.63$
PNRR-IIbatch = 1.46 + 0.35 × ΔHOT-2 + 0.31 × SubMot + 0.51 × PMot - 0.57 × GMot + 13.96 × ALH - 0.07 × LSM + 0.06 × MTDR-H

These models were used to calculate the predicted fertility outcomes at bull and batch level. The highest correlation was found between PNRR-II and NRR at bull level ($r = 0.96$; $P < 0.001$).

A positive correlation was also found between PNRR-I and NRR at batch level ($r = 0.84$; $P < 0.001$) and at bull level ($r = 0.87$; $P < 0.001$), and between PNRR-II and NRR at batch level ($r = 0.78$; $P < 0.001$; **IV**).

5. DISCUSSION

The objective of this study was to determine the usefulness of different semen quality evaluation methods for use in commercial AI industry, and develop prediction models for the estimation of the potential fertility of bull semen. The effects of the grade of Holstein genes, age, and the season of semen collection on sperm motility, membrane integrity, membrane lipid architecture status, and mitochondrial membrane potential characteristics in FT semen of EHF bulls were also studied (**I**–**IV**).

As the use of sexed semen has increased in dairy cattle industry, we also investigated the structural and functional characteristics of flow-cytometrically sexed semen in comparison with unsexed semen from the same bulls (**V**).

Assessment of the effect of age on the quality of semen is important keeping in view the production of semen straws for AI, and effective use of bulls.

Our earlier studies (Padrik and Jaakma, 2002) showed that the age of breeding bulls significantly affected the percentage of morphologically abnormal sperm in fresh semen. Similar results have been obtained by other researchers (Söderquist et al., 1996; Hallap et al., 2005; Carreira et al., 2017).

In the current study, we examined the age-related differences in sperm motility, plasma membrane intactness and stability, and mitochondrial activity. The general and progressive motility of sperms in FT semen tended to be higher in the groups of 3–4- and 5–7-year-old bulls compared to 1–2-year-old bulls, whereas difference in GMot at batch level was significant ($P < 0.05$). Similarly, the specific motility parameters VCL, LIN and ALH increased in 3–4- and 5–7-year-old bulls. The highest percentage of sperms with LSM and MTDR-H was in the group of 5–7-year-old bulls. A similar relationship between the age and sperm motility has been found by Hallap et al. (2004) and Devkota et al. (2008). Hallap et al. (2005) also found that the mitochondrial activity of sperms was associated with age, but in terms of LSM, there was no difference between age groups (Hallap et al., 2006).

The age-related increase in the percentage of GMot and PMot of sperms may result from the continuous growth and development of the body which is accompanied by the growth of testicles. Devkota et al. (2008), Lozano et al. (2008), Fontoura et al. (2016) and Waite et al. (2018) found that the size of scrotal circumference is strongly associated with body mass and age. Forsberg (1996), Andrade et al. (2008) and Mahmood et al. (2018) noted that increase in testicular size results in an increase in the testosterone level in blood plasma. The increase in testicular size and blood testosterone levels affects the ejaculate volume, morphological quality and motility of sperms (Pinho et al*.,* 2008; Devkota et al., 2008; Rajak et al., 2014). The decrease tendency of some single semen motility characteristics in the group of 5–7-year-old bulls compared to the group of 3–4-year-old bulls may be due to a decline in hormone levels regulating spermatogenesis – follicle-stimulating hormone, luteinizing hormone and testosterone (Hafez and Hafez, 2000), or wavering hormone levels (Forsberg, 1996) associated with aging.

The hormonal changes described above are just some of the possible reasons why sperm quality parameters may change during aging. As hormone levels were not examined in this research, further studies are needed to find relationships between hormonal changes and sperm quality.

The seasonal variation in semen quality is also a well-known phenomenon but the magnitude of the changes and the quality parameters most subjected to the variation were not much studied in Estonia. In our earlier studies we focused on sperm morphology and found more morphologically abnormal sperms in bull fresh semen in spring and summer, compared to autumn and winter (Padrik and Jaakma, 2002). In the current study, motility parameters, mitochondrial activity, and plasma membrane intactness and stability were studied. VCL and ALH were lower in spring in comparison to other seasons. There was no significant difference in GMot and PMot, however, the numerical values were higer in autumn and winter. Higher MTDR-H values were found in the autumn-winter period compared to the spring-summer period.

Recently, Malama et al. (2017) and Westfalewicz et al. (2018) examined the effect of seasonality on the sperm quality in mature Holstein breeding bulls and revealed even more pronounced seasonal effects. Both CASA (GMot, PMot, VSL, VAP, ALH) and flow cytometry (percentage of

sperm with intact plasma and acrosome, percentage of sperm with high mitochondrial membrane potential) parameters were significantly higher ($P < 0.01$) in the ejaculates collected in winter, compared to those collected in summer.

Interestingly, the same phenomenon has been observed in buffalo semen. Koonjaenak et al. (2007 b) found that VCL of buffalo sperms was higher in July-October (rainy period) than in the summer period (March-June; $P < 0.05–0.001$). The same study showed a statistical difference between plasma membrane integrity and stability which was better in the winter period (November-February) than in summer and the rainy season. Mandal et al. (2003) noted that the percentage of motile sperms in buffalo semen was higher in winter (November-March) than in other seasons.

Seasonal changes in sperm quality parameters are explained with the temperature rise in the spring-summer period that might cause fluctuations in hormone levels. Shubbur et al. (1989) detected the highest blood testosterone levels in December. Park and Yi (2002), observed the highest testosterone levels in spring ($P < 0.05$), accompanied by the highest concentration of sperms with normal morphology and motility.

Higher ambient temperature may result in increased testicular temperatures and thus decrease semen quality. Shojaei Saadi et al. (2013) and Lucio et al. (2016) have studied the effect of an increase in temperature due to scrotal insulation. Their work has revealed that short-term scrotal insulation affects the morphological quality of sperm as well as the sperm chromatin stability. Newton et al. (2010) reported a 1 to 2.3°C increase in testicular temperature due to scrotal insulation in an experiment.

The proportion of the Holstein breed in the dairy cattle population has shown a rising trend over the years. In 1965, the proportion of the Estonian Black-and-White breed was 30.2% (EPJ, 2015-2019). In 2018, however, it was as high as 82.5% of the total dairy cattle population (EPJ, 2015-2019). The holsteinization of the Estonian Black-and-White dairy cattle population has positively influenced the productivity of cows and raised the average annual milk production per cow by more than 6,700 kg – from 3,280 kg in 1965 to 10,059 kg in 2018 (EPJ, 2015-2019). However, this may result in an increase in the inbreeding coefficient, and

possibly affect female fertility. Many researchers have noted the effect of inbreeding on the fertility parameters of cows, such as the calving interval, the number of inseminations, calving difficulties, stillborns, and NRR (Wall et al., 2005; González-Recio et al., 2007; Mc Parland et al., 2007; 2009).

The effect of the increase in the grade of Holstein genes on the quality of semen has been studied relatively little. In a previous study, we have found that an increase in the grade of Holstein genes in the descendant results in an increase in the percentage of abnormal sperms in fresh semen ($P < 0.001$) (Padrik, 2001). We also found that breeding bulls with 100% grade of Holstein genes are more sensitive to seasonal temperature changes due to a significant increase in the the percentage of abnormal sperms in fresh semen in the spring-summer period compared to the bulls with less than 96.9% grade of Holstein genes (Padrik and Jaakma, 2001). Remarkable changes in the shape of sperm heads occurred most often in the group with 100% grade of Holstein genes. These types of abnormalities were seldom found in the semen of the bulls with a lower grade of Holstein genes. The results of this study also showed a significant difference in the incidence of GMot and PMot, ALH, LSM, and MTDR-H ($P < 0.05$) between the groups of bulls with a different grade of Holstein genes at batch level (**II**).

We propose that the homogeneity of the ancestors of the bulls is one of the reasons why an increase in the grade of Holstein genes significantly affects the quality of sperm. The global Holstein population is becoming more inbred that can be illustrated by the fact, that 13 top bulls have been as a sire or as a maternal grandsire 41 times (106 potential chances) to 53 famous bulls from whom over 1,000.000 semen straws per bull have been produced and sold (Bierma and Hopman, 2016). Most EHF bulls used in the Holstein cow population are descended from only 10 progenitors (Animal Breeders' Association of Estonia, database).

Traditionally, AI stations examine sperm motility, that is, the proportion of motile spermatozoa in fresh semen expressed as percentage of the total sperm count. Depending on sperm concentration and total ejaculate volume, semen is diluted with a commercial extender and frozen. Sperm motility is also assessed after freezing. Adequate attention shall be paid to the assessment of sperm quality in the processing and handling of FT semen to ensure sustainability of dairy cattle breeding systems utilising

AI. For an AI station to be able to produce high-quality insemination doses, accurate data is required about both fresh and FT sperm quality and the factors affecting it. This is why it is important to upgrade the production process at AI stations by implementing new, more precise and reliable tests for evaluating the quality of bull semen.

HOT and its modifications can effectively be used by AI industry due to reliability and low cost. HOT has been used for assessing the quality of human semen (Moskovtsev et al., 2005; Cincik et al., 2007) and semen from various species of domestic animals, including cattle (Correa et al., 1994; Mandal et al., 2003; Hu et al., 2010; Zubair et al., 2015; Ntemka et al., 2016), horses (Neild et al., 1999), and pigs (Gadea and Matas, 1998). As the test gives a consistent estimate of the percentage of spermatozoa with a physiologically active membrane, it can be used to predict the fertilizing capacity of spermatozoa in animals (Rota et al., 2000; Brito et al., 2003; Panmei et al., 2015). Correa et al. (1997 b) and Neild et al. (1999) found that spermatozoa show different swelling patterns and not all spermatozoa with intact plasma membrane react to moderate osmotic pressure. Therefore, in our experiments, the traditional HOT was modified by shortening the incubation time of sperm cells in highly hypotonic, 0.2% and 0.4% NaCl (HOT-2), and adding the sperm survival aspect (HOT-3). The decrease in osmotic pressure from 130 to 66 mOsm kg^{-1} resulted in a significant difference in sperm behaviour among different semen batches. The percentage of spermatozoa with swollen tails increased in some batches, while it remained stable or decreased in others. The latter can be explained by a rapid rupture of the sperm membrane due to the influx of water under highly hypo-osmotic conditions. In contrast, the increase in the percentage of spermatozoa with swollen tails after short-term exposure to low osmotic pressure showed the maintenance of sperm membrane functional integrity. A strong correlation was found between the results of HOT in fresh semen and GMot and PMot of spermatozoa in FT semen (**III**). Thus, there is a high probability that spermatozoa with intact membranes before extension and cryopreservation maintain good motility after the FT procedure. This finding allows the application of individual semen processing methods to different semen batches for obtaining an optimal number of semen doses, by taking into account the proportion of spermatozoa with functionally intact membranes when diluting the semen.

The results of HOT combine well with the results of other sperm quality tests, thereby providing a broad and solid foundation for the general assessment of the quality of semen. A positive correlation was observed between HOT results and sperm GMot and PMot in FT semen, which is similar to earlier findings (Neild et al., 1999; Mandal et al., 2003).

Januskauskas et al. (1996) have observed associations between ATP content and sperm membrane integrity, assessed using fluorophore probes. Zuge et al. (2008) have reported a high positive correlation between the proportion of sperm cells with high mitochondrial activity and the proportion of intact membranes, determined using HOT.

Assessment of FT semen quality was also carried out by using flow cytometry. In addition, we determined the relationships between the results of conventional laboratory tests and FCM analysis, as well as the suitability of these tests for developing a fertility prediction model for FT semen. FCM analysis enables evaluation of a considerably larger number of sperms, thereby providing a more objective assessment. The disadvantage is, however, that the technique is costly and more time-consuming. The importance of FCM for the AI industry should be viewed from two perspectives. First, FCM could be used for testing the suitability, efficiency, and reliability of conventional laboratory tests carried out daily at AI stations to evaluate the quality of sperm. Second, the routine use of FCM analysis for the evaluation of sperm quality at an AI station would be feasible when the production volumes are relatively low, so that FCM could be used cost-effectively, without hiring additional laboratory staff. FCM is beneficial by providing a high-quality product and thereby ensuring effective use of the bulls with the highest genetic value for the cattle population.

The study showed a positive correlation between the results of conventional laboratory tests (SubMot, PMot, GMot, VCL, ΔHOT-2, ΔHOT-3) and sperm quality variables LSM (Figure 5A) and MTDR-H (Figure 6A) obtained by FCM analysis (**IV**). In our study, a strong positive correlation was found between subjectively assessed motility (SubMot) and the proportion of spermatozoa with stable plasma membrane (LSM) ($P < 0.01$) at bull level. However, a medium positive correlation between PMot and LSM and between PMot and MTDR-H ($P < 0.001$) must be considered even more important because of the lack of subjectivity. The results of our study also showed a significant positive correlation

between the percentage of cells with intact membrane estimated by conventional HOT and MTDR-H ($P < 0.01$). Similarly, Zuge et al. (2008) observed a significant positive correlation between sperm cells with full mitochondrial activity and the percentage of cells with intact membrane assessed by HOT. Hallap et al. (2006) and Hua et al. (2006) reported a positive correlation between sperm motility and high mitochondrial activity and between the percentage of cells with stable membranes and motility in fresh and FT semen. Some earlier studies described a significant association between the ATP content in FT semen and sperm motility (Januskauskas et al*.,* 1996; Alam-Kristiansen et al., 2018). In our study, a strong positive correlation was found between PMot and MTDR-H ($P < 0.001$) which indirectly indicates the importance of ATP energy produced by the intact mitochondria (Vishwanath et al*.,* 1986; Rajender et al*.,* 2010). A high positive correlation, found between LSM and MTDR-H ($P < 0.001$; **IV**), supports earlier findings that a stable sperm plasma membrane is the prerequisite for the functioning of mitochondria that power sperm movement (Kasai et al*.,* 2002; Hua et al*.,* 2006; Zuge et al*.,* 2008).

Determining the relationships between different functional parameters of FT spermatozoa is important, because it provides us some evidence for the selection of few of them for the routine use in AI practice. If due to limited time or economical reasons only some sperm parameters can be determined, we should use those parameters, for example sperm motility parameters, which have strong correlation with other sperm functional parameters such as mitochondrial activity and plasma membrane stability, to evaluate the fertility of the FT semen. Thus, the higher the percentage of motile sperm and VCL in FT semen, the greater the probability that the sperms in FT semen batch have also a higher mitochondrial activity and plasma membrane stability, and better NRR of cows and heifers after inseminations.

In the preceding chapters, we showed the impact of age of the bulls and the seasons on the quality of fresh and FT semen. These important factors as well as the individuality of bulls are taken into consideration in the AI station's production process on a daily basis to ensure the production of high-quality insemination doses which, in turn, facilitate timed conception of female animals. The fertility of FT sperms, in addition to female-dependent factors, farm management and oestrus detection, is crucial to ensure timed conception of female animals. This

is why it is important to determine the parameters of FT sperms that characterize sperm functionality, and choose the tests which would best relate to the non-return rates or pregnancy rates.

Evaluation of sperm motility characteristics has traditionally been the most frequently used semen quality test in the AI industry. As had been expected, a strong correlation was observed between GMot and PMot of FT spermatozoa and NRR ($P < 0.01$) in this study, which confirms previous findings (Holt et al., 1997; Farell et al., 1998; Januskauskas et al., 2003; Hallap et al., 2006; Morrell et al., 2017; Morrell et al., 2018; Nogueira et al., 2018). Similarly to Revell and Mrode (1994) and Correa et al. (1997 a), a significant positive correlation was found between the results of HOT-1 in FT semen and NRR at batch level ($P < 0.01$). The correlation coefficients were even higher between the results of HOT-2, HOT-3, and NRR at both batch ($P < 0.001$) and bull level ($P < 0.01$), which is an evidence that HOT-2 and HOT-3 are even more suitable for semen quality testing than HOT-1. HOT-2 appears to be especially attractive for the AI industry as it is neither expensive nor time-consuming. In our study, a strong correlation was recorded also between SubMot, VCL, and ALH of FT spermatozoa and NRR ($P < 0.01$) at bull level, similarly to Zhang et al. (1998) and Januskauskas et al. (2003). Also, a significant positive correlation was observed between LSM, MTDR-H in FT semen, and NRR at batch level ($P < 0.001$), and between MTDR-H and NRR at bull level ($P < 0.05$; **I–IV**).

Evaluation of FT semen quality is often based on subjective evaluations by experienced professionals rather than the use of objective methods to ensure validity of the results. Although many AI laboratories are nowadays equipped with CASA that enables objective measurement of motility, a combination of multiple sperm attributes would explain in more detail the variations in fertility among bulls than the variables of motility alone (Christensen et al., 1999). The single tests for the evaluation of FT semen quality have not been sufficiently discriminative (Christensen et al., 1999) because each of them measures only a single attribute necessary for the fertilization. Therefore, it would be rational to combine different single parameters into a prediction model (Rodriguez-Martinez, 2006; Sellem et al., 2015; Kumaresn et al., 2017).

We tested 3 models: PNRRage, based on conventional laboratory tests where the age of the bulls was taken into consideration (**III**); PNRR-I,

based on conventional laboratory tests; PNRR-II, based on conventional tests in combination with flow cytometric measurements of sperm membrane stability and mitochondrial activity (**IV**).

A high positive correlation was found between predicted and actual NRRs if, in addition to HOT and sperm motility parameters, the age of the bulls was taken into consideration in developing the model. In the first study, using a regression equation with five parameters (HOT-1; ΔHOT-3; GMot, PMot, and LIN), the predicted NRRs were highly correlated with the actual NRRs (**III**). The high correlation coefficient observed was between PNRRage and NRR for young bulls ($P < 0.001$). HOT has previously been successfully incorporated into a regression equation as a predictor of *in vitro* fertility of FT bull and boar semen by Gadea and Matas (2000), Pérez-Llano et al. (2001), and Tartaglione and Ritta (2004).

In the second study, we tested two models, one (PNRR-I) based on conventional laboratory tests only, and the other (PNRR-II) that aditionally included flow cytometric measurements of sperm membrane stability and mitochondrial activity. The highest positive correlation was found between PNRR-II and NRR at bull level ($P < 0.001$; **IV**). A combination of conventional laboratory tests and flow cytometric analysis of membrane stability and mitochondrial activity in FT spermatozoa allowed better prediction of the potential fertility of bull semen in comparison to the conventional AI laboratory tests alone. Motility parameters and membrane integrity of FT spermatozoa were also included into the models proposed earlier by Januskauskas et al. (2003), Tartaglione and Ritta (2004), Phillips et al. (2004) and Ahmed et al., 2016). Similarly, they reported a strong correlation between predicted and actual NRRs. Gliozzi et al. (2017) reported, similarly to our study findings, that a combination of single semen quality parameters and FCM analysis of sperm quality results allowed better prediction of the potential fertility of bull semen (R^2 adjusted = 0.84 $P < 0.05$) compared to single semen quality parameters alone (R^2 adjusted = 0.47, $P < 0.05$). Our proposed prediction model (PNRR-I) could be suitable for use in commercial AI, provided that the determination of all the required parameters would not be too time-consuming and expensive.

Today, beside genomic selection, the use of sexed semen is one of the most important achievements effective in breeding practice. The only

commercially feasible method for increasing the desired gender up to 90% is based on the difference in the DNA content between X- and Y-chromosomes.

Many studies have shown that pregnancy rates after AI with sex-sorted semen make 60-80% of that of unsexed semen (Seidel et al., 1997; 1999; Schenk, 2000; DeJarnette et al., 2008; Schenk et al., 2009).

We investigated the structural and functional characteristics of flow-cytometrically sexed semen in comparison with unsexed semen from the same bulls (**V**). The mean HOT-1 results and several CASA parameters such as GMot, PMot and linear motility were higher in unsexed semen ($P < 0.01$). At the same time, more nonlinear and immotile sperms were found in sexed semen ($P < 0.05$ and $P < 0.01$, respectively; **V**). No difference in the chromatin stability was found between sexed and unsexed sperm. Lower sperm motility and HOT-1 parameters in sexed semen could be explained by sperm membrane damage caused by the sorting process. The qualitative difference between sexed and unsexed semen is one of the reasons for the difference in the pregnancy rates between cows and heifers.

Consequently, similarly to unsexed sperm, the model that combines the parameters obtained by using conventional laboratory tests (e.g., HOT-1 and CASA), could be used to assess the fertility of sexed semen, as it has been successfully applied for the routine evaluation of FT sperm fertility.

The implementation of sex-selection technologies should include assessment of fresh sperm quality parameters and selection of bulls whose sperm is suitable for sex-selection. Sperm functionality parameters such as HOT-1 and motility could be good candidates for this, because a high proportion of sperms with intact membranes in fresh semen predicts a greater proportion of PMot sperm and sperm with intact membranes after FT (**III**). A medium to high correlation ($r = 0.65–0.81$; $P < 0.05$) was observed between these parameters at bull level. It can be hypothesized that the more motile sperms with intact membrane in fresh semen, the more motile sperms with intact membrane in sexed semen after sorting.

6. CONCLUSIONS

The objective of this study was to determine the usefulness of different semen quality evaluation methods for the AI industry, and to develop prediction models for the estimation of the potential fertility of bull semen. The effect of the grade of Holstein genes, age, and season of semen collection on FT sperm motility, membrane integrity, membrane lipid architecture status, and mitochondrial membrane potential characteristics in EHF bulls were also determined in this study.

Based on the results, the following conclusions were drawn:

- The age of bulls affected semen quality. The increase in age of bulls from 1–2 years to 5–7 years was accompanied by an increase in sperm motility parameters and mitochondrial activity measured in FT semen (**I**).

- The season of semen collection affected semen quality. The sperm motility parameters of FT semen were significantly higher in winter, autumn, and summer, compared to those collected in spring (**I**). The sperm mitochondrial activity of FT semen was significantly higher in the semen collected in winter and autumn, compared to that in the semen collected in spring (**I**).

- The grade of Holstein genes had an effect on semen quality and field fertility parameters. An increase in the proportion of Holstein genes was accompanied by a decrease in sperm motility parameters, mitochondrial activity and membrane stability (**II**).

- HOT-1 of fresh semen correlated well with the sperm motility parameters of FT semen, which makes the test suitable for the prediction of post-thaw semen quality (**III**).

- The results of the conventional laboratory tests (subjectively assessed motility, and the parameters measured by CASA and HOT) correlated well with flow-cytometrically measured membrane stability and mitochondrial activity (**IV**).

- For FT semen, the use of HOT-2 was found to be effective for the assessment of the quality of FT semen samples, as it is relatively quick and inexpensive (**III**).

- Strong correlations were found between single semen quality parameters PMot, HOT-3, and field fertility estimated as NRR. Medium correlations were found between HOT-1, GMot, HOT-2; sperm membrane stability and mitochondrial activity results and NRR (**III**).

- The proportion of sperms with intact membranes and GMot and linear motility were higher in unsexed semen, compared with sexed semen (**V**).

- A combination of conventional laboratory tests and flow cytometric analysis of membrane stability and mitochondrial activity in FT spermatozoa allowed better prediction of the potential fertility of bull semen in comparison to the conventional AI laboratory tests alone. The proposed prediction model could be suitable for use in commercial AI (**IV**).

7. IMPLEMENTATION OF THE RESULTS IN ESTONIA

Our study showed a strong positive correlation between the results of HOT in fresh semen and the motility of spermatozoa in FT semen. Thus, there is a high probability that spermatozoa with intact membranes before extension and cryopreservation show high motility after FT. This correlation makes it possible to apply an individual semen processing approach to different semen batches to obtain an optimal quantity of semen straws. The percentage of motile spermatozoa per straw after FT can be optimized if the proportion of spermatozoa with functionally intact membranes is taken into account when diluting semen. The results of the study were used to improve the semen dilution procedure and sperm freezing technology used at the Kehtna AI station of the Animal Breeders' Association of Estonia (Figure 7; Padrik et al*.,* 2009; 2012 a, b).

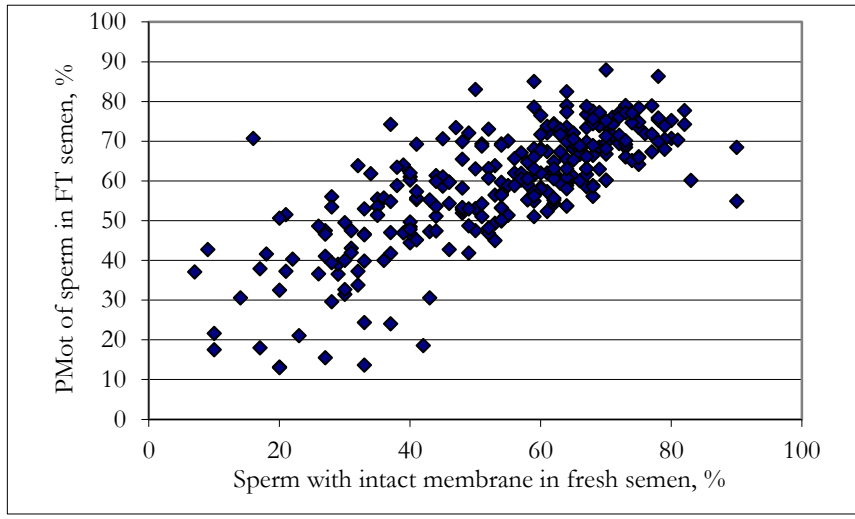


Figure 7. Relationship between sperm with intact membrane in fresh semen and PMot of sperm in FT semen (Padrik et al*.,* 2009).

Another key factor that enabled improvements in the sperm freezing technology was the integration of various measurements and analyses into the production process – for example, morphological quality of spermatozoa, CASA sperm motility parameters, and membrane integrity.

All aspects of the production cycle were placed under bioanalytical control; considering of seasonal variations and the age of the bulls are examples on how an evidence-based production process was implemented.

In 2010-2011, before the research-based innovations were implemented into practice, at the Kehtna AI station, the average number of semen straws produced per ejaculate was 414 (Padrik et al., 2012 b), while in 2016, after the implementation of the improvements, the average number of semen straws per ejaculate increased to 476, i.e., by 15% (Figure 8).

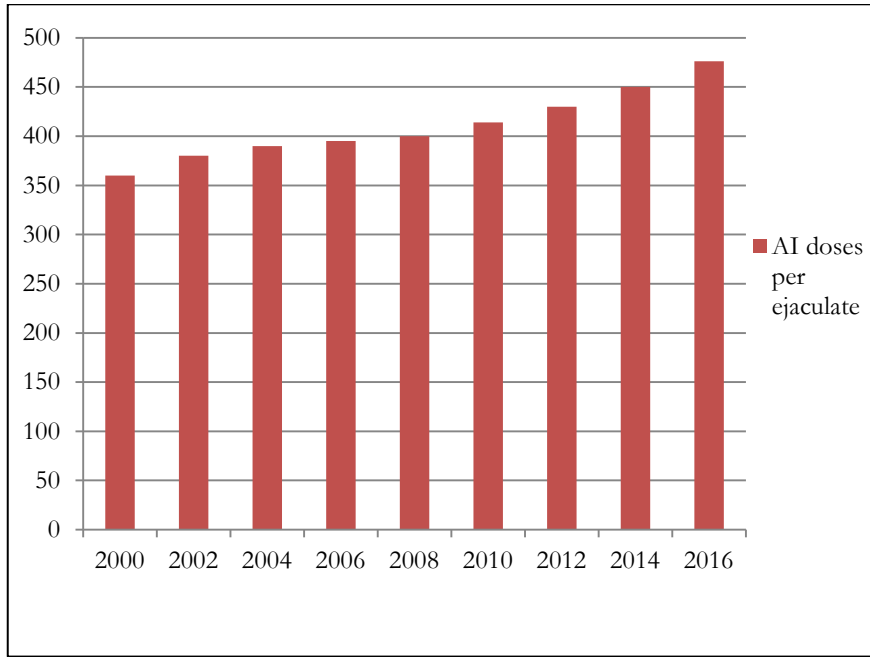


Figure 8. Average number of AI doses produced per ejaculate at Kehtna AI station, 2000-2016 (Animal Breeders' Association of Estonia, database).

The total number of sperms in semen straw decreased by 2×10^6 sperms as an average, while the number of PMot sperms increased by about 20% (Figure 9). The decrease in the concentration of sperms in semen straws had no negative effect on the NRR of cows and heifers.

Thanks to the proposed improvements in the technology, FT semen of higher quality can be produced which results in better NRR. This is economically beneficial for dairy cattle farmers due to lower AI costs.

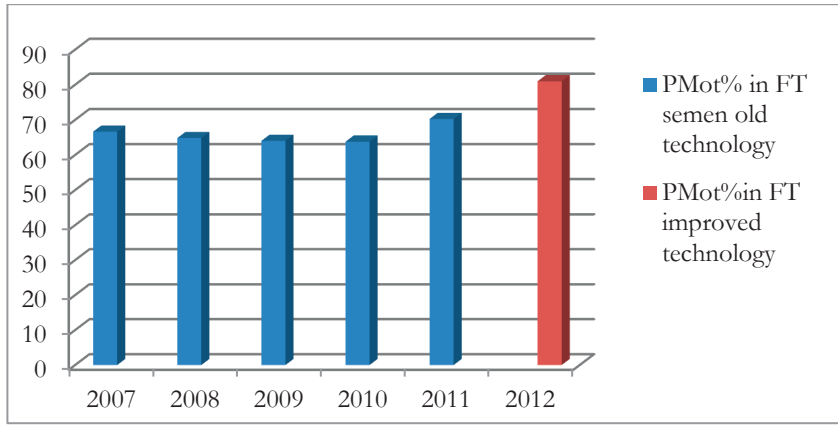


Figure 9. Proportion of PMot sperms after FT. (Old technology: during this period, innovative methods to determine semen quality parameters were not used for semen and sperm evaluation, and semen collecting regimen was not optimized depending on individual characteristics, sexual behaviour, age, breed, pedigree of the bulls and seasonality; Padrik et al., 2012 a, b).

The average cost of an AI procedure is €25 in Estonia. At 41.6% conception rate, an average of 2.4 insemination procedures are required to conceive a cow, while at 52.2% conception rate, 1.9 procedures are required (i.e., 0.5 procedures less), thus about €12.5 per cow would be saved (Padrik et al., 2012 b; Figure 10).

Our study showed that an increase in the age of bulls was accompanied by an increase in the proportion of motile sperm in FT semen (**I**, **III**). These results were used at Kehtna AI Station to reorganise the production process so that each bull, depending on its age, would have an optimal semen collecting regimen (Padrik et al., 2012 b). This enables more effective use of breeding bulls, production of the maximal number of insemination straws, and achieving a higher quality of FT semen (Padrik et al., 2012 b).

The season of semen collection had a significant effect on sperm motility characteristics (**I**). Based on the research findings, the production of FT semen at Kehtna AI Station was reorganised to produce a larger portion of frozen semen during the autumn-winter period (Padrik et al., 2010).

After this improvement, the average PMot of FT has increased year by year (Padrik et al., 2012 b).

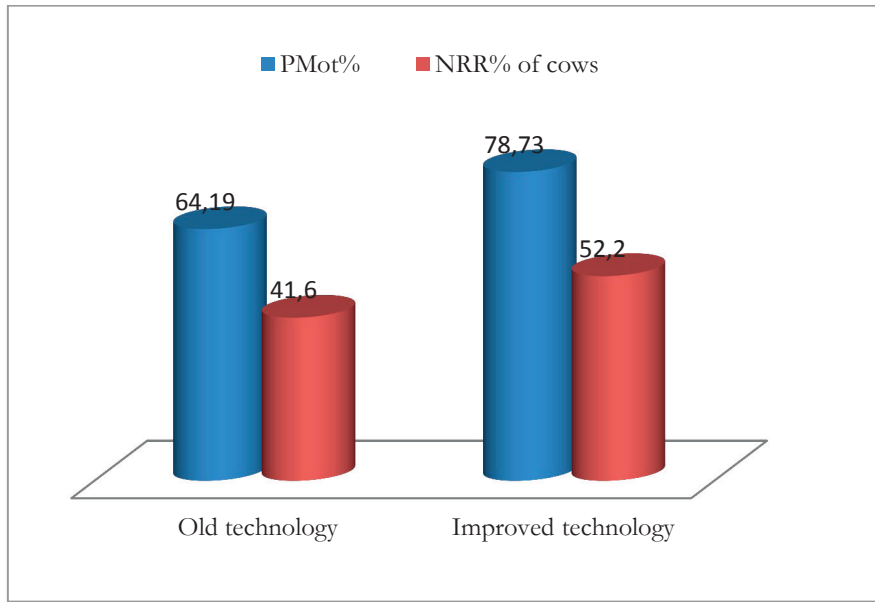


Figure 10. PMot and respective NNR of FT sperm – old vs improved technology (Padrik et al., 2012 b).

The results that showed a difference between unsexed and sexed sperms (**V**) appeared valuable for specifying the details of handling sexed semen and AI with sexed semen. The research findings have also been used in the provision of in-service training for AI technicians.

The implementation of the research findings had a more extensive impact on the entire AI sector. A well-known fact is that an increase in the milk yield has a significant effect on the pregnancy rate of cows after first AI after calving (Figure 11). The NRR declined steadily between 2006 and 2010 along with an increase in the yearly milk yield per cow. Between 2011 and 2013, NRR of cows and heifers showed an increase, and remained unchanged afterwards. We believe that the steady increase in NRR is at least partly due to the implementation of the improved research-based technology at the AI station.

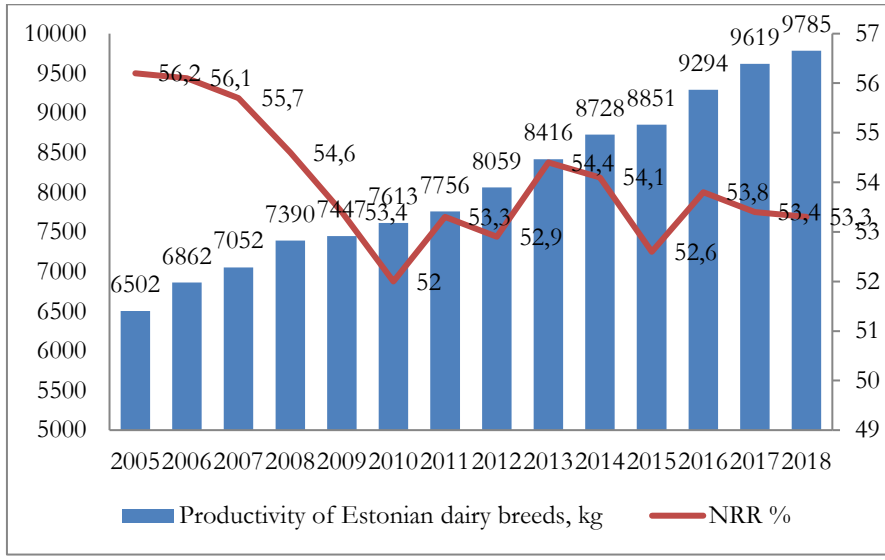
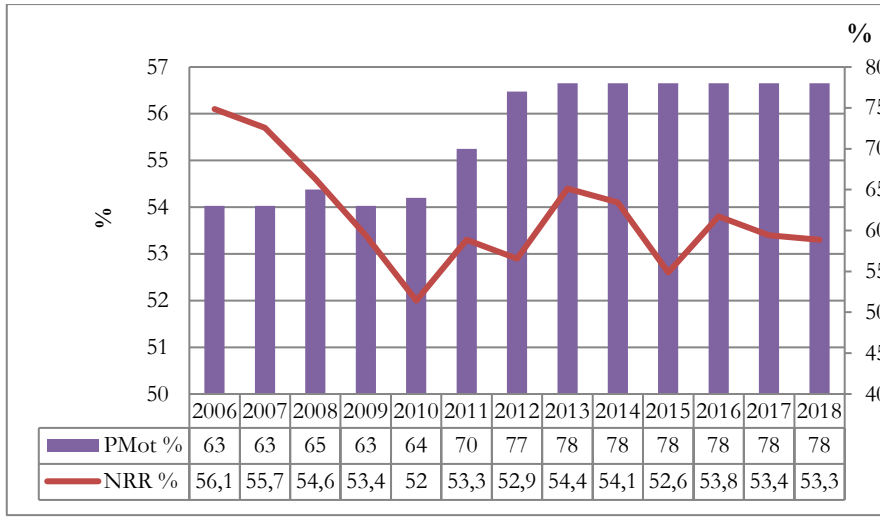


Figure 11. Relationships between NRR% and productivity of Estonian dairy breeds (kg) (JKK, 2006-2013; JKK, 2014; EPJ, 2015-2019).

The improvements have also contributed to an increase in the number of insemination straws produced per ejaculate and PMot of the FT sperm population in semen straws, which, in turn, has correlation with an increase in the NRR of cows and heifers (Figure 12).



	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018
PMot %	63	63	65	63	64	70	77	78	78	78	78	78	78
NRR %	56,1	55,7	54,6	53,4	52	53,3	52,9	54,4	54,1	52,6	53,8	53,4	53,3

Figure 12. Relationships between PMot% of FT semen and NNR% of cows and heifers (JKK, 2006-2013; JKK, 2014; EPJ, 2015-2019; Animal Breeders' Association of Estonia, database).

Further studies are needed to determine additional fresh semen quality parameters that would enable prediction of sperm motility and fertility of FT semen even more precisely.

The accuracy, speed, cost, and practical applicability of different semen quality evaluation methods should be studied in more detail.

Novel, more advanced methods for sperm quality assessment should be developed along with the development and implementation of new sex-selection technologies.

Recommendations for AI stations based on the results of the studies and AI station practice

- When developing a sperm collection regimen for the bulls, consider the individual characteristics, sexual behaviour, pedigree, breed, and age of the bull.

- Fresh semen from young bulls (12–16 months old) can be used for producing semen straws when the morphological quality of the sperm has stabilised (generally after 10–12 ejaculates, from the beginning of sperm collection).

- Most semen straws should be produced in the autumn-winter period, if possible.

- During the spring-summer period, semen straws should be produced only from the bulls that are in great demand for breeding purposes, or when there is a shortage of semen straws in the storage.

- In bull fresh semen, the morphological quality and motility of sperm as well as the intactness of the sperm membrane must be determined. These are highly relevant parameters for predicting FT semen quality.

- When determining the FT semen quality, the sperm motility parameters must be taken into account because they are related to the NRR of cows and heifers.

- For more information on the fertility of spermatozoa in both unsexed and sexed semen, it would be useful to combine the evaluation model with the sperm motility parameters and membrane integrity indicators.

- If the AI station laboratory is equipped with CASA and FCM equipment and the manufacturing process allows it, the results of the parameters derived from these technologies can be used to determine the fertility of spermatozoa even more accurately.

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9. SUMMARY IN ESTONIAN

Eesti holsteini tõugu sugupullide sperma kvaliteet, seda mõjutavad tegurid ning seos *in vivo* viljakusega

Kokkuvõte

Tänapäeval on Eesti veisekasvatuses kõige tulusam pidada piimakarja. Selline olukord ei ole välja kujunenud *eo ipso*, vaid toetub põllumajanduse kultuurilis-ajaloolisel põhjale, sest Eesti veisekasvatusel on pikk traditsioon.

Piimatootmise tõhusus ei sõltu tänapäeval ainult väärtusliku geneetilise materjali olemasolust, vaid ka paljudest teistest teguritest, mille hulgas on tähtsal kohal lehmade optimaalne poegimisvahemik. Pärast poegimist mõjutab lehmade edukat tiinestamist oluliselt sügavkülmutatud pullisperma kvaliteet. Sügavkülmutatud pullisperma kvaliteeti võivad mõjutada omakorda mitu tegurit, näiteks aastaaeg, millal sperma varuti (Malma et al., 2017), sugupulli vanus (Helbig et al., 2007) jne. Samuti on selgunud, et mida rohkem kvaliteedinäitajaid on sügavkülmutatud/sulatatud spermide kohta, seda täpsemalt võib nende põhjal prognoosida spermide viljastamisvõimet (Rodriguez-Martinez, Barth, 2007). Kuigi paljud seemendusjaamade laborid on juba varustatud uuema spermide funktsionaalsete omaduste hindamise tehnoloogiaga, nagu liikuvuse kompuuteranalüüs (CASA), mis muudab labori töö palju tõhusamaks ja objektiivsemaks, jääb ainult selle kasutamine siiski ühekülgseks, sest põhineb vaid spermide liikumisomadustel, samuti on hinnatavate spermide arv väike (*ca* 500 rakku). Uute, suurema jõudlusega meetodite juurutamine (näiteks voolutsütomeetriline analüüs (FCA)) nõuab aga kõigepealt võrdlemist seniste, juba kasutusel olevate meetoditega. Vajalik on uurida uute meetodite sobivust nii seemendusjaama igapäevatöös kui ka nende perspektiivikust emasloomade tiinestumise prognoosimisel.

Käesoleva uurimistöö peamine eesmärk oli leida nii värske kui ka sügavkülmutatud/sulatatud spermide kvaliteeti määravate meetodite seast sellised, mida saaks rakendada seemendusjaama tööprotsessis ja sobiksid samas ka emasloomade tiinestumise prognoosimiseks nii üksiktestina kui ka matemaatilise mudeli komponendina. Selleks uuriti, milline on sügavkülmutatud/sulatatud spermide membraani terviklikkus,

liikumiskarakteristikud, spermimembraani stabiilsus ja mitokondriaalne aktiivsus ning nende näitajate seost emasloomade tiinestumisega. Selgitati, kas on võimalik sügavkülmutatud/sulatatud spermide kvaliteedinäitajate põhja koostada matemaatiline mudel spermide viljastamisvõime hindamiseks. Täiendavalt uuriti, kuidas mõjutavad sügavkülmutatud/sulatatud spermide kvaliteeti pulli vanus ja sperma kogumise aastaaeg ning holsteini verelisuse suurenemine sugupulli põlvnemises. Samuti selgitati, millisel määral erineb samade pullide suguselekteeritud spermide kvaliteet selekteerimata spermide omast.

Sugupulli vanuse mõju selgitamiseks sügavkülmutatud/sulatatud sperma kvaliteedinäitajatele uuriti 15 sugupulli 45 ejakulaati. Pullid jaotati kolme rühma: 1–2-aastased (7 pulli), 3–4-aastased (6 pulli) ja 5−7-aastased (2 pulli).

Sperma kogumise aastaaja mõju kindlakstegemiseks uuriti 15 pulli 45 ejakulaati. Sügavkülmutatud/sulatatud spermide kvaliteedinäitajaid määrati talvel (detsember, jaanuar, veebruar), kevadel (märts, aprill, mai), suvel (juuni, juuli, august) ja sügisel (september, oktoober, november).

Holsteini verelisuse suurenemise mõju sügavkülmutatud/sulatatud spermide kvaliteedile uuriti noorpullidel, kes olid jagatud kahte rühma: I rühm 87,5–93,8% ning II rühm 100% holsteini veresusega.

Spermide liikumiskarakteristikute, osmoresistentsuse, spermimembraani stabiilsuse, mitokondrite aktiivsuse ja tiinestumise vaheliste seoste kindlakstegemiseks uuriti 17 sugupulli 49 ejakulaati. Nendest ejakulaatidest valmistatud seemendusdoosidega tegid neli erinevat seemendustehnikut ühe kalendriaasta jooksul neljas erinevas karjas 3850 katseseemendust (keskmiselt 226 seemendust pulli kohta ja 77 seemendust ejakulaadi kohta). Tiineks loeti emasloomad, kes ei innelnud uuesti 60 päeva jooksul pärast seemendamist (NRR-60). Tiinestusandmeid ei korrigeeritud aastaaja, piirkonna ega karja järgi.

Spermide morfoloogilise kvaliteedi hindamiseks kasutati Spermaci (Stain Enterprises, Inc., Wellington, USA) värve ja tootjafirma soovitatud metoodikat. Pärast preparaadi kuivatamist uuriti seda valgusmikroskoobi abil 1000-kordse suurendusega ja igast preparaadist analüüsiti kokku 100 spermi. Spermidel fikseeriti järgmised defektid: pea kuju, sabata sperm,

akrosoomi ja kaela defektid, proksimaalse ja distaalse tsütoplasma tilgakese olemasolu, keha- ja sabaosa defektid (joonised 2, 3).

Spermide membraani terviklikkuse kindlakstegemiseks värskes ja sügavkülmutatud spermas kasutati nii traditsioonilist hüpoosmootset testi HOT-1 (Jeyendran et al., 1984) kui ka selle modifikatsioone HOT2 (Padrik, 1999) ning HOT-3 (Padrik, Jaakma, 2000). Valmistatud märgpreparaadist loendati pundunud sabaga spermid (joonis 4) 1000-kordsel suurendusel faaskontrastmikroskoobiga (Olympus BX40, Jaapan). Igast preparaadist loendati 100 spermi ja pundunud spermide osakaal avaldati protsentides kahe preparaadi keskmisena.

Spermide liikumiskarakteristikud sügavkülmutatud/sulatatud pullispermas määrati kompuuteranalüüsi (Computer Assisted Cell Motion Analyser, Sperm Vision, Minitüb GmbH&CO, Saksamaa) abil. Määrati järgmised näitajad: liikuvate spermide (LS) protsent, otseliikuvate spermide (OLS) protsent, spermide kiirus liikumisteekonnal (SKL) (µm/s), spermide kiirus sirglõigul (SKS) (µm/s), spermide otseliikuvus – SOL (SOL = SKS/SKL), spermide kõrvalekaldeamplituud liikumistrajektoorist (SKA) (µm), spermide ristumissagedus liikumistrajektooriga (SRL) (Hz), võnkuva liikumisega spermid – VLS (VLS = SKT/SKL; spermide kiirus trajektooril – SKT µm/s). Visuaalselt hinnati mikroskoobi all ka subjektiivselt liikuvate spermide protsent (subjektiivselt hinnatud spermide liikuvus protsentides – SubL%).

Spermimembraani stabiilsuse määramiseks kasutati voolutsütomeetriat ja spermid värviti Merocyanine 540 värvinguga (Harrison et al., 1996). Detektorite FL 1 / FL 2 (Yo-PRO 1 / M-540) kohta koostati punktdiagrammide ala, et diferentseerida elusad stabiilse plasmamembraaniga spermid – ESM-id (Yo-PRO 1 negatiivne ja M-540 negatiivne), elusad ebastabiilse plasmamembraaniga spermid - EVM-id (Yo-PRO 1 negatiivne ja M-540 positiivne) ja surnud spermid (Yo-PRO 1 positiivne; Harrison et al., 1996).

Spermide mitokondriaalse aktiivsuse määramiseks kasutati Hallapi et al. (2005) kirjeldatud metoodikat. Punktdiagrammil SYBR14 / MitoTracker Deep Red (FL 1 / FL 3) määrati kindlaks väikse (VMA) ja suure mitokondriaalse aktiivsusega spermid (SMA).

Spermide kromatiini (DNA) stabiilsuse määramiseks kasutati Evensoni et al. (1980) metoodikat. Spermid värviti akridiinoranžiga (Merck, Kebo Lab, Stockholm, Rootsi). Voolutsütomeetri abil määrati roheline fluorestsents (FL1, anormaalne DNA) ja punane fluorestsents (FL3, normaalne DNA), mis väljendati DNA fragmentatsiooni indeksina DFI%.

Eestis aretatavate sugupullide vanuse mõju selgitamine sperma ja spermide kvaliteedile on sellepärast tähtis, et sugupulli ealiste iseärasuste tõttu saab ja peab korrigeerima seemendusdooside tootmise intensiivsust ja kvaliteeti ning seeläbi on võimalik tõhustada sugupulli kasutamist.

Meie uuringud näitasid, et kõige enam oli liikuvaid ja otseliikuvaid sperme sügavkülmutatud/sulatatud spermas 3–4- ja 5–7-aastaste pullide vanuserühmas võrreldes 1–2-aastaste pullidega, kuigi oluline erinevus ilmnes ainult liikuvate spermide osas ejakulaatide lõikes ($P < 0{,}05$). Ka spermide spetsiifilised liikuvusparameetrid SKL ja SKA kasvasid pulli vanuse suurenedes 1 aastast kuni 3–4 aastani ja sealt edasi kuni 5–7 aastani. Väikest vähenemistendentsi 5–7-aastastel pullidel võrreldes 3–4-aastastega näitas SKL. Samuti oli stabiilse membraaniga ja suure mitokondriaalse aktiivsusega spermide osakaal kõige suurem 5–7-aastaste pullide vanuserühmas. Mitmed autorid on ka varem täheldanud sugupulli vanuse ja spermide liikuvuse vahelist seost (Hallap et al., 2004; Devkota et al*.,* 2008). Hallap et al. (2005) leidsid oma uuringus, et pullide vanus mõjutab ka spermide mitokondriaalset aktiivsust, kuid nad ei tuvastanud spermide plasmamembraani stabiilsuse puhul pullide vanuserühmade vahel erinevust (Hallap et al*.,* 2006). Liikuvate ja otseliikuvate spermide osakaalu suurenemine pulli vanuse tõustes võib olla tingitud sugupulli jätkuvast kasvust ning arengust, millega kaasneb ka munandite kasv ja ümbermõõdu suurenemine. Devkota et al. (2008) ja Lozano et al. (2008) leidsid oma uurimustes, et munandite ümbermõõt on tugevalt seotud sugupulli kehamassi ja vanusega. Forsberg (1996) ja Andrade et al. (2008) märkisid, et munandi ümbermõõdu suurenedes suureneb ka vereplasma testosteroonisisaldus. Üksikute sperma liikuvuskarakteristikute vähenemistendents 5–7-aastaste sugupullide rühmas võrreldes 3–4-aastaste pullidega võib olla tingitud spermatogeneesi reguleerivate hormoonide, folliikuleid stimuleeriva hormooni, luteiniseeriva hormooni ja testosteroonisisalduse (Hafez and Hafez, 2000) vähenemisest või kõikumisest (Forsberg, 1996) sugupulli vananedes (artikkel **I**).

Sperma kvaliteedi sõltuvust aastaaegadest iseloomustasid spetsiifiliste liikuvusparameetrite suuremad väärtused sügistalvisel perioodil võrreldes kevadsuvise perioodiga ($P < 0,05$). Samas selgus, et kõige rohkem suure mitokondriaalse aktiivsusega sperme esines sügisel kogutud sügavkülmutatud/sulatatud pullispermas, erinedes kõige enam kevadel varutud ejakulaatidest (16,0 protsendipunkti võrra; $P < 0,001$).

Muutused spermide kvaliteedinäitajate osas aastaaegade lõikes võivad olla tingitud asjaolust, et kevadsuvine temperatuuri tõus võib põhjustada mitmete hormoonide sisalduse kõikumist. Temperatuuritõusust põhjustatud kuumastressi mõju spermatogeneesile on uuritud munandite lühiajalise kinnikatmise katsetes (Newton et al., 2010; Shojaei Saadi et al., 2013). Lühiajalise munandite kinnikatmise tagajärjel tõusis munandites temperatuur 1–2,3 °C võrra (Newton et al., 2010). Selgus, et lühiajaline temperatuuritõus mõjutas oluliselt spermatogeneesi, põhjustades ebanormaalse peakujuga spermide osakaalu suurenemist. Selle üheks põhjuseks võib olla Leydigi rakkudes sünteesitava hormooni testosterooni sisalduse kõikumine, mille tagajärjel vähenes spermatogeneesi käigus raku struktuurvalgu aktiini produktsioon. Nii leidsid Shojaei Saadi et al. (2013), et pärast lühiajalist munandite kinnikatmist varutud ejakulaatides oli morfoloogiliselt ebanormaalse peakujuga spermide populatsioonis raku struktuurvalgu aktiini katteproteiini (CAPZB) oluliselt vähem kui enne munandite kinnikatmist.

Oleme oma varasemas uuringus (Padrik, 2001) leidnud, et koos holsteini verelisuse suurenemisega sugupulli põlvnemises suureneb ka patoloogilise morfoloogiaga spermide osakaal värskes pullispermas ($P < 0,001$). Samuti tegime kindlaks (Padrik, Jaakma, 2001), et 100% holsteini verelisusega sugupullid on aastaaegadest tuleneva välistemperatuuri kõikumise suhtes vastuvõtlikumad, sest patoloogilise morfoloogiaga spermide osakaal värskes pullispermas suurenes kevadsuvisel perioodil palju rohkem kui pullidel, kelle põlvnemises jäi holsteini verelisus alla 96,9%. Käesolevast uuringust selgus, et 87,5−93,8% holsteini verelisusega noorpullide rühmas olid spermide spetsiifilisemad funktsionaalsed kvaliteedinäitajad sügavkülmutatud/sulatatud pullispermas paremad kui 100% holsteini verelisusega pullide rühmas. Rühmi võrreldi ejakulaatide lõikes. Statistiline erinevuste tõenäosus ilmnes LS-i, OLS-i, ESM-i ja SMA osas ($P < 0,05$; artikkel **II**). Holsteini verelisuse osakaalu suurenemist sugupulli põlvnemises ning selle mõju sperma ja spermide kvaliteedile on küll suhteliselt vähe uuritud, kuid holsteini

verelisuse osakaalu suurenemist emasloomade põlvnemises ning seega ka võimalust inbriidingu koefitsiendi suurenemiseks ja fertiilsusnäitajate mõjutamiseks on täheldanud mitmed uurijad. Nii leidsid Cassell et al. (2003), et holsteini verelisus ei mõjuta pärast poegimist oluliselt päevade arvu lehmade esmakordse seemenduseni. Paljud uurijad täheldasid inbriidingu mõju mitmetele lehmade fertiilsusnäitajatele nagu poegimisvahemik, seemenduste arv, poegimisraskused, surnultsünnid ja ümberindlemine (Wall et al*.,* 2005; González-Recio *et al*., 2007; Mc Parland et al*.,* 2007*;* 2009).

Üks mitte eriti töömahukas ja lihtne meetod spermide funktsionaalsete omaduste hindamiseks on spermimembraanide terviklikkuse kindlakstegemine hüpoosmootse testi (HOT) abil (Jeyendran et al., 1984). Hüpoosmootse testi sobivust erinevate põllumajandusloomade värske ja sügavkülmutatud sperma kvaliteedi hindamisel on rõhutanud mitu autorit (Engel, Petzoldt, 1994; Mladenovic et al*.,* 1995; RodriguesMartinez, 1998; Neild et al*.,* 1999; Lagares et al*.,* 2000). Test annab hea ülevaate sellest, kui suurel osal spermidest on pärast sügavkülmutamist ja sulatamist säilinud füsioloogiliselt normaalsed membraanid. Selle näitaja alusel võib kaudselt hinnata sperma viljastusvõimet nii veistel (Correa et al*.,* 1994; Mandal et al*.,* 2003; Hu *et al.,* 2010), hobustel (Neild et al*.,* 1999), sigadel (Gadea and Matas, 1998) kui ka inimestel (Avery et al*.,* 1990; Check et al*.,* 1992; Hossain *et al.,* 1999; Moskovtsev et al*.,* 2005). Sügavkülmutatud sperma HOT testi tulemuste ja tiinestumise vahel on mitu autorit leidnud positiivse korrelatsiooni (Revell, Mrode, 1994; Januskauskas et al*.,* 1996; Correa et al*.,* 1997 a, b; Trojacanec et al*.,* 2000).

Spermide kvaliteedi hindamiseks HOT testi sobivust uurides selgus, et värskes spermas intaktse membraaniga spermide osakaalu ja sügavkülmutatud/sulatatud spermide liikuvusparameetrite vahel oli tugev positiivne seos (joonis 8, artikkel **III**). Sellise seose olemasolu oli peamine initsiaator, mille põhjal täiustati seemendusjaamas tootmisprotsessi. Seemendusjaamas saadi täiustatud tehnoloogia rakendamisel ühest ejakulaadist rohkem seemendusdoose, milles oli ka enam otseliikuvaid sperme (Padrik et al*.,* 2012 a, b).

Meie uuringus näitasid HOT testi tulemused, et sügavkülmutatud/sulatatud spermid käituvad hüpotoonilises lahuses (150 mOsm/kg +37 °C juures 60 minutit) erinevalt. Pärast inkubeerimist esines lahuses nii liikumatuid pundumata sabaga sperme, liikumatuid ja tugevalt pundunud

sabaga sperme, liikuvaid ja vähepundunud sabaga sperme kui ka liikuvaid pundumata sabaga sperme. Vähepundunud ja pundumata sabaga liikuvate spermide esinemine näitas, et katses kasutatud hüpotooniline lahus ei põhjustanud veel kõigi eeldatavalt terve membraaniga spermide pundumist. Ka pundumisaste ei olnud ühesugune ja sellele on varem viidanud ka teised autorid (Correa et al., 1994), näidates samuti, et spermid punduvad hüpotoonilistes lahustes erinevalt (Neild et al., 1999; Amorim et al., 2009). Saadud tulemused andsid tõuke HOT testi modifitseerimiseks. Traditsioonilise hüpoosmootse testi modifitseerimise vajalikkusele on varem viidanud ka Engel ja Petzoldt (1994) ning Neild et al. (1999). Meie modifitseeritud HOT-2 ja HOT-3 testi kasutamisel suurenes NaCl kontsentratsiooni vähendamisel 0,7−0,4% kõikides ejakulaatides pundunud sabaga spermide hulk lineaarselt. Edasisel NaCl kontsentratsiooni vähendamisel 0,4–0,2% NaCl lahuses (130–66 mOsm kg^{-1}) ilmnesid aga ejakulaatide vahel suured erinevused: osas ejakulaatides suurenes pundunud sabaga spermide hulk, teistes jäi see stabiilseks või isegi vähenes (artikkel **III**). Viimane variant on seletatav teatud osa spermimembraanide purunemisega vee sissevoolu toimel, kui osmootne rõhk langes. Vastupidi, pundunud sabaga spermide osakaalu suurenemine annab tunnistust spermimembraanide vastupidavusest. Nendes ejakulaatides, kus osmootse rõhu alanedes suurenes pundunud sabaga spermide hulk, olid ka seemendustulemused paremad. Nii selgus, et HOT-1 testi puhul oli tervikliku membraaniga spermide osakaalu ja emasloomade tiinestumise vahel ejakulaatide lõikes keskmine positiivne korrelatsioon (r = 0,37, P < 0,01), kuid pullide lõikes selline seos ei ilmnenud. Sama näitaja HOT-2 testi puhul ejakulaatide lõikes oli (r = 0,63–0,66; P < 0,01–0,001) ja pullide lõikes (r = 0,63–0,73; P < 0,01–0,001; **III**, **IV**). HOT-3 testi tulemuste ja emasloomade tiinestumise vaheline korrelatsioon ejakulaatide lõikes oli (r = 0,65–0,66; P <0,01–0,001) ja pullide lõikes (r = 0,57–0,71; P < 0,05–0,01; **III**, **IV**). Seega on spermimembraanide vastupidavus hüpoosmootsetes lahustes otseselt seotud spermide viljastusvõimega. Meie uuringust selgus, et spermimembraani terviklikkus määratuna HOT-2 testiga sõltub suurel määral sugupulli vanusest (P < 0,05). Sügavkülmutatud/sulatatud pullispermas määratud liikuvate spermide osakaalu ja emasloomade tiinestumise vahel ilmnes keskmine ning tugev positiivne korrelatsioon, seda nii ejakulaatide kui ka pullide lõikes (r = 0,50–0,73; P < 0,05–0,001). Teiste hinnatud kvaliteedinäitajate (LS, OLS, SKL, SKA, ESM ja SMA) ja emasloomade tiinestumise vahel esines keskmine positiivne korrelatsioon. Ka Correa et al. (1997 a, b) ja Januskauskase et al. (2003) läbi viidud

uurimustest selgus, et liikuvate spermide osakaal sügavkülmutatud/sulatatud spermas ja emasloomade tiinestumine on omavahel seotud (vastavalt $r = 0{,}53$ ja $r = 0{,}61$). Nii spermimembraani stabiilsuse kui ka mitokondriaalse aktiivsuse ja emasloomade tiinestumise vahel ilmnes statistiliselt oluline positiivne korrelatsioon. Nagu meie uuringus, leidsid ka Kasai et al. (2002) inimeste spermide *in vitro* viljastamisvõimet uurides, et suure mitokondriaalse aktiivsusega spermidel on parem viljastamisvõime. Erinevalt meie uuringu tulemustest ei leidnud Hallap et al. (2005, 2006) statistiliselt olulist korrelatsioon ESM-i ja SMA spermide ega emasloomade tiinestumise vahel. Seega on teadusandmed tihti vastuolulised ja tulemused sõltuvad uuritud populatsioonist, loomade ning spermapartiide arvust ja variatsioonist.

Sügavkülmutatud/sulatatud pullispermas olevate tervikliku membraaniga spermide osakaalu ja emasloomade tiinestumise vahel oli meie uuringus nõrk positiivne korrelatsioon. Tervikliku membraaniga spermide osatähtsust sügavkülmutatud/sulatatud pullispermas tuleb lugeda siiski tähelepanuväärseks, sest see annab hea ülevaate spermimembraanide võimest täita oma funktsiooni (Pommer et al*.,* 2002), korreleerudes hästi teiste kvaliteedinäitajate (Moskovtsev et al*.,* 2005; Zuge et al., 2008) ja ka emasloomade tiinestumisega (Revell, Mrode, 1994; Correa et al*.,* 1997 a, b; Lagares et al*.,* 2000; Perez-Liano *et al.,* 2001). Lihtsa metoodika tõttu sobib see hästi ka sperma viljastamisvõime prognoosimiseks seemendusjaamas. Meie uurimusest selgus, et emasloomade tiinestumise prognoosimiseks sobis kõige paremini sügavkülmutatud/sulatatud spermide seitsme funktsionaalse parameetri põhjal koostatud mudel. Sellesse mudelisse kuulusid ΔHOT-2, SubL, OL, OSL, SKA, ESM ja SMA. Mudeli põhjal prognoositud tiinestumise ja tegeliku tiinestumise vahel ilmnes tugev positiivne korrelatsioon ($r = 0{,}96$; $P < 0{,}001$; artikkel **IV**). Erinevaid mudeleid on varem koostanud ka mitmed teised uurijad (Zhang et al*.,* 1999; Januskauskas et al., 2000; Phillips et al*.,* 2004), lähtudes sügavkülmutatud/sulatatud spermide liikuvuse ja membraani terviklikkuse parameetritest. Zhang et al. (1999) leidsid oma uuringutes, et spermide liikuvus pärast sügavkülmutamist/sulatamist sobib hästi sperma viljastamisvõime hindamise mudelisse, kusjuures korrelatsioon emasloomade tegeliku tiinestumise ja prognoosimismudeli vahel oli $r = 0{,}94$ ($P < 0{,}001$) ning kohaldatud determinatsioonikordaja $R^2 = 0{,}71$. Ka Januskauskas et al. (2000) märkisid oma uuringus, et spermide liikuvus ja membraani funktsionaalne terviklikkus pärast sügavkülmutamist/sulatamist sobisid hästi emasloomade

tiinestumise prognoosimismudelisse ning selle mudeli korrelatsioon emasloomade tiinestumisega oli r = 0,74 (P < 0,002; kohaldatud determinatsioonikordaja: R^2 = 0,55). Phillips *et al.* (2004) leidsid austraalia piimatõugu sugupullide sügavkülmutatud/sulatatud spermat uurides, et morfoloogiliselt normaalsete ja tervikliku membraaniga spermide põhjal koostatud viljastamisvõime prognoosimismudel kirjeldas emasloomade tiinestumise variatsiooni 76,5% ulatuses. Zhangi et al. (1999) koostatud emasloomade tiinestumise prognoosimismudeli ja emasloomade tegeliku tiinestumise vaheline tugev korrelatsioon (r = 0,94; P < 0,001) tuleneb tõenäoliselt sellest, et mudel koosnes kaheksast erinevast sperma funktsionaalsest kvaliteedinäitajast.

Tänapäeval on genoomselektsiooni kõrval suguselekteeritud sperma kasutamine üks tõhusamaid aretusmeetodeid. Ainuke tänapäeval kommertsiaalselt kasutatav meetod sugupoole 90% kallutamiseks põhineb spermi DNA-s olevate X- ja Y-kromosoomide massi erinevusel. Esimesed teated edukast X- ja Y-kromosoomide massi alusel spermide eraldamisest pärinevad 1983. aastast (Johnson *et al.,* 1987). Meie uuringust selgus, et HOT-1 ja SubL spermide osakaal on suguselekteeritud spermas palju väiksem kui tavaspermas (P < 0,01). Samas selgus, et LS-i ja OLS-i spermide osakaal on tavaspermas palju suurem kui suguselekteeritud spermas (P < 0,01; artikkel **V**).

See, et spermide liikumisparameetrite ja HOT-1 väärtused olid suguselekteeritud spermas väiksemad, võib olla tingitud sellest, et sorteerimise käigus saavad spermide membraanid kahjustada. Samas selgus, et võrreldes kahjustatud DNA-ga spermide osakaalu suguselekteeritud ja tavaspermas, statistilist erinevust ei olnud. Selline spermide kvalitatiivne erinevus tava- ja suguselekteeritud spermas võib põhjustada erinevust ka emasloomade tiinestumisel. Meie uuringust selgus, et suguselekteeritud spermas liikuvate spermide (LS) osakaalu ja mullikate tiinestumise vahel ilmnes tugev positiivne korrelatsioon (r = 0,82; P < 0,09). Mitmest uuringust on selgunud, et suguselekteeritud spermaga seemendades on tiinestumine 60–80% tavaspermaga seemendamise tulemusest (Seidel et al*.,* 1997; 1999; DeJarnette *et al.,* 2008; Shenk et al*.,* 2009).

Pulli vanus avaldab mõju sügavkülmutatud/sulatatud spermide kvaliteedile. Sugupulli vanuse mõju sperma ja spermide kvaliteedile on tähtis arvesse võtta muuhulgas sellepärast, et sugupulli ealiste iseärasuste

tõttu saab ja peab korrigeerima seemendusdooside tootmise intensiivsust ning seeläbi on võimalik tõhustada sugupulli kasutamist. Pulli sperma varumise intensiivsust korrigeeritakse Kehtna seemendusjaamas sõltuvalt pulli east, aastaaegadest ja pulli individuaalsetest iseärasustest.

Sperma varumise aastaaeg ja pulli holsteini verelisus avaldavad mõju sügavkülmutatud/sulatatud spermide kvaliteedile, seepärast ongi Kehtna kunstlikus seemendusjaamas korraldatud tootmisprotsess nii, et enamik seemendusdoose toodetakse sügistalvisel perioodil, mis mõjutab positiivselt toodangu kvaliteeti.

Värskes pullispermas tervikliku membraaniga spermide (HOT1) ja otseliikuvate spermide osakaalu vahel sügavkülmutatud/sulatatud spermas leiti tugev positiivne korrelatsioon. Sellise seose olemasolu oli seemendusjaamas tootmisprotsessi täiustamise peamine katalüsaator. Täiustatud tehnoloogia rakendumisel oli võimalik toota ühest ejakulaadist ~15% võrra enam seemendusdoose ning ühtlasi suurenes otseliikuvate spermide osakaal sügavkülmutatud/sulatatud spermas ~20 % (joonised 8, 9, 10).

Hüpoosmootse testi modifikatsioon HOT-2 on sobiv sügavkülmutatud/sulatatud sperma hindamiseks oma odavuse, lihtsuse ja kiiruse poolest, seega igati sobiv kasutamiseks seemendusjaama igapäevatöös.

Hüpoosmootse testi ja selle modifikatsioonide ning spermide liikuvusparameetrite põhjal moodustatud mudel sobib spermide viljastamisvõime hindamiseks. Tänu selliste mudelite väljatöötamisele on ka seemendusdooside kvaliteedi üle polemiseerimisel võimalik tuua piisavalt argumente, et vaidlusaluses seemendusdoosis on emaslooma õigeaegse tiinestumise tagamiseks piisavalt viljastamisvõimelisi sperme.

Seemendusjaamas spermide hindamiseks kasutatavate testide ja voolutsütomeetriga määratud spermide mitokondriaalse aktiivsuse ning membraani stabiilsuse vahel leiti positiivne korrelatsioon. Tänu selliste seoste väljaselgitamisele on võimalik garanteerida seemendusjaamas toodetud seemendusdooside tippkvaliteet, mis on vajalik emasloomade õigeaegseks tiinestumiseks. Pärast sügavkülmutamist/sulatamist hinnatakse spermidel peamiselt kompuuteranalüüsiga 2–3 parameetrit. Kompuuteranalüüsi tulemusel saadud parameetrid seostuvad väga hästi

voolutsütomeetriliste testide tulemustega, andes seega laiapõhjalise ja kindla aluse üldhinnangu kujundamisel spermide kvaliteedi kohta.

Spermide liikuvusparameetrite, HOT-2 ning HOT-3 tulemuste ja emasloomade tiinestumise vahel leiti tugev positiivne korrelatsioon. Keskmise tugevusega korrelatsioon leiti spermide mitokondriaalse aktiivsuse, membraani stabiilsuse ja emasloomade tiinestumise vahel,

Kõige parem mudel spermide viljastamisvõime hindamiseks moodustus kombinatsioonist, mis hõlmas nii seemendusjaamas kasutatavaid teste kui ka voolutsütomeetriga määratud spermide kvaliteeti.

Nii tervikliku membraaniga spermide osakaal kui ka spermide liikuvusparameetrid olid tavaspermas paremad kui suguselekteeritud spermas. Tavaliste laboritestide abil määratud spermide kvaliteeti tavaspermas võrreldi samade näitajatega suguselekteeritud spermas ja ilmnes, et erinevused olid selgepiirilisemad ning nende erinevuste statistiline tõenäosus suurem kui voolutsütomeetrilise analüüsi puhul. See tunnistab, et seemendusjaama labori lihtsad ja kiired tavatestid iseloomustavad spermi membraanikahjustusi suguselekteerimisprotsessi käigus küllaltki hästi. Seepärast on otstarbekas ja jätkusuutlik kasutada suguselekteeritud sperma viljastamisvõime hindamiseks seemendusjaama laboris matemaatilist mudelit, mis on kombinatsioon HOT-1 ja spermide liikuvuse kompuuteranalüüsi parameetritest, mida on sügavkülmutatud/sulatatud sperma viljastamisvõime hindamisel edukalt kasutatud.

Suguselekteerimistehnoloogiate rakendamisega või uute väljatöötamisega tulevikus on seemendusjaamas tööprotsessi käigus otstarbekas selgitada värskes pullispermas välja need spermide kvaliteedinäitajad, mis vastaksid selekteerimisnõuetele, ja markeerida pullid, kelle sperma sobib suguselekteerimiseks. Meie uuringud on näidanud, et selleks sobivad hästi nii CASA-ga hinnatud spermide funktsionaalsusparameetrid kui ka HOT-1.

Uuringutest saadud teadmiste ja kogemuste rakendamine seemendusjaama tehnoloogilises protsessis on mõjutanud veiste kunstliku seemenduse süsteemi tulemusi Eestis laiemalt. Seda, et lehmade piimatoodangu suurenemine mõjutab oluliselt nende tiinestumist pärast poegimist, on ammu teada fakt. Aastatel 2005-2010 langes lehmade ja mullikate tiinestumine 56,2%-lt 52,0%-ni (JKK 2006-2013), samal ajal suurenes

lehmade piimatoodang 6502 kg-lt 7613 kg-ni. Seejärel tõusis lehmade tiinestumine aastatel 2011-2013 52,0%-lt 54,4%-ni, mis on püsinud suhteliselt stabiilsena (üle 52,6%) siiani (2018), samal ajal suurenes lehmade piimatoodang 7756 kg-lt 9785 kg-ni (joonised 11, 12; JKK, 2006-2013; JKK, 2014; EPJ 2015-2019).

Üks tähtsamaid lehmade ja mullikate tiinestumise stabiliseerumise põhjusi on kindlasti see, et Kehtna seemendusjaamas rakendati seemendusdooside tootmiseks teadusuuringute põhjal täiustatud tehnoloogiat ehk teisisõnu teaduspõhist tootmisprotsessi.

Tänu täiustatud tehnoloogia rakendamisele tõusis seemendusdoosides otseliikuvate spermide osakaal aastatel 2010-2013 64%-lt 78 %-ni ning on püsinud stabiilsena siiani. Tänu sellele suurenes nii ejakulaadist toodetud seemendusdooside arv kui ka otseliikuvate spermide osakaal pärast sügavkülmutamist/sulatamist, mis omakorda on lehmade ja mullikate tiinestumise hoidnud stabiilsena (üle 52,6%).

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I

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SÜGAVKÜLMUTATUD/SULATATUD SPERMIDE KVALITEEDINÄITAJATE SEOS SESOONSUSE JA SUGUPULLI VANUSE NING EMASLOOMADE TIINESTUMISEGA

Peeter Padrik [a,b*], Triin Hallap[b], Tanel Bulitko[a], Ülle Jaakma[b]

[a] *Eesti Tõuloomakasvatajate Ühistu, 79005 Keava, Eesti*
[b]*Sigimisbioloogia osakond, Veterinaarmeditsiini ja Loomakasvatuse Instituut, Eesti Maaülikool, Kreutzwaldi 62, 51014 Tartu, Eesti*
**tel.+3727313466; fax +3727313706; e-mail: peeter.padrik@mail.ee*

ABSTRACT. Influence of bull age and season to frozen-thawed semen quality and fertility. *The aim of the current study was to investigate the influence of season and bull age to sperm motility, membrane integrity, membrane lipid architecture status and mitochondrial membrane potential characteristics in frozen-thawed (FT) semen, collected from Estonian Holstein (EHF) bulls. Forty five ejaculates from 15 (1–7 years) EHF bulls were examined for motility (objectively using a computer assisted motility analyzer (CMA)), hypo-osmotic swelling (HOS), membrane lipid architecture status (Merocyanine 540 staining) and mitochondrial membrane potential (Mitotracker Deep Reed 633 staining). Stained spermatozoa were assessed by FCM (flow cytometry). The results of the study showed that the increase in bulls' age from 1–2 years to 5–7 years was accompanied by the increase in general motility (GMot), membrane stability (LSM) and mitochondrial activity (MTDR-H)(P<0.05), curve line velocity (VCL; P<0.01), linearity (LIN) and amplitude of lateral head displacement (ALH; P<0.001) on batch level. The quality of spermatozoa in FT samples varied between the seasons. The mean values for VCL, ALH and MTDR-H were higher in winter and autumn on both batch and bull level (P<0.05). In relationship with NRR (non-return rate 60-day), strongest correlation was obtained for the ALH results on bull level (P<0.001). We conclude that the bulls' age and season of semen collection have an effect on sperm quality parameters. Frozen/thawed sperm motility parameters, LSM and MTDR-H are related to NRR of cows and heifers and could be used for the prediction of bull's fertility.*

Key words: *Bull, Sperm quality, Age, Season, Fertility*

Sissejuhatus

Piimatootmise efektiivsus ei sõltu ainult väärtusliku geneetilise materjali olemasolust, vaid ka paljudest teistest faktoritest, mille hulgas on tähtsal kohal lehmade optimaalne poegimisvahemik. Lehmade edukat tiinestamist pärast poegimist mõjutab oluliselt sügavkülmutatud pullisperma kvaliteet. Sügavkülmutatud pullisperma kvaliteeti mõjutavad omakorda mitmed tegurid nagu aastaaeg, millal sperma varuti (Mandal *et al*., 2003; Padrik, Jaakma 2004; Koivisto *et al.,* 2009), sugupulli vanus (Pant *et al.,* 2003; Hallap *et al*., 2004; Helbig *et al*., 2007) jt. Samuti on selgunud et, mida rohkem on kvaliteedinäitajaid sügavkülmutatud/sulatatud spermide kohta, seda täpsemalt võib nende põhjal prognoosida spermide viljastamisvõimet (Rodriguez-Martinez, 2006; Rodriguez-Martinez and Barth, 2007). Kuigi paljud seemendusjaamade laborid on juba varustatud uuemate spermide funktsionaalsete omaduste hindamise tehnoloogiatega nagu liikuvuse kompuuteranalüüs (*CASA),* mis muudab labori töö oluliselt efektiivsemaks ja objektiivsemaks, jääb ainult selle kasutamine siiski ühekülgseks, sest põhineb vaid spermide liikumisomadustel, samuti on hinnatavate spermide arv väike (ca 500 rakku). Uute, suurema jõudlusega meetodite juurutamine nõuab aga kõigepealt seniste, juba kasutusel olevate meetodite võrdlust uutega. Vajalik on testida uute meetodite sobivust nii seemendusjaama igapäevatöösse kui ka nende perspektiivikust emasloomade tiinestumise prognoosimisel.

Paljud autorid on oma uuringutes näidanud voolutsütomeetria efektiivsust ja täpsust spermide kvaliteedi hindamisel (Hallap *et al.,* 2006; Kasimanickam et al., 2006; Peña et al., 2007). Selle tehnoloogia puhul hinnatakse ühe analüüsiga keskmiselt 10000 rakku kiirusega 200 rakku/s. Kui spermide liikuvuse hindamine CASA abil annab objektiivse ülevaate nii värske kui ka sügavkülmutatud/sulatatud sperma kvaliteedist (Muiño et al., 2008) ning selle tulemused korreleeruvad hästi emasloomade tiinestumisega (Januskauskas et al., 2003), siis voolutsütomeetriliseks analoog-testiks spermide funktsionaalsuse hindamiseks oleks spermi keskosas asuvate mitokondrite membraanipotentsiaali (MMP) määramine. Kõrge MMP viitab intensiivsele energia tootmisele, mida spermatosoid vajab edasiliikumiseks teel viljastuspaika (Vishwanath et al., 1986; Hua et al., 2006; Wu et al., 2006). Hallap et al. (2005) täheldas oma uurimistöös, et spermide mitokondriaalse aktiivsuse määramine MitoTracker Deep Red 633 ja voolutsütomeetri abil oli igati sobilik spermide liikumisvõime kaudseks hindamiseks kuna korreleerus hästi *CASA* tulemustega.

Spermimembraani funktsionaalse terviklikkuse hindamiseks on mitmeid võimalusi*:* traditsiooniline hüpoosmootne test (Jeyendran et al., 1984), selle modifikatsioonid (Padrik, 1999; Petrunkina *et a*l., 2001; Amorim *et al*., 2009), spermide värvimine fluorestseeruvate kemikaalidega (Brito *et al*., 2003) jne. Sügavkülmutatud/sulatatud spermide membraanide terviklikkuse kõrval on mebraani stabiilsus oluliseks kvaliteedinäitajaks spermide funktsionaalsuse hindamisel (Hallap *et al*., 2005). Selle määramise üheks võimaluseks on kasutada Merocyanine 540 (M540*)* värvingut ja voolutsütomeetriat, mis võimaldab kindlaks teha, kui suurel määral on toimunud fosfolipiidide ümberpaigutumine

spermimembraani siseselt. Merocyanine 540 värvimisel suureneb fluorestsents vastavalt sellele, mida rohkem on membraanis ümberpaigutunud fosfolipiide (Harrison *et al*., 1996).

Kui eelnevates uuringutes (Padrik *et al.,* 2000; Padrik, 2001; Padrik, Jaakma 2002; 2004) oleme välja selgitanud pulli vanuse ja aastaaja mõju spermide morfoloogilisele kvaliteedile ning liikuvusparameetritele, siis käesoleva uuringu põhirõhk on suunatud uutele, suurema jõudlusega sügavkülmutatud/sulatatud spermide kvaliteeti hindavatele meetoditele ning spermi teistele funktsionaalsusparameetritele nagu membraani stabiilsus ja mitokondriaalne aktiivsus.

Käesoleva töö eesmärgiks oli välja selgitada pulli vanuse ja sperma kogumise aastaaja mõju eesti holsteini tõugu pullide sügavkülmutatud/sulatatud spermide membraani terviklikkusele, liikumiskarakteristikutele, spermimembraani stabiilsusele ja mitokondriaalsele aktiivsusele ning nende näitajate seost emasloomade tiinestumisega

Materjal ja metoodika

Pullid, varutud ejakulaadid ja sperma töötlemine

Selgitamaks sugupulli vanuse mõju sügavkülmutatud/sulatatud sperma kvaliteediparameetritele uuriti 15 sugupulli 45 ejakulaati. Pullid jaotati kolme gruppi: 1–2 aastased (7 pulli), 3–4 aastased (6 pulli) ja 5–7 aastased (2 pulli).

Sperma kogumise aastaaja mõju kindlakstegemiseks uuriti 15 pulli 45 ejakulaati. Sügavkülmutatud/sulatatud spermide kvaliteedinäitajaid määrati talvel (detsember, jaanuar, veebruar), kevadel (märts, aprill, mai), suvel (juuni, juuli, august) ja sügisel (september, oktoober, november).

Spermide liikumiskarakteristikute, osmoresistentsuse, spermimembraani stabiilsuse, mitokondrite aktiivsuse ja tiinestumise vaheliste seoste kindlakstegemiseks uuriti 13 sugupulli 36 ejakulaati. Nendest ejakulaatidest valmistatud spermadoosidega tehti 2828 katseseemendust (keskmiselt 218 seemendust pulli kohta ja 79 seemendust ejakulaadi kohta) 4 erinevas karjas 4 erineva seemendustehniku poolt ühe kalendriaasta jooksul (1999 ja 2001 aastal). Tiineks loeti emasloomad, kes ei innelnud uuesti 60 päeva jooksul pärast seemendamist (NRR-60). Tiinestusandmeid ei korrigeeritud olenevalt aastaajast, piirkonnast ja karjast. Erinevate ejakulaatide NRR-60 varieerus 22.8 kuni 80.0%-ni.

Sperma lahjendamiseks kasutati Triladyl'i (*Minitüb GmbH&CO, Germany*) ja munarebu (Kehtna Mõis OÜ, Eesti) lahjendit. Värske sperma lahjendati pärast viieminutilist temperatuuride ühtlustamist lahjendi ja sperma vahel (+35°C vesivannis) vahekorras 1:1. Teine lahjendamine toimus 15 minutit hiljem toatemperatuuril (+ 20°C). Lahjendit lisati niipalju, et ühte seemendusdoosi jääks ~30×10^6 spermi. Seejärel asetati lahjendatud sperma külmikusse (+4°C). Kahetunnilise jahutamise järel pakendati sperma 0.25 ml spermakõrrekestesse (*Minitüb GmbH&CO, Germany).* Pärast kahetunnilist ekvilibreerumist spermakõrrekesed sügavkülmutati ning säilitati vedelas lämmastikus –196°C juures.

Spermide membraani terviklikkuse määramine

Funktsionaalselt tervikliku membraaniga spermide osakaalu määramiseks kasutati traditsioonilist hüpoosmootset testi HOT (Jeyendran *et al.,* 1984). Kaks spermakõrrekest sulatati +35°C juures vesivannil 20 sekundi jooksul ja tühjendati katseklaasi 1 ml HOT lahusesse (0.735 g naatriumtsitraati (*Sigma-Aldrich Laborchemikalien GmbH, Germany*), 1.351 g fruktoosi (*Merck KGaA, Germany*), 100 ml destilleeritud vett; lahuste osmootne rõhk 150m0s, kg^{-1}). Pärast hoolikat segamist vorteksi loksutis (*VORTEX, Europe*) asetati katseklaas termostaati (*Memmert GmbH, Germany*) ning inkubeeriti 60 minutit +37°C juures. Seejärel lisati katseklaasi 0.3 ml eosiini (*0.99%, Pioneer Research Chemicals, Ltd. England*), valmistati märgpreparaat ja loendati pundunud sabaga spermid 1000–kordsel suurendusel faaskontrastmikroskoobis (*Olympus BX40, Japan*). Igast preparaadist loendati 100 spermi ning pundunud spermide osakaal avaldati protsentides kahe preparaadi keskmisena.

Spermide liikumiskarakteristikute määramine CASA abil

Spermide liikumiskarakteristikud sügavkülmutatud/ sulatatud pullispermas määrati kompuuteranalüüsi (*Computer Assisted Cell Motion Analyser, Sperm Vision*, *Minitüb GmbH&CO, Germany*) abil. Spermakõrreke sulatati +35°C juures 20 sekundi jooksul ja uuriti Makleri kambris (*Makler Counting Chamber, Sefi-Medical Instruments, Israe*l) 400× suurendusel iga proovi 4–5 erinevalt väljalt kokku ~400 spermi. Määrati järgmised näitajad: liikuvate spermide (LS) % */ Motility (GMot %)*; otseliikuvate spermide % (OLS; %)/ *Progressive Motility (PMot %);* spermide kiirus liikumisteekonnal (SKL, µm/s) /*Velocity Curve Line (VCL, µm/s);* spermide otseliikuvus (SOL, SKS/SKL)/ *Linearity LIN (VSL/VCL);* spermide kõrvalekaldeamplituud liikumistrajektoorist (SKA, µm)/ *Amplitude of Lateral Head Displacement (ALH, µm).*

Spermimembraani stabiilsuse määramine

Spermimembraani stabiilsuse (ESM) määramiseks valmistati 1mM *Merocyanine 540 (M-540; Molecular Probes, M24571, Leiden, Holland) ja 25 µM Yo-PRO 1 (Molecular Probes, Y3603 Leiden, The Netherlands)* põhilahused dimetüülsulfoksiidis (*DMSO; AppiliChem; Germany).* Pestud spermidele lisati 25 nM *Yo-PRO* 1 ja inkubeeriti 38°C juures pimedas 9 min (Harrison et al., 1996). Seejärel lisati 10 µL 40 µM *M-540 lahust SP-TALP*-is, et saada lõplik *M-540* kontsentratsioon 2.7 µM ja segati 10 s. enne voolutsütomeetris *(FacsCalibur, Becton Dickinson, San Jose, USA)* analüüsimist. Andmete kogumist alustati 60 sekundit pärast *M-540* lisamist. Mõõtmised tehti voolutsütomeetriga, mis oli varustatud standardsete optiliste laseritega. Merocyanine-*540* ja *Yo-PRO 1* ergastati argoonioon 488 laseriga 15 mW juures. Otse- ja kõrvalhajuvuse väärtused toodi lineaarskaalale ja

fluorestseeruvad väärtused logaritmskaalale. Maksimaalse tundlikkuse jaoks sätestati neeldunud kiirguse ala, et saavutada L–kujuline otsevalgus (hajuv/külgsuunaline valgus hajutab spermide jaotumise). *Yo-PRO 1* fluorestsents määrati detektoris *FL* 1 (530/28nm *BP*), samal ajal kui *M-540* fluorestsents määrati detektoris *FL 2* (585/2 nm *BP*). Igast spermaproovist tehti 10000 mõõtmist, voolukiirusega ca 200 rakku/s. Kasutati *CellQuest Pro* tarkvara *(Becton Dickinson, San Jose, USA)*. Punkt-diagrammid autonoomseteks analüüsideks tehti *WinMDI 2.8.* abil *(free software by J. Trottre, available at http://facs.scripps.edu./software. html*). Detektorite *FL 1/FL 2 (Yo-PRO 1/M-540)* kohta koostati punktdiagrammide ala, et diferentseerida elusad stabiilse plasmamembraaniga ESM *(Yo-PRO 1* negatiivne ja *M-540* negatiivne); elusad ebastabiilse plasmamembraaniga EVM (*Yo-PRO 1* negatiivne ja *M-540* positiivne) ja surnud spermid (*Yo-PRO 1* positiivne).

Spermide mitokondriaalse aktiivsuse määramine

Spermide mitokondriaalse aktiivsuse (KMA) määramiseks kasutati Hallap *et al.* (2005) poolt kirjeldatud metoodikat. Mõõdistamised tehti *FacsCalibur* voolutsütomeetris *(Becton Dickinson, San Jose, USA). SYBR-14* (*Sperm Viability Kit L-7011, Molecular Probes Inc., Eugene, OR, USA)* värvain ergastati 15 mW argoonioon 488 nm laseriga, samal ajal kui *MitoTracker Deep Red* ergastati 17 mW *HeNe* 633 nm laseriga. *SYBR-14* fluorestsents (tervikliku plasmamembraaniga rakud) määrati kindlaks detektoris *FL 1* (530/28 nm) ja *MitoTracker Deep Red* fluorestsents (kõrge mitokondriaalne aktiivsus) määrati kindlaks detektoris *FL 3* (670 *LP*). Otse- ja kõrvalhajuvuse väärtused toodi lineaarskaalal ja fluorestseeruvad väärtused logaritmskaalal. Tasakaalustamine tehti vastavalt Roedererile (2000). Kasutati *CellQuest Pro* tarkvara *(Becton Dickinson, San Jose, USA*). Voolutsütomeetrit kasutati madalal voolukiirusel (6–24 µL/min). Tehti ~10000 *SYBR-14*-positiivset mõõtmist ja andmed salvestati tulevasteks analüüsideks. Punktdiagrammil FL1/FL2 eristati spermid muudest partiklitest *SYBR-14* fluorestsentsi (*DNA* sisaldus) alusel. Punktdiagrammil *SYBR-14/MitoTracker Deep Red (FL 1/FL 3)* määrati kindlaks madala (MMA) ja kõrge mitokondriaalse aktiivsusega spermid (KMA).

Statistiline analüüs

Uuringute tulemuste statistilises analüüsis kasutati erinevuste olulisuse hindamiseks *t*-testi ja dispersioonanalüüsi. Tunnustevahelised erinevused loeti tõenäoseks, kui $P<0.05$ (* kui $P<0.05$; ** kui $P<0.01$; *** kui $P<0.001$). Tunnustevaheliste seoste hindamiseks kasutati Pearsoni korrelatsioonikordajat. Tunnustevahelist seost loeti järgnevalt: nõrk seos, kui $|r| \leq 0.3$; keskmine seos, kui $0.3 < |r| < 0.7$; tugev seos, kui $|r| \geq 0.7$.

Tulemused

Pulli vanuse mõju sügavkülmutatud/sulatatud spermide liikumiskarakteristikutele

Erinevas vanuses sugupullidelt kogutud sperma analüüs näitas, et vanuse suurenedes paranesid oluliselt mitmed spermide liikumisparameetrid (tabel 1). Nii oli liikuvate spermide osakaal ejakulaatide lõikes 5–7 ja 3–4 aastastel pullidel oluliselt suurem võrreldes 1–2 aastaste pullidega ($P<0.05$). Spermide kvaliteedinäitajad SKL, SOL ja SKA olid samuti kõrgemad 3–4 ja 5–7-aastaste pullide vanusegrupis, erinedes oluliselt (vastavalt; $P<0.01$; $P<0.001$; $P<0.001$) 1–2-aastaste pullide sügavkülmutatud/sulatatud sperma samadest näitajatest. Samuti selgus, et ka sperma plasmamembraani stabiilsus ja mitokondrite aktiivsus olid ejakulaatide lõikes oluliselt kõrgemad 5–7 aastastel pullidel erinedes 1–2 aastaste pullide samadest näitajatest ($P<0.05$) ning 3–4 aastaste pullide tulemustest ESM osas ($P<0.05$).

Funktsionaalselt tervikliku membraaniga spermide osas ei leitud vanusegruppide vahel statistilist erinevust, kuigi näitaja oli parem 1–2 aastastel pullidel. Uurimistulemustest selgus, et pullide lõikes olid vanusegruppide vahelised erinevused statistiliselt nõrgemini väljendunud, kuid järgisid üldjoontes samu trende kui ejakulaatide vahelises võrdluses**.** Oluliselt kõrgemad olid SKL ja SKA väärtused 3–4 ja 5–7-aastaste pullide vanusegrupis võrreldes 1–2-aastaste pullidega kusjuures statistiline erinevuste tõenäosus oli vastavalt $P<0.05$ ja $P<0.01$. Samas oli spermide SOL oluliselt suurem 1–2 aastaste pullidel võrreldes 3–4 aastastega ($P<0.01$) ja 5–7-aastastega ($P<0.05$).

Tabel 1. Pulli vanuse mõju sügavkülmutatud/sulatatud spermide kvaliteedile ejakulaatide lõikes
***Table 1**. Influence of bulls' age on frozen/thawed sperm quality characteristics; batch level (means±S.D.)*

Spermide kvaliteedinäitajad	Pulli vanus aastates/*Age of bulls (year)*		
Sperm quality characteristics	1–2	3–4	5–7
Ejakulaate/*No of ejaculates*	*n*=19	*n*=17	*n*=9
HOT(%)/ *HOS(%)*	36.4 ± 9.3	31.2 ± 10.0	33.9 ± 9.8
LS(%)/ *GMot(%)*	71.5 ± 12.3[b]	78.9 ± 7.6[a]	78.4 ± 9.2[a]
OLS(%)/ *PMot(%)*	55.1 ± 15.2	62.7 ± 7.7	61.7 ± 9.3
SKL (µm/sek)/ *VCL(µm/sec)*	88.3 ± 7.9[c]	100.7 ± 10.2[d]	93.3 ± 3.9[d]
SOL/*LIN*	0.51 ± 0.04[f]	0.45 ± 0.03[e]	0.45 ± 0.03[e]
SKA(µm)/ *ALH(µm)*	2.7 ± 0.3 [c,f]	3.1 ± 0.3[e]	3.0 ± 0.5[d]
ESM(%)/ *LSM(%)*	51.3 ± 20.8[a]	55.6 ± 7.6[a]	65.1 ± 11.8[b]
KMA(%)/ *MTDR-H(%)*	66.9 ± 23.7[a]	78.4 ± 12.9	82.6 ± 7.6[b]

HOT–funktsionaalselt tervikliku membraaniga spermid LS–liikuvad spermid; OLS–otseliikuvad spermid; SKL–spermide kiirus liikumisteekonnal; SOL–spermide otseliikuvus, SKA–spermide kõrvalekaldeamplituud liikumistrajektoorist; ESM–elusad stabiilse membraaniga spermid; KMA–kõrge mitokondriaalse aktiivsusega spermid;
HOS –intact plasma membranes; Gmot–general motile; PMot–progressively motile; VCL–curve line velocity; LIN–linearity; ALH–amplitude of lateral head displacement; LSM–live stable membrane; MTDR-H–high mitochondrial activity;
[a, b, c, d, e ,f] Erinevate ülaindeksitega väärtused samas reas on statistiliselt erinevad/Values with different superscripts in a row are significantly different ([a, b] –P<0.05; [c, d] –P<0.01; [e, f]–P<0.001).

Aastaaegade mõju sügavkülmutatud/sulatatud spermide liikumiskarakteristikutele.
Spermide liikumiskiirused SKL ja SKA olid ejakulaatide lõikes suve-, sügise- ja talvekuudel (P<0.05) oluliselt kõrgemad võrreldes kevadega, mitokondrite aktiivsus oli kõrgem sügis-talvisel perioodil võrreldes kevad-suvise perioodiga (P<0.05–P<0.001). Samuti näitasid sarnast tendentsi liikuvate ja otseliikuvate spermide mõõtmistulemused.

Pullide lõikes ilmnes taas, et enamus mõõdetud kvaliteediparameetritest olid sügis-talvisel perioodil kõrgemad võrreldes kevad-suvise perioodiga. Statistiliselt oluline erinevus ilmnes ainult sügisel varutud sperma kõrge mitokondriaalse aktiivsusega spermide osakaalu puhul võrreldes kevadel varutud spermide sama näitajaga (P<0.001).

Tabel 2 Aastaaegade mõju sügavkülmutatud/sulatatud spermide kvaliteedile ejakulaatide lõikes
***Table 2.** Seasonal variation in frozen/thawed sperm motility characteristics on batches level (means*±S.D.)

Spermide kvaliteedinäitajad / *Sperm quality characteristics*	Aastaaeg/*Season*			
	Kevad Spring	Suvi *Summer*	Sügis *Autumn*	Talv *Winter*
Ejakulaate/*No of ejaculates*	*n*=26	*n*=7	*n*=3	*n*=9
HOT(%)/ *HOS(%)*	34.8 ± 8.9	33.5 ± 11.5	30.0 ± 0	31.1 ± 11.6
LS(%)/ *GMot(%)*	74.8 ± 10.9	71.4 ± 14.5	83.0 ± 6.3	79.3 ± 7.0
OLS(%)/ *PMot(%)*	58.3 ± 12.5	55.1 ± 15.1	65.0 ± 7.5	63.4 ± 8.6
SKL(µm/sek)/ *VCL(µm/sec)*	91.7 ± 10.0[b]	98.03 ± 10.8[a]	97.2 ± 4.4[a]	100.0 ± 8.7[a]
SOL/*LIN*	0.49 ± 0.04	0.48 ± 0.07	0.44 ± 0.01	0.47 ± 0.03
SKA(µm)/ *ALH(µm)*	2.8 ± 0.3[b]	3.0 ± 0.4[a]	3.0 ± 0.2[a]	3.1 ± 0.3[a]
ESM(%)/ *LSM(%)*	56.1 ± 19.3	48.7 ± 20.6	67.5 ± 7.6	56.9 ± 10.9
KMA(%)/ *MTDR-H(%)*	72.5 ± 18.8[b,e]	63.9 ± 18,2[b]	89.5 ± 1.5[a,f]	82.9 ± 7.1[a]

HOT–funktsionaalselt tervikliku membraaniga spermid LS–liikuvad spermid; OLS–otseliikuvad spermid; SKL–spermide kiirus liikumisteekonnal; SOL–spermide otseliikuvus, SKA–spermide kõrvalekaldeamplituud liikumistrajektoorist; ESM–elusad stabiilse membraaniga spermid; KMA–kõrge mitokondriaalse aktiivsusega spermid;
HOS –intact plasma membranes; Gmot–general motile; PMot–progressively motile; VCL–curve line velocity; LIN–linearity; ALH–amplitude of lateral head displacement; LSM–live stable membrane; MTDR-H–high mitochondrial activity;
[a, b, c, d, e ,f] Erinevate ülaindeksitega väärtused samas reas on statistiliselt erinevad/Values with different superscripts in a row are significantly different ([a, b] –P<0.05; [c, d] –P<0.01; [e, f]–P<0.001).

Emasloomade tiinestumise ja sügavkülmutatud/sulatatud spermide kvaliteedikarakteristikute vaheline seos
Sügavkülmutatud/sulatatud spermas määratud liikuvate ja otseliikuvate spermide osakaalu ja emasloomade tiinestumise vahel oli keskmise tugevusega positiivne korrelatsioon nii pullide kui ka ejakulaatide lõikes (tabel 3). Spermide spetsiifiliste liikumiskarakteristikute osas leiti keskmise tugevusega korrelatsioon SKA ja SKL ning emasloomade tiinestumise vahel. Kõige tugevam oli see näitaja SKA ja emasloomade tiinestumise vahel pullide lõikes r=0.77 (P<0.001). Uurimusest selgus, et ESM ning KMA ja emasloomade tiinestumise vahel ilmnes samuti oluline positiivne korrelatsioon (P<0.05; tabel 3) ejakulaatide lõikes.

Tabel 3. Emasloomade tiinestumise ja sügavkülmutatud/ sulatatud spermide kvaliteedinäitajate vaheline seos
Table 3. *Correlations between sperm quality characteristics in frozen/thawed semen and 60-days NRR*

Spermide kvaliteedinäitajad/ *Sperm quality characteristics*	Tiinestumise %/*60-days non-return rate (NRR)%*			
	Ejakulaatide lõikes/ *Batch level*		Pullide lõikes/ *Bull level*	
	n=36		*n*=13	
	r		*r*	
HOT(%)/ *HOS(%)*	0.10		0.01	
LS(%)/ *GMot(%)*	0.70	***	0.73	**
OLS(%)/ *PMot(%)*	0.64	***	0.64	*
SKL(µm/sek)/ *VCL (µm/sec)*	0.67	***	0.75	**
SOL/*LIN*	–0.49		–0.66	
SKA(µm)/ *ALH(µm)*	0.63	***	0.77	***
ESM(%)/ *LSM(%)*	0.45	**	0.32	
KMA(%)/ *MTDR-H(%)*	0.49	**	0.51	

HOT–funktsionaalselt tervikliku membraaniga spermid; LS–liikuvad spermid; OLS–otseliikuvad spermid; SKL–spermide kiirus liikumisteekonnal; SOL–spermide otseliikuvus, SKA–spermide kõrvalekalde-amplituud liikumistrajektoorist; ESM–elusad stabiilse membraaniga spermid; KMA–kõrge mitokondriaalse aktiivsusega spermid;
HOS–intact plasma membranes; Gmot–general motile; PMot–progressively motile; VCL–curve line velocity; LIN–linearity; ALH–amplitude of lateral head displacement; LSM–live stable membrane; MTDR-H–high mitochondrial activity
*(*P*<0.05), **(*P*<0.01), ***(*P*<0.001).

Arutelu

Käesoleva töö eesmärgiks oli välja selgitada pulli vanuse ja sperma kogumise aastaaja mõju eesti holsteini tõugu pullide sügavkülmutatud/sulatatud spermide membraani terviklikkusele, liikumiskarakteristikutele, spermimembraani stabiilsusele ja mitokondrite aktiivsusele ning määrata nende näitajate seos emasloomade tiinestumisega.

Meie uuringud näitasid, et kõige enam oli liikuvaid ja otseliikuvaid sperme sügavkülmutatud/sulatatud spermas 3–4- ja 5–7-aastaste pullide vanuserühmas võrreldes 1–2-aastaste pullidega, kuigi statistiline erinevus ilmnes ainult liikuvate spermide osas ejakulaatide lõikes (*P*<0.05). Mitmed autorid on ka varem täheldanud sugupulli vanuse ja spermide liikuvuse vahelist seost (Hallap *et al*., 2004; Devkota *et al.,* 2008). Meie uuringust selgus, et ka spermide spetsiifilised liikuvusparameetrid SKL, SOL ja SKA kasvasid pulli vanuse suurenedes 1-aastast kuni 3–4-aastani ja sealt edasi kuni 5–7-aastani. Väikest langustendentsi 5–7-aastastel pullidel võrreldes 3–4-aastastega näitas SKL. Uuringust selgus samuti, et stabiilse membraaniga ja kõrge mitokondriaalse aktiivsusega spermide osakaal oli kõige suurem 5–7-aastaste pullide vanusegrupis. Ka Hallap *et al*. (2005) leidis oma uuringus, et pullide vanus mõjutab spermide mitokondriaalset aktiivsust, kuid ESM puhul ei olnud pullide vanusegruppide vahelist erinevust (Hallap *et al.,* 2006). Liikuvate ja otseliikuvate spermide osakaalu suurenemine pulli vanuse suurenedes võib olla tingitud sugupulli jätkuvast kasvust ja arengust, millega kaasneb ka munandite kasv ja ümbermõõdu suurenemine. Devkota *et al*. (2008) ja Lozano *et al.* (2008) leidsid oma uurimustes, et munandite ümbermõõt on tugevalt seotud sugupulli kehamassiga ja vanusega. Forsberg (1996) ja Andrade *et al.* (2008) märkisid, et munandi ümbermõõdu suurenedes tõuseb ka testosterooni tase vereplasmas. Munandi ümbermõõdu suurenemine sugupulli kasvades ja vereplasma testosteroonisisaldus mõjutab omakorda nii spermide morfoloogilist kvaliteeti kui ka spermide liikuvust (Pinho *et al.,* 2008; Devkota *et al*., 2008). Üksikute sperma liikuvuskarakteristikute langustendents 5–7-aastaste sugupullide grupis võrrelduna 3–4-aastaste pullidega võib olla tingitud spermatogeneesi reguleerivate hormoonide – folliikuleid stimuleeriva hormooni, luteiniseeriva hormooni ja testosterooni (Hafez and Hafez, 2000) taseme langusest või kõikumisest (Forsberg, 1996) sugupulli vananedes.

Sperma kvaliteedi sõltuvust aastaaegadest iseloomustasid spetsiifiliste liikuvusparameetrite suuremad väärtused sügis-talvisel perioodil võrreldes kevad-suvise perioodiga (*P*<0.05). Samas selgus, et kõige rohkem kõrge mitokondriaalse aktiivsusega sperme esines sügisel kogutud sügavkülmutatud/sulatatud pullispermas, erinedes kõige enam kevadel varutud ejakulaatidest (16.0% võrra; *P*<0.001). Aastaaegade mõju spermide kvaliteedile on märkinud paljud autorid (Mandal *et al.,* 2003; Janett *et al.,* 2003a,b; Koonjaenak *et al*., 2007a,b; Koivisto *et al*., 2009). Janett *et al.* (2003a,b) leidis oma uuringus, et suvel oli liikuvaid ja morfoloogiliselt normaalseid sperme täkuspermas vähem kui teistel aastaaegadel (*P*<0.05). Koonjaenak *et al*. (2007b), uurides vesipühvlite spermat, leidis, et spermide liikumiskiirus SKL oli juulis-oktoobris (vihmaperioodil) suurem kui suvisel perioodil (märts-juuni; *P*<0.05–0.001). Samast uurimusest selgus statistiline erinevus ka spermi plasmamembraani terviklikkuse ja stabiilsuse osas, mis oli talvisel perioodil (november-veebruar) parem võrreldes nii suvise kui ka vihmaperioodiga. Mandal *et al*. (2003) märkis oma uurimuses, et pühvlite spermas oli liikuvate spermide osakaal talvisel perioodil (november-märts) suurem kui teistel aastaegadel. Muutused spermide kvaliteedinäitajate osas olenevalt aastaajast võivad olla tingitud asjaolust, et kevad-suvine temperatuuri tõus võib põhjustada mitmete hormoonide taseme kõikumist. Shubbu*r et al*. (1989) leidsid oma uuringus, et testosterooni tase sugupulli vereplasmas oli kõige kõrgem detsembris. Park Yi, (2002), uurides kultide sperma kvaliteeti täheldas, et testosterooni tase oli kõige kõrgem kevadel (*P*<0.05) ning leidis samas, et ka spermide morfoloogia, liikuvus ja kontsentratsioon oli samuti kõrgeimad kevadel. A*x et al*. (1987) andmeil põhjustab suvine kõrge temperatuur patoloogiliste spermide esinemissageduse suurenemist ja spermide liikuvuse vähenemist. Ka Godfre*y et al.* (1990) leidis, et testosterooni-taseme kõikumine sugupulli vereplasmas sõltub aastajast, kuid Koivis*to et al.* (2009) ei leidnud aastaaegade mõju testosteroonitaseme kõikumisele.

Meie uurimusest selgus, et sügavkülmutatud/sulatatud pullispermas määratud liikuvate spermide osakaalu ja emasloomade tiinestumise vahel esines tugev korrelatsioon, seda nii ejakulaatide kui ka pullide lõikes (vastavalt *r*=0.70 ja *r*=0.73; *P*<0.001). Teiste hinnatud kvaliteedinäitajate (otseliikuvate spermide osakaal, SKL, SKA, ESM ja KMA) ja emasloomade tiinestumise vahel esines keskmine positiivne korrelatsioon. Ka

Correa *et al*. (1997) ja Januskauskase *et al.* (2003) poolt läbiviidud uurimustest selgus, et liikuvate spermide osakaal sügavkülmutatud/sulatatud spermas ja emasloomade tiinestumine on omavahel seotud (vastavalt r=0.53 ja r=0.61). Meie uuringust selgus, et nii spermimembraani stabiilsuse kui ka mitokondriaalse aktiivsuse ja emasloomade tiinestumise vahel ilmnes statistiliselt oluline positiivne korrelatsioon. Sarnaselt meie uuringule, leidis ka Kasai *et al*. (2002), uurides inimeste spermide *in vitro* viljastamisvõimet, et kõrge mitokondriaalse aktiivsusega spermidel on parem viljastamisvõime Erinevalt meie uuringust selgunule ei leidnud Hallap *et al.* (2005, 2006) statistiliselt olulist korrelatsioon ESM ja KMA spermide ja emasloomade tiinestumise vahel. Seega on teadusandmed tihti vastuolulised ja tulemused sõltuvad uuritud populatsioonist, loomade ja spermapartiide arvust ning variatsioonist. Sügavkülmutatud/sulatatud pullispermas olevate tervikliku membraaniga spermide osakaalu ja emasloomade tiinestumise vahel oli meie uuringus nõrk positiivne korrelatsioon. Tervikliku membraaniga spermide osatähtsust sügavkülmutatud/sulatatud pullispermas tuleb siiski lugeda oluliseks, kuna see annab hea ülevaate spermimembraanide võimest oma funktsiooni täita (Pommer *et al.,* 2002) korreleerudes hästi teiste kvaliteedinäitajatega (Moskovtsev *et al.,* 2005; Zuge *et al*., 2008) ja ka emasloomade tiinestumisega (Revell, Mrode, 1994; Correa *et al.,* 1997; Lagares *et a.l,*. 2000; Perez-Llano *et al.,* 2001) ning lihtsa metoodika tõttu sobib hästi sperma viljastamivõime prognoosimisks seemendusjaamades (Brito *et al*., 2003; Tartaglione, Ritta, 2004).

Kokkuvõte

Sugupulli vanus mõjutab oluliselt liikuvate spermide osakaalu ja spetsiifiliste liikumiskarakteristikute SKL, SOL, SKA, väärtusi sügavkülmutatud/sulatatud pullispermas. Nimetatud parameetrite keskmised väärtused on kõrgeimad 3–4-aastastel pullidel. ESM ning KMA näitajad on aga kõige suuremad 5–7-aastastel pullidel.

Liikuvate ja otseliikuvate spermide osakaal, spetsiifiliste liikumiskarakteristikute näitajad (SKL, SOL, SKA) ja ESM ning KMA sügavkülmutatud/sulatatud pullispermas sõltuvad sperma kogumise aastaajast.

Sügavkülmutatud/sulatatud spermas määratud liikuvate ja otseliikuvate spermide osakaalu SKL, SOL, SKA, ESM ning KMA ja emasloomade tiinestumise vahel olid keskmise tugevusega positiivne korrelatsioon ejakulaatide lõikes.

Sugupulli vanus ning aastaaeg, millal sperma varuti, mõjutab oluliselt spermide kvaliteeti sügavkülmutatud/sulatatud spermas. Optimaalne oleks deponeerimiseks suunatav sügavkülmutatud pullisperma varuda 3–7-aastatelt pullidelt ning sügis-talvisel perioodil. Sügavkülmutatud/sulatatud spermide liikumisparameetrite, mitokondriaalse aktiivsuse ning membraani stabiilsuse ja emasloomade tiinestumise vahel oli postiivne seos, seepärast sobivad need parameetrid emasloomade tiinestumise prognoosimiseks

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Influence of bull age and season to frozen-thawed semen quality and fertility

P. Padrik, T. Hallap, T. Bulitko and Ü. Jaakma

Summary

The aim of the current study was to determine the effects of bulls' age and season of semen collection on frozen/thawed sperm motility, membrane integrity, membrane lipid architecture status and mitochondrial membrane potential characteristics in frozen-thawed (FT) semen of Estonian Holstein bulls and estimate the relations between sperm quality characteristics and bulls' in vivo fertility expressed as 60-days non-return rate (NRR) of the dairy cows and heifers.

Semen was collected from 13 Estonian Holstein (EHF) bulls (1–7years) using an artificial vagina. Two consecutive ejaculates were pooled (hereafter referred to as a "batch"), extended with a commercial extender (Triladyl®, Minitüb, Germany), packed in 0.25 ml plastic straws, each containing ~30–40x10^6 spermatozoa, and frozen using biological freezer. Semen evaluation was performed immediately after thawing. The semen from two straws of the same batch was thawed by immersion in water +35°C 20 seconds then pooled and used for testing. Following preservation, a post-thaw motility ≥50% was set up as threshold. Forty five ejaculates were used for the test inseminations. Altogether, 2828 cows and heifers (average 79 inseminations per ejaculate and 218 inseminations per bull) were inseminated by 4 AI technicians in 4 different herds according to the breeding program. Inseminations were performed routinely within 1 year of freezing on heifers and cows of different parity during all seasons of the year. Non-return rates (NRRs) 60 days after AI were recorded for each batch but not corrected for season, area, and parity. Fertility values NRR-60 days ranged from 22.8 to 80,0%.

Traditional hypo-osmotic test (Jeyendran, 1984), labelled as HOS, was performed by incubating a contents of 2 frozen/thawed semen straws together with 1 ml of a 150mOsm kg^{-1} hypoosmotic solution at 37°C for 60 min. After incubation, 0.3 ml of eosin was added into the test tube. Wet preparation was evaluated under the phase contrast microscope (×1000) and the ratio of spermatozoa with swollen tails was expressed in % as an average of two replicates. One hundred spermatozoa were assessed in each replicate.

Sperm motility characteristics were determined with a computer assisted motility analyzer (Computer Assisted Cell Motion Analyzer (CMA), Sperm Vision, Minitüb GmbH&Co, Germany). Samples of 5µl were placed in Makler chamber where ~400 post-thaw spermatozoa were tracked and assessed (x 400) at +37°C. The following parameters were determined: the percentage of general motile (GMot) and progressively motile (PMot) spermatozoa, curve line velocity (VCL, µm/sec), linearity LIN(VSL/VCL) and amplitude of lateral head displacement (ALH, µm).

Sperm plasma membrane stability. The following working solutions were prepared: Merocyanine 540 (M-540; Molecular Probes, M24571, Leiden, The Netherlands) 1mM in dimethyl sulfoxide (DMSO) and Yo-PRO 1 (molecular Probes, Y3603) 25 µM in DMSO. Washed spermatozoa were stained with 25 nM Yo-PRO 1 and incubated at 38°C for 9 min in the dark as previously described (Harrison et al.1996). Thereafter 10 µL of a 40 µM solution of M-540 in SP-TALP was added to give a final M-540 concentration of 2.7 µM and vortexed for 10 s before analysis on a flow cytometer (FacsCalibur, Becton Dickinson, San Jose, USA). Data collection was started at 60 s after M-540 addition. Measurements were made with a flow cytometer, equipped with standard optical lasers as excitation sources. The M-540 and Yo-PRO 1 dyes were excited by an Argon ion 488 nm laser running at 15 mW. Forward and side scatter values were recorded on a linear scale; while florescent values were recorded on a logarithmic scale.

Obscuration bars were set for maximum sensitivity in order to obtain L-shaped forward light – scatter/sideways light scatter distribution of sperm cells. Fluorescence of Yo-PRO 1 was detected on detector FL 1 (530/28nm BP), while M-540 fluorescence was detected on detector FL 2 (585/2 nm BP). From each sample, a total of 10,000 events were measured with flow rate of approx. 200 cells/s. Acquisitions were made using CellQuest Pro software (Becton Dickinson, San Jose, USA). Dot plots for offline analyses were drawn by WinMDI, version 2.8. Events accumulated in the lower left corner correspond to sample debris and were excluded from the analysis by gating. On FL 1/FL 2 (Yo-PRO 1/M-540) dot plots regions were set to differentiate viable, stabile plasma membrane LSM (Yo-PRO 1 negative and M-540 negative); viable, scrambled plasma membrane (Yo-PRO 1 negative and M-540 positive); and dead (Yo-PRO 1 positive) events.

Sperm mitochondrial activity. The staining protocol was identical to that described by Hallap et al. (2005). The measurements were made using a FacsCalibur flow cytometer

(Becton Dickinson, San Jose, USA). The SYBR-14 dye was excited by a 15 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 by a 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 made using the CellQuest Pro software (BD).Non-sperm events were gated out based on SYBR-14 fluorescence (DNA content). The FC was used at a 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. On SYBR-14 (FL 1/FL 2) dot plots, regions were drawn around the SYBR-14-positive cluster, and these events were classified as spermatozoa. In SYBR-14/ MitoTracker Deep Red dot plots sperm cells with low MTDR-L) and high (MTDR-H) Deep Red fluorescence were specified.

Results. The results of the study showed that the increase in bulls' age from 1–2 years to 5–7 years was accompanied by the increase in the proportions of motile sperm (GMot), sperm with stable membrane (LSM) and high mitochondrial activity (MTDR-H) (Table 1; $P<0.05$), curve line velocity (VCL), amplitude of lateral head displacement (ALH) ($P<0.01$) and linearity (LIN) ($P<0.001$) on batch level. Season of semen collection had significant influence on frozen/thawed sperm motility characteristics (Table 2). The overall sperm motility was significantly higher in ejaculates collected in autumn and winter. The means of VCL, ALH and MTDR-H were higher in winter and autumn on batch and bull level ($P<0.05$). Medium correlations were observed between the GMot, PMot, VCL, ALH, LSM, MTDR-H and NRR. ($r=0.49–0.70$; $P<0.01$ Table 3) on batches level and ($r=0.64–0.77$; $P<0.01$) on bull level. Strongest correlation was obtained between the ALH results and NRR on bull level ($r=0.77$; $P<0.001$).

Conclusion. We conclude that the bulls' age and season of semen collection have an effect on sperm quality parameters. Frozen/thawed sperm motility, progressive motility, VCL ALH, LSM and MTDR-H are related to NRR of cows and heifers and could be used for the prediction of bull's fertility.

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THE QUALITY OF FROZEN-THAWED SEMEN OF YOUNG A.I. BULLS AND ITS RELATION TO THE GRADE OF HOLSTEIN GENES AND FERTILITY

Peeter Padrik[1, 2, *], Triin Hallap[2], Tanel Bulitko[1], Aloyzas Januškauskas[3], Tanel Kaart[2], Ülle Jaakma[2]
[1]*Animal Breeders Association of Estonia, 79005 Keava, Estonia*
[2]*Department of Reproductive Biology, Institute of Veterinary Medicine and Animal Sciences Estonian University of Life Sciences, Kreutzwaldi 1, 51014 Tartu, Estonia*
[3]*Department of Non-Infectious Diseases, Lithuanian Veterinary Academy, Kaunas, Lithuania*
**Corresponding author: tel. +3727313466; fax +3727313706; e-mail: peeter.padrik@mail.ee*

Abstract. The aim of the current study was to investigate relationships between the grade of Holstein genes and sperm motility, membrane integrity, membrane lipid architecture status and mitochondrial membrane potential in frozen-thawed (FT) semen, collected from Estonian Holstein (EHF) dairy bulls. Nineteen ejaculates from seven young (age from 14 to 22 MO) EHF bulls were examined for motility using a computer assisted motility analyzer (CMA), hypo-osmotic swelling (HOS). Membrane lipid architecture status (Merocyanine 540 staining) and mitochondrial membrane potential (Mitotracker Deep Reed 633 staining) was assessed by flow cytometry (FCM). Fertility results were available as 60 days non-return rates (NRR). The results showed that there was a significant difference in the incidence of general motile (GMot) and progressively motile spermatozoa (PMot), viable sperms with stable membrane (LSM) and high mitochondrial activity (MTDR-H) between the bull groups with the different grade of Holstein genes at batch level. The positive correlation between PMot, LSM, MTDR-H and NRR was recorded at batch level ($P<0.05$). The strongest correlation was obtained between the curve line velocity (VCL) and NRR at bull level ($P<0.01$). A strong positive correlation was found between predicted non-return rates (PNRR) and NRR ($P<0.001$).

Keywords: dairy bull, grade of Holstein genes, semen quality.

VEISLINIŲ BULIUKŲ KRIOKONSERVUOTOS SPERMOS KOKYBĖS RYŠIO SU HOLŠTEINŲ BULIAUS GENOTIPO GENŲ DALIMI ĮVERTINIMAS IR APVAISINIMO GALIA

Peeter Padrik[1, 2, *], Triin Hallap[2], Tanel Bulitko[1], Aloyzas Januškauskas[3], Tanel Kaart[2], Ülle Jaakma[2]
[1]*Estijos gyvulių veisėjų asociacija, 79005 Keava, Estija*
[2]*Reprodukcinės biologijos katedra, Veterinarinės medicinos ir gyvulininkystės institutas Estijos gyvybės mokslų universitetas, Kreutzwaldi 1, 51014 Tartu, Estija*
[3]*Neužkrečiamųjų ligų katedra, Lietuvos veterinarijos akademija, Kaunas, Lietuva*
**Adresas susirašinėti: tel.+372 731 3466; fax +372 731 3706; el paštas: peeter.padrik@mail.ee*

Santrauka. Šio eksperimento tikslas – išanalizuoti Estijos Holšteino veislės (EHF) bulių kriokonservuotos spermos kokybinių rodiklių – spermatozoidų judrumo, membranų vientisumo, lipidų pasiskirstymo spermatozoidų membranose ir mitochondrijų membranų potencialo ryšį su Holšteino veislės buliaus genotipo genų dalimi. Buvo analizuojama 17 ejakuliatų sperma, gauta iš septynių 14–22 mėn. EHF veislės bulių. Spermatozoidų judrumas buvo vertinamas kompiuterizuota judrumo vertinimo sistema (CMA), membranų vientisumas – hipoosmotiniu testu (HOS). Lipidų pasiskirstymas spermatozoidų membranose (nudažius merocianinu 540) ir mitochondrijų membranų potencialas (nudažius „Mitotracker Deep Reed 633“ dažais) buvo vertinamas tėkmės citometru (FCM). Apvaisinimo rezultatai vertinti išvedant sėklintų ir nesurujojusių per 56 dienas po sėklinimo santykį (NRR). Gauti rezultatai byloja, kad tarp skirtingą Holšteino veislės genų dalį savo genotipe turinčių bulių statistiškai reikšmingai skiriasi bendrasis spermatozoidų judrumas (GMot), progresyvusis judrumas (PMot) gyvybingų, stabilią membraną turinčių spermatozoidų procentas (LSM) ir spermatozoidų, turinčių didelį mitochondijų aktyvumą procentas (MTDR-H). Taip pat spermos partijos lygmeniu nustatyta statistiškai reikšminga teigiama koreliacija tarp PMot, LSM, MTDR-H ir NRR ($p<0,05$). Buliaus lygmeniu didžiausio patikimumo statistiškai reikšminga koreliacija nustatyta tarp spermatozoidų greičio (VCL) ir NRR ($p<0,01$). Statistiškai reikšminga teigiama koreliacija nustatyta tarp prognozuojamo apvaisinimo (PNRR) ir eksperimento metu nustatyto apvaisinimo (NRR) rezultatų ($p<0,001$).

Raktažodžiai: bulius, Holšteino veislės genų dalis, spermos kokybė.

Introduction. Breeding success depends on the efficient use of bulls with high breeding value. At the same time, semen quality imposes restrictions on the use of bulls in AI. During the last decade, extensive use of purebred Holstein bulls in breeding of Estonian Black & White dairy cattle has improved milk yield substantially, however, the fertility of cows has declined. The fertility of Holstein dairy cows has declined mostly due to high metabolic load and negative energy balance post partum (Leroy et al., 2004). However, there are few studies published on relations between the grade of Holstein genes in a bull`s pedigree and his semen production and quality. In our earlier study (Padrik and Jaakma, 2001) the increase of grade of Holstein genes was accompanied by a decline

in the morphological quality of fresh semen in AI bulls and the sensitivity to heat stress of the bulls. As with any other dairy breed, the semen quality of Holstein bulls is affected by several different factors: season of semen collection (Mathevon et al., 1998, Padrik and Jaakma, 2004), age (Padrik et al., 2008; Devkota et al., 2008) and bull variation (Muiño et al., 2008). In most of these studies, among a variety of parameters measurable in fresh and frozen-thawed semen, sperm membrane integrity and sperm motility correlated well with the female fertility (Zhang et al. 1998; Vererckmoes et al., 2002). Sperm membrane integrity can be evaluated with several methods such as light or fluorescence microscopy combined with vital stains (Brito et al., 2003) or flow cytometry (Hallap et al., 2004). One of the simplest methods to evaluate the intactness of plasmalemma of bovine sperm is the hypo-osmotic swelling test (HOS) described by Jeyendran (1984) where intact spermatozoa "swell" under hypo-osmotic conditions due to the influx of water, and the expansion of the membranes causes the tails to coil. Another method based on estimation of sperm plasma membrane stability includes the use of the hydrophobic dye Merocyanine 540 (M540) and FCM, and results in determining the percentage of scrambling of the phospholipids in the plasma membrane lipid bilayer (Harrison et al., 1996). Estimation of sperm motility (subjectively, using light microscope or objectively, using CMA) gives a good overview of the quality of fresh and FT semen (Muiño et al., 2008) and the results usually correlate well with female fertility (Januskauskas et al., 2003). Also a measurement of mitochondrial membrane potential (organelles where ATP is synthesized) with MitoTracker Deep Red 633 using FCM has been found to be useful as another indirect measure of sperm motility (Hallap et al., 2005). The aim of the current study was to investigate relationships between the grade of Holstein genes and sperm motility, membrane integrity, membrane lipid architecture status and mitochondrial membrane potential characteristics in frozen-thawed (FT) semen, collected from young Estonian Holstein bulls, and establish how these characteristics correlate to NRR.

Materials and Methods

Animals, semen collection and processing. Nineteen ejaculates from seven Estonian Holstein (EHF) bulls (aged from 14 to 22 MO) were used for the test inseminations. To study the influence of the grade of Holstein genes on FT sperm quality the bulls were divided into two groups according to their grades as following: Group I - 87.5-93.8% of Holstein genes (three bulls, seven batches; age from 14 to 19 MO) and Group II - 100% of Holstein genes (four bulls, 12 batches; age from 14 to 22 MO). In total, tested batches were used to inseminate 1,338 cows and heifers (average 70 inseminations per batch and 191 inseminations per bull) by four AI technicians, on four different herds, according to the breeding program. Inseminations were performed routinely within one year of semen freezing on heifers and multiparous cows throughout the year. Non-return rates (NRRs) 60 days after AI were recorded for each batch but not corrected for season, area, or parity. Fertility values presented as NRR-60 days ranged from 22.8 to 69.2% at batch and 37.5 to 57.0% at bull level. Semen was collected using an artificial vagina. Two consecutive ejaculates were pooled (hereafter referred to as a "batch"), extended with a commercial extender (Triladyl®, Minitüb, Germany), packed in 0.25 ml plastic straws, each containing ~30-40×10^6 spermatozoa, and frozen in a manually regulated biological freezer according to a conventional bull semen freezing curve. The frozen straws were stored in liquid nitrogen until tested. Semen evaluation was performed immediately after thawing. Two straws of the same batch were thawed by immersion in water at +35°C for 20 s., and pooled for further analysis. Following preservation, a post-thaw motility threshold of ≥50% was set up for semen to be used for field AI.

Hypo-osmotic swelling (HOS) test Hypo-osmotic test was performed by incubating 50 µl of FT semen with 1 ml of a 150mOsm/kg hypoosmotic solution at 37ºC for 60 min as described by Jeyendran (1984). The semen straw was thawed and, following incubation at +37ºC for 60 min was mixed with 0.2 ml of Eosin (0.99%, Pioneer Research Chemicals, Ltd. England) in a test tube. Wet preparation was evaluated under the phase contrast microscope (×1000) and one hundred spermatozoa were assessed in each of three replicates. The ratio of spermatozoa with swollen tails was expressed in % as an average of three replicates.

Sperm motility. Sperm motility characteristics were determined with a computer assisted motility analyzer (Computer Assisted Cell Motion Analyzer (CMA), Sperm Vision, Minitüb GmbH&Co, Germany). Samples of 5µl were placed in Makler chamber where ~400 post-thaw spermatozoa were tracked and assessed (×400) at +38ºC. The following parameters were determined: the percentage of general motile (GMot) and progressively motile (PMot) spermatozoa, curve line velocity (VCL, µm/sec), linearity LIN (VSL/VCL) and amplitude of lateral head displacement (ALH, µm).

Sperm plasma membrane stability. The following working solutions were prepared: Merocyanine 540 (M-540; Molecular Probes, M24571, Leiden, The Netherlands) 1mM in dimethyl sulfoxide (DMSO); Yo-PRO 1 (Molecular Probes, Y3603, Leiden, The Netherlands) 25 µM in DMSO. Washed spermatozoa were stained with 25 nM Yo-PRO 1 and further incubated at 38°C for 9 min in the dark as previously described (Harrison et al.1996). Thereafter 10 µL of a 40 µM solution of M-540 in SP-TALP was added to give a final M-540 concentration of 2.7 µM and vortexed for 10 s before analysis in a flow cytometer (FacsCalibur, Becton Dickinson, San Jose, USA). Data collection was started at 60 s after M-540 addition.

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Statistical Analyses. The characteristics of observed traits were expressed as means ±S.D. The Pearson correlation test was used to calculate the correlation between different sperm parameters in fresh and FT semen and between the sperm parameters measured and field fertility (60-days NRR). The bulls were divided into two groups: Group I 87.5-93.8% grade of Holstein genes (three bulls, seven batches) and Group II 100% (four bulls, 12 batches).

The grade of Holstein genes was calculated from the pedigrees of the bulls (Новиков et al., 1969). The general linear models analyses for repeated measurements with the SAS System (version 9.1.3; SAS Institute Inc., Cary, NC) were performed to compare the mean sperm quality characteristics between bulls groups at batch level. The stepwise regression analyses were applied to find the optimal combination of sperm quality characteristics for a predictive model of non-return rates.

Results

Relations between the grade of Holstein genes and quality of FT semen at batch level

The increase in the grade of Holstein genes was accompanied by a decrease in GMot, PMot, LSM and MTDR-H in FT semen (Table 1).

Table 1. **Influence of the grade of Holstein genes, batch level**

Sperm parameters	Grade of Holstein genes				
	Group I 87.5-93.8% (n=7)		Group II 100.0% (n=12)		
	means ±S.D	range	means ±S.D	range	P
HOS	41.1±7.2	29.0-50.0	33.3±9.4	12.0-50.0	0.068
General motile (%)	79.3 ± 7.1	69.2-85.0	66.9±12.6	49.0-82.4	0.014
Progressively motile (%)	65.1± 7.6	54.1-73.1	49.3±15.7	27.8-67.7	0.009
VCL (μm/sec)	92.2±4.5	86.7-100.0	86.1±8.7	72.0-99.2	0.057
Linearity	0.51±0.02	0.49-0.54	0.51±0.04	0.47-0.60	0.870
ALH (μm)	2.7±0.2	2.4-3.1	2.7±0.4	2.1-2.7	0.910
LSM%	65.3± 9.9	48.7-81.5	43.1±20.8	17.6-75.6	0.008
MTDR-H%	81.0 ± 7.6	73.1-88.8	58.7±26.1	24.7-85.2	0.015

Relations between grade of Holstein genes and quality of FT semen at bull level

As shown in Table 2, the increase of grade of Holstein genes was accompanied by a similar tendency of decrease in HOS, GMot, PMot, VCL, LSM and MTDR-H in FT semen, however, the differences were not significant (P>0.05).

Effect of grade of Holstein genes on fertility estimated as NRR

The average non-return-rates were higher in the bull group with 87.5-93.8% grade of Holstein genes - 52.3% (range from 42.9 to 69.2%.) than in the bull group with 100.0% grade of Holstein genes - 43.9% (range from 22.8 to 56.8%). GMot, PMot, VCL, LSM and MTDR-H correlated significantly with NRR at batch level, whereas only GMot and VCL correlated significantly with NRR at bull level (Table 3).

Relationship between predicted (PNRR) and observed non-return rates (NRR) for young bulls

The most optimal PNRR for young bulls at batch level was obtained with the model including five parameters: GMot, PMot, LIN, ALH and LSM, with the following formula:

PNRR= –136.4–0.72×Pmot+1.397×GMot+174.27×LIN+9.407×ALH+0.165× LSM,

where R^2=0.73 and adjusted R^2=0.62.

A strong positive correlation was found between PNRR and NRR on batch level (r=0.86; P<0.001).

Discussion

The aim of this study was to investigate relationships between the grade of Holstein genes and sperm motility, membrane integrity, membrane lipid architecture status and mitochondrial membrane potential in frozen-thawed semen, collected from young Estonian Holstein bulls. We also looked at relationships between the different sperm quality parameters and bull fertility.

Table 2. **Influence of the grade of Holstein genes of FT semen quality, bull level**

Sperm parameters	Grade of Holstein genes				
	Group I 87.5-93.8% (n=3)		Group II 100.0% (n=4)		
	means±S.D	range	means±S.D	range	P
HOS	40.8±4.1	34.5-44.5	34.9±5.5	29.4-36.5	0.195
General motile (%)	78.5 ± 4.6	75.1-83.7	70.7±10.8	55.7-79.8	0.268
Progressively motile (%)	64.5± 4.0	61.0-64.0	54.1±14.1	34.1-66.5	0.234
VCL (μm/sec)	91.6±4.0	88.5-96.1	88.3±6.8	80.4-91.4	0.466
Linearity	0.51±0.01	0.51-0.52	0.51±0.03	0.49-0.53	0.748
ALH (μm)	2.7±0.2	2.5-2.8	2.7±0.2	2.4-2.8	0.947
LSM%	66.7±21.3	56.9-79.8	49.9±11.8	21.9-73.9	0.242
MTDR-H%	83.2±5.3	77.5-87.5	67.4±25.4	24.6-82.8	0.312

Table 3. **Correlations between the quality parameters of spermatozoa in FT semen and 60-NRR**

Sperm parameters	60-days non-return rate			
	Batch level		Bull level	
	r (n=19)		r (n=7)	
HOS	0.34	ns	0.59	
General motile (%)	0.71	***	0.85	*
Progressively motile (%)	0.64	**	0.74	ns
VCL (μm/sec)	0.58	*	0.92	**
Linearity	-0.01	ns	0.33	ns
ALH (μm)	0.35	ns	0.57	sn
LSM%	0.54	*	0.55	ns
MTDR-H%	0.57	*	0.67	ns

*(P<0.05),**(P<0.01),***(P<0.001).

The results of our earlier study (Padrik Jaakma, 2001) showed that there was a significant difference in the incidence of abnormal sperms between the bull groups with a different grade of Holstein genes (P<0.0001). The bulls with 100% of Holstein genes had the highest incidence of sperms with abnormal heads and bulls with 75.0-87.5% of Holstein genes had the lowest incidence of sperm abnormalities. The same study found that the incidence of abnormal sperms in semen of bulls with 100% of grade Holstein genes increased by 4% in summer compared to winter. The respective increase for the bulls with 75.0-87.5% and 87.5-96.9% of Holstein genes was 2.56 and 3.10% (P<0.0001), showing that an increase in the grade of Holstein genes might be accompanied by an increase in sensitivity of spermatogenesis to heat stress (Padrik, Jaakma, 2001).

We have chosen HOS as a basic test to evaluate the differences between the bulls and the prognostic value of the test for fertility. Several authors have emphasized the suitability of the hypo-osmotic test for evaluation of the quality of human semen (Moskovtsev et al., 2005) as well as the semen of different farm animal species such as cattle (Mandal et al., 2003), horses (Lagares et al., 2000) pigs (Gadea et al., 1998), and rabbits (Amorim et al., 2009). The test gives a good overview of the proportion of spermatozoa with functionally active membranes in fresh or FT semen correlated with the fertilizing capacity of the spermatozoa (Rota et al., 2000; Brito et al., 2003). In our study, the increase in the grade of Holstein genes was accompanied by a tendency for lower HOS values, however, the difference was not significant. In earlier studies, a positive correlation between the results of the HOS test and NRR was observed (Revell and Mrode, 1994; Correa et al., 1997). Some authors suggested to use the results of the HOS test of post-thaw semen for prognosis of the potential fertility of bovine semen samples used for A.I (Brito et al., 2003; Tartaglione and Ritta, 2004). In the current study we were not able to demon-

strate a significant correlation between HOS and NRR, however, this has been demonstrated previously in another study where the bull numbers in the experimental groups were larger (Jaakma and Padrik, 2000; 2004).

Another parameter related to sperm membrane status and function, LSM, measures the changes in the scrambling of plasma membrane phospolipids. We found a significant difference between the two groups, whereas the proportion of viable sperm cell with stable membranes was lower in purebred Holstein young bulls.

The differences between the bull groups also became obvious when sperm motility parameters were compared. The bulls with 87.5-93.8% of Holstein genes had higher percentages of general and progressively motile sperms and VCL than the bulls with 100.0% of Holstein genes when compared at the batch level.

Several authors have emphasized the suitability of computer-assisted sperm motility analysis for the objective evaluation of the quality of fresh and frozen-thawed semen in different farm animals (Rodriques-Martinez, 1998; Tartaglione et al., 2004; Padrik and Jaakma, 2004; Hallap et al., 2005; Hoflack et al., 2007; Volpe et al., 2009). Sperm motility traits have been shown to have high heritabilities in Holstein bulls, indicating that selection for these traits can be efficient (Druet et al., 2009).

As spermatozoa need ATP energy for the maintenance of flagellar movement, then the measurement of mitochondrial activity could be useful as an additional parameter of sperm viability. Hallap et al. (2005) and Hua et al. (2006) have recently observed positive correlations between sperm motility and high mitochondrial activity in their recent studies. Our results from the present investigation showed that there was a significant difference in the incidence of MTDR-H between the groups with a different grade of Holstein genes at batch level. This finding is also in good accord with the sperm motility measurements. Also, the mean NRR in the bulls group with 87.5-93.8% grade of Holstein genes NRR was higher than in the bulls group with 100.0% grade of Holstein genes, which is in agreement with the measured sperm quality parameters. Altogether, a positive correlation was recorded between the GMot, VCL and NRR at bull level confirming the earlier data (Zhang et al., 1998; Al-Quarawi et al., 2002). At batch level, we obtained a positive correlation between the GMot, PMot, VCL, LSM, MTDR-H and NRR. As fertilization is a complex series of events, then fertility of FT spermatozoa cannot be evaluated sufficiently precisely using a single parameter. Therefore, it would be rational to combine different parameters to develop a suitable predictive model (PNRR model) for AI laboratories. In the present study, we obtained a positive correlation between PNRR and NRR at batch level ($r=0.86$; $p<0.001$). Several authors (Zhang et al., 1999; Januskauskas et al., 2003; Padrik et al., 2008) have found strong correlations between predicted and actual non-return rates, while the number and character of parameters included into the models varied. Phillips et al. (2004) found that such post-thaw sperm parameters as morphologically normal sperm, the proportion of intact sperm and cleavage of embryos can be used to predict field fertility of dairy sires. In the study of Tartaglione and Ritta (2004) eosin-nigrosin supravital stain combined with HOS-test has been included in a regression equation as predictors of *in vitro* fertility of FT bull semen. Padrik et al. (2008) has found a high value statistical model of seven parameters for PNRR, which included membrane integrity, motility, LSM and MTDR-H of spermatozoa in FT semen. Similarly to Zhang et al., 1999; Januskauskas et al., 2003; Tartaglione and Ritta, 2004; Phillips et al., 2004, motility parameters estimated by CASA and membrane stability of FT spermatozoa were included in the best PNRR model in the current study.

Conclusion

The results demonstrate the relationship between the grade of Holstein genes and semen quality, which was clearly confirmed by the field fertility data and showed lower semen quality in 100% pure Holstein young bulls in comparison to the bulls with 87.5-93.8% of Holstein genes. Sperm motility parameters measured by CASA and LSM as sperm membrane stability parameters were shown to be valuable for the prediction of fertility of young Holstein bulls semen batches.

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Relationships between the results of hypo-osmotic swelling tests, sperm motility, and fertility in Estonian Holstein dairy bulls

P. Padrik [1,2], T. Hallap[1], T. Kaart[1], T. Bulitko[2], Ü. Jaakma[1]

[1]Department of Reproductive Biology, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia
[2]Animal Breeders' Association of Estonia, Keava, Estonia

ABSTRACT: As an attempt to find an inexpensive and simple laboratory method for artificial insemination (AI) bull semen quality assessment, the osmotic resistance of spermatozoa was measured using the hypo-osmotic swelling (HOS) test, developed by Jeyendran et al. (1984) (labelled HOS-1), and its modifications (HOS-2, HOS-3), with decreased osmotic pressure aimed at challenging sperm survival ability. The test results were benchmarked against sperm viability measurements performed using the Computerized Motility Analyzer (CMA), and field fertility was calculated as non-return rate (NRR). Two age groups of Estonian Holstein bull sires were included in this study to test possible age effect on semen quality parameters. The HOS-1 test in fresh bull semen correlated well with sperm general motility (GMot) (r = 0.63, P < 0.001 at batch level and r = 0.77, P < 0.001 at bull level) as well as with progressive motility (PMot) in frozen-thawed (FT) semen (r = 66, P < 0.001 at batch level and r = 0.81, P < 0.001 at bull level), which makes the test suitable for the prediction of post-thaw semen quality. However, the HOS-2 and HOS-3 values in FT semen had high correlations with NRR (r = 0.65, r = 0.66, P < 0.001 at batch level and r = 0.63, r = 0.71, P < 0.01 at bull level), which was comparable to those between GMot and NRR or PMot and NRR. A combination of motility parameters and the results of the HOS-1 and HOS-3 tests provided a good model for predicting the potential fertility of bull semen. Values of sperm membrane post-thaw intactness, assessed using HOS-2, as well as of sperm motility measurements were higher in mature bulls compared to those in young bulls. Short conclusion: different modifications of the hypo-osmotic swelling test are useful for routine bovine semen quality assessment at AI stations.

Keywords: bull fertility; semen quality; sperm membrane intactness; bull age

Laboratory analyses have been used over many decades to evaluate semen quality. Despite the fact that none of the single assays developed provide results that consistently and highly correlate with fertility, the laboratory evaluation of semen samples remains an important procedure for the artificial insemination (AI) industry to eliminate low fertility bulls or semen from being used in artificial insemination programmes (Graham and Mocé, 2005).

Post-thaw viability and fertility of cryopreserved sperm are often reduced due to accumulated cellular damage during the cryopreservation phases (Muiño et al., 2008). Decrease in temperature, cold shock, and intracellular ice formation can affect sperm plasma membrane, acrosomal and mitochondrial membrane integrity (Thomas et al., 1998; Defoin et al., 2008), and cause the loss of intracellular components (Graham and Mocé, 2005), which can initiate cell death.

Sperm membrane integrity can be evaluated by several methods, e.g. light or fluorescent microscopy combined with vital stains (Brito et al., 2003), and flow cytometry (Januskauskas et al.,

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1999; Hallap et al., 2004). One of the simplest methods to evaluate the plasmalemma of sperm cells is the hypo-osmotic swelling (HOS) test. The traditional HOS test, originally presented by Jeyendran et al. (1984), enables reproduction specialists to determine the functional intactness of sperm membranes as spermatozoa "swell" under hypo-osmotic conditions due to the influx of water, and the expansion of the membranes causes the tails to coil. Several authors (Rodríguez-Martínez, 1998; Neild et al., 1999; Imam et al., 2008) have emphasized the suitability of the HOS test for assessing the quality of fresh and frozen-thawed semen in different farm animal species. A positive correlation was found between the results of the HOS test or its modifications and the non-return rate of female animals (Revell and Mrode, 1994; Correa et al., 1997a), which potentially makes it one of the most appropriate and simple methods for semen quality evaluation for the AI industry. Several authors have found a positive correlation between plasmalemmal integrity and sperm motility characteristics (Neild et al,. 1999; Mandal et al., 2003). Mandal et al. (2003) reported that in Murrah buffalo bulls ejaculates with less than 50% HOS-positive spermatozoa showed lower motility as compared with those with > 50% HOS-positive spermatozoa. Stanger et al. (2010) observed that in humans low HOS test values of neat semen samples were significantly associated with increased DNA damage identified by the sperm chromatin structure assay as well as TUNEL assay.

Reaction of spermatozoa to the HOS solution varies depending on animal species, solution, osmolality, and time of incubation (Correa et al., 1997b; Neild et al., 1999; Amorim et al., 2009). Not all spermatozoa with intact plasma membrane react to moderate osmotic pressure, however the swelling response of spermatozoa occurred mostly during the first minute of incubation in a hypotonic medium (Correa et al., 1997a). Therefore, for the maximum osmotic challenge, along with the traditional HOS test, the usefulness of the modified versions of the test was examined using 0.2% and 0.4% NaCl solutions immediately after thawing of the semen samples, or after 6 h of incubation of the samples at 37°C.

In earlier studies, a relationship between semen quality and the age of a bull was found (Padrik and Jaakma, 2004). The increase in age from 1–2 years to 3–4 years was accompanied by an increase in the proportions of motile and progressively motile spermatozoa, whereas an opposite tendency was recorded from 3–4 to 5–7 years of age (Padrik and Jaakma, 2004). A study by Hallap et al. (2004) showed that the post-thaw sperm membrane integrity estimated using fluorophores SYBR-14 and propidium iodide (PI) was higher in the semen of four-year-old bulls compared with that of younger bulls.

The aim of this study was to assess sperm membrane intactness in young and mature Estonian Holstein AI bulls using three different modifications of the HOS test, and to reveal the relationships between the HOS test and sperm motility characteristics as well as the field fertility estimated by the NRR of cows and heifers.

MATERIAL AND METHODS

Animals, semen collection, and processing

The first experiment was carried out using fresh semen from 91 Estonian Holstein (EHF) bulls aged 14 to 72 months, kept at the AI station of the Animal Breeders' Association of Estonia at Kehtna.

An artificial vagina was used to collect semen. Two consecutive ejaculates were pooled (hereafter referred to as a batch), extended with a commercial extender (Triladyl®; Minitüb GmbH, Tiefenbach, Germany), packed in 0.25 ml plastic straws, each containing ~ 25×10^6 spermatozoa for mature bulls, and $30–40 \times 10^6$ spermatozoa for young bulls, and frozen in a manually adjustable biological freezer. The frozen straws were stored in liquid nitrogen until testing. Following preservation, post-thaw motility ≥ 50% was set up as a threshold.

A total of 683 ejaculates, collected over sixteen months, were examined for sperm membrane hypo-osmotic swelling and motility in fresh and frozen-thawed semen. During this period, 4–6 ejaculates from proven bulls and 2–4 ejaculates from young bulls were collected per month. The bulls were a part of a breeding programme; all collections as well as freezing of semen were performed at the AI station under commercial conditions.

In the second experiment, FT semen (49 ejaculates) from 10 mature (Group I) and 7 young (Group II) bulls were examined and used for test inseminations. These ejaculates were used to inseminate 3850 cows and heifers (78 inseminations per ejaculate and 226 inseminations per bull on average) by four AI technicians on four different herds according to the established breeding programme. Inseminations were performed routinely,

within one year of freezing, on heifers and cows of different parity in the course of all seasons of the year. A 60-day non-return rate was recorded for each batch, but not corrected for season, location, or parity.

Hypo-osmotic swelling test

HOS-1 test. The HOS test was conducted under three modifications. A traditional hypo-osmotic test (Jeyendran et al., 1984), labelled HOS-1, was performed by incubating 100 µl of fresh semen with 1 ml of 150 mOsm/kg hypo-osmotic solution (7.35g sodium citrate and 13.51 g fructose per 1 l of distilled water) at 37°C for 60 min. The semen straw was thawed at 35°C in a water bath for 20 s before emptying into a test tube containing the hypo-osmotic solution. After incubating at 37°C for 60 min, 0.2 ml of eosin (0.99%) (Pioneer Research Chemicals, Ltd., Colchester, UK) was added to the test tube. The wet preparation was examined under a phase contrast microscope (Olympus BX40, ×1000). The ratio of spermatozoa with swollen tails was expressed as a percentage of the total count (mean of 3 replicates). A total of 100 spermatozoa were assessed in each replicate.

HOS-2 test. In the HOS-2 test (Padrik, 1999), the proportion of FT spermatozoa with swollen tails was determined in 0.2% and 0.4% NaCl solutions (osmotic pressure 66 and 130 mOsm/kg, respectively). The semen straws were thawed in a water bath at 35°C for 20 s, and emptied into a test tube containing 1 ml of 0.2% and 0.4% NaCl solution. After incubating at room temperature (20–22°C) for 2 min, 0.2 ml of eosin was added to each test tube. A wet preparation of each concentration was examined under a phase contrast microscope. The ratio of spermatozoa with swollen tails was expressed as a percentage of the total count (mean of 3 replicates). A total of 100 spermatozoa were assessed in each replicate. ΔHOS-2 was estimated by subtracting the ratio of spermatozoa with intact membranes in the 0.2% NaCl solution from the similar value in the 0.4% NaCl solution.

HOS-3 test. In the HOS-3 test (Padrik and Jaakma, 2000), three straws of frozen semen were thawed at 35°C for 20 s, emptied into a test tube containing 3 ml of 2.9% sodium citrate solution (Tallinn Pharmacy Ltd., Tallinn, Estonia), mixed and incubated at 37°C for 6 h. A 100 µl fraction of the sperm suspension was pipetted into each of two solutions, 1 ml of 0.2% and 1 ml of 0.4% NaCl. After 2 min incubation at room temperature (20–22°C), 0.2 ml of eosin was added into each test tube and wet preparations were made. A total of 100 spermatozoa were assessed in each preparation, and the ratio of the spermatozoa with swollen tails was given as a percentage of the total count (mean of 3 replicates). ΔHOS-3 was estimated by subtracting the ratio of spermatozoa with intact membranes in the 0.2% NaCl solution from the similar value in the 0.4% NaCl solution.

Sperm motility

Sperm motility characteristics were determined using Computer Assisted Cell Motion Analyzer, Sperm Vision (Minitüb GmbH, Tiefenbach, Germany). Samples of 5 µl were placed in the Makler chamber (Makler Counting Chamber, Sefi-Medical Instruments, Haifa, Israel) to track and assess ~ 400 fresh or post-thaw sperm (×400). The following parameters were determined: percentage of general motile (GMot) and progressively motile (PMot) sperms, curve line velocity (VCL, µm/s), linearity (LIN, straight line velocity (VSL)/VCL), and amplitude of lateral head displacement (ALH, µm).

Statistical analysis

The characteristics of the observed traits were expressed as means ± SD. The Pearson's correlation test was used to calculate correlations between different sperm parameters in fresh and FT semen, and between the measured sperm parameters and field fertility (60-day NRR). The bulls were divided into two age groups: mature bulls (Group I, aged 36–72 months, n = 10) and young bulls (Group II, aged 14–22 months, n = 7). General linear model analyses were performed using SAS® software (Version 9.1.3, 1999) to assess the influence of different sperm quality characteristics and bull age on the pregnancy rate, and to develop a predictive model for non-return rates.

RESULTS

Experiment 1

HOS-1 in fresh semen. The percentage of swollen spermatozoa was 57.9 ± 13.6% (range

7.0–85.0%) in batches, and 57.6 ± 10.2% (range 31.0–77.4%) in bulls. A medium correlation was found between the results of the HOS-1 test and GMot and PMot at both the bull and batch levels in fresh semen (Table 1). A stronger correlation was recorded between the HOS-1 results in fresh semen, and GMot and PMot in FT semen at both the batch and bull levels.

HOS-1 in frozen-thawed semen. The mean HOS-1 test scores in FT semen were 35.2 ± 6.8% (range 22.0–45.5%) at bull, and 33.3 ± 9.7% (range 13–50%) at batch level. A significant positive correlation was determined between the HOS-1 and PMot (r = 0.55, P < 0.05) at bull level, between the HOS-1 and PMot (r = 0.47, P < 0.01), and the HOS-1 and GMot (r = 0.55, P < 0.01) at batch level.

HOS-2 in frozen-thawed semen. The mean ΔHOS-2 for bulls was 7.1 ± 6.5% (range –4.6 to 22.0%) and for batches 6.0 ± 8.9% (range –15.0–25.0%). Strong correlations were found between ΔHOS-2 and GMot, and ΔHOS-2 and PMot (r = 0.66, 0.55, respectively; P < 0.05) at bull level. Significant positive correlations were also observed between ΔHOS-2 and GMot, PMot, VCL, ALH at batch level (r = 0.42–0.64, P < 0.01).

HOS-3 in frozen-thawed semen. The mean ΔHOS-3 score for bulls was –0.3 ± 3.7% (range –6.5–7.3%), and for batches –0.7 ± 6.1% (range –11.0–10.0%). The ΔHOS-3 was positively correlated with GMot and PMot at bull level (r = 0.61, 0.52 respectively; P < 0.05). Medium correlations were also found between ΔHOS-3 and GMot, PMot, VCL, ALH at batch level (r = 0.36–0.55, P < 0.01).

Relationships between HOS-1, HOS-2, and HOS-3 in frozen-thawed semen. A positive correlation was found between HOS-1 and ΔHOS-2 both at bull (r = 0.28, P > 0.05) and batch (r = 0.49, P < 0.05) level, and also between HOS-1 and ΔHOS-3 at bull (r = 0.28, P > 0.05) and batch (r = 0.38, P < 0.05) level. ΔHOS-2 related to ΔHOS-3 both at bull (r = 0.81, P < 0.001) and batch (r = 0.72, P < 0.001) level.

Experiment 2

Effect of bull age on the results of hypo-osmotic swelling test and sperm motility in frozen-thawed semen. The results of the HOS-1 and HOS-3 tests on FT semen were not different between mature and young bulls, either at bull or batch level, whereas the HOS-2 showed an improvement in semen quality of mature bulls compared to semen quality of young bulls at batch level (Table 2). The results also showed a significant influence of the age of the bulls on sperm motility parameters.

Relationships between laboratory tests and *in vivo* fertility. Fertility, expressed as 60-days NRR, ranged 37.5–57.0% for young, and 60.0–71.5% for mature bulls. A medium positive correlation was found between the HOS-1 results in FT semen and NRR at batch level (r = 0.37, P < 0.01), but not at bull level. A strong positive correlation was detected between both ΔHOS-2 and NRR, and ΔHOS-3 and NRR at batch (r = 0.65, 0.66, respectively; P < 0.001) and bull (r = 0.63, 0.71, respectively, P < 0.01) level, which was comparable to that between GMot and NRR, PMot and NRR at batch (r = 0.69, 0.66, respectively; P < 0.001) and bull (r = 0.71, 0.63, respectively; P < 0.01) level. Positive correlations were also observed between VCL and ALH in FT semen and NRR at batch (r = 0.59, 0.50, respectively; P < 0.001) and bull (r = 0.59, 0.56, respectively; P < 0.05) level.

Table 1. Correlations (r) between HOS-1 test in fresh semen and sperm motility characteristics in fresh and frozen-thawed semen (batch and bull level)

Sperm motility parameters, fresh semen	HOS-1 in fresh semen		Sperm motility parameters, post-thaw	HOS-1 in fresh semen	
	683 batches	91 bulls		683 batches	91 bulls
GMot (%)	0.22*	0.33**	GMot (%)	0.63***	0.77***
PMot (%)	0.20*	0.32**	PMot (%)	0.66***	0.81***
VCL (μm/s)	0.09	–0.04	VCL (μm/s)	0.23*	0.22*
LIN	0.02	0.10	LIN	0.04	0.26*
ALH (μm)	0.04	–0.01	ALH (μm)	0.13	0.14

HOS = hypo-osmotic swelling, GMot = general motile, PMot = progressively motile, VCL = curve line velocity, LIN = linearity, ALH = amplitude of lateral head displacement

*P < 0.05, **P < 0.01, ***P < 0.001

Table 2. Influence of age of bulls on hypo-osmotic swelling and motility of spermatozoa in frozen-thawed semen, bull and batch level (means ± SD)

Sperm parameters	Bull level		Batch level	
	mature (n = 10)	young (n = 7)	mature (n = 30)	young (n = 19)
HOS-1	34.3 ± 6.6	36.4 ± 7.5	32.3 ± 9.6	34.4 ± 10.1
Δ HOS-2	9.1 ± 6.1	4.2 ± 6.5	8.3 ± 8.7[a]	2.6 ± 8.5[b]
Δ HOS-3	0.6 ± 3.3	–1.5 ± 4.1	0.3 ± 6.3	–2.2 ± 5.4
GMot (%)	79.1 ± 4.9	74.0 ± 9.1	79.1 ± 7.6[a]	71.5 ± 12.3[b]
PMot (%)	63.1 ± 4.9	58.4 ± 11.6	62.8 ± 7.4[a]	55.1 ± 15.0[b]
VCL (μm/s)	92.9 ± 10.1[a]	89.7 ± 5.6[b]	99.1 ± 9.2[c]	88.3 ± 5.6[d]
LIN	0.4 ± 0[c]	0.5 ± 0[d]	0.4 ± 0[c]	0.5 ± 0[d]
ALH(μm)	3.0 ± 0.2[c]	2.5 ± 0.3[d]	3.1 ± 0.3[c]	2.7 ± 0.3[d]

HOS = hypo-osmotic swelling, GMot = general motile, PMot = progressively motile, VCL = curve line velocity, LIN = linearity, ALH = amplitude of lateral head displacement

[a,b]values with different superscripts in a row are significantly different ($P < 0.05$)

[c,d]values with different superscripts in a row are significantly different ($P < 0.001$)

Relationship between predicted (PNRR) and observed non-return rates (NRR) of mature and young bulls. Based on the results, the predictive model for NRR (PNRRage) included five parameters: HOS-1, ΔHOS-3, general motility (GMot, in %), progressive motility (PMot, in %), and linearity (LIN).

R^2 = 0.80 in mature bulls at batch level

PNRRage = 36.423 + 0.174 HOS-1 + 0.634 ΔHOS-3 – 0.388 GMot + 0.652 PMot + 16.32 LIN

R^2 = 0.79 in young bulls at batch level

PNRRage = –0.88 + 0.648 HOS-1 + 0.466 ΔHOS-3 + 1.291 GMot – 0.887 PMot + 135.3 LIN

A strong positive correlation between PNRR (predicted NRR in %) and NRR (60-day non-return rate in %) was found at batch level (r = 0.89, $P < 0.001$) and at bull level (r = 0.92, $P < 0.001$) in mature bulls. A medium positive correlation between PNRRage and NRR was observed at batch level (r = 0.64, $P < 0.001$), and a strong positive correlation was found between PNRRage and non-return rates at bull level (r = 0.95, $P < 0.001$) in young bulls.

DISCUSSION

The objective of this investigation was to study the hypo-osmotic swelling of spermatozoa in fresh and FT semen of young and mature Estonian Holstein bulls, and assess relationships between the HOS test results, sperm motility characteristics, and fertility of bulls expressed as 60-days NRR.

Several authors have emphasized the suitability of the HOS test for assessing the quality of human semen (Moskovtsev et al., 2005; Cincik et al., 2007) and also that of various domestic animals including cattle (Correa and Zavos, 1994; Mandal et al., 2003; Hu et al., 2010), horses (Neild et al., 1999), and pigs (Gadea et al., 1998). As the test gives a consistent estimate of the percentage of spermatozoa with a physiologically active membrane, it can be used to predict the fertilizing capacity of spermatozoa in animals (Rota et al., 2000; Brito et al., 2003).

Correa et al. (1997b) and Neild et al. (1999) found that spermatozoa show different swelling patterns and not all spermatozoa with intact plasma membrane react to moderate osmotic pressure. Therefore, the traditional HOS test was modified by shortening the incubation time of sperm cells in hypotonic solution, using hypo-osmotic concentrations of 0.2% and 0.4% NaCl (HOS-2), and adding sperm survival (HOS-3). The decrease in osmotic pressure from 130 to 66 mOsm/kg resulted in a significant difference in sperm behaviour in different semen batches. The percentage of spermatozoa with swollen tails increased in some batches, while it remained stable or decreased in others. The latter can be explained by the fast breakage of the sperm membrane due to the influx of water under highly hypo-osmotic con-

ditions. Contrarily, the increase in the percentage of spermatozoa with swollen tails after short-term exposure to low osmotic pressure showed the maintenance of sperm membrane functional integrity.

This study found a strong correlation between the results of the HOS test in fresh semen and GMot and PMot of spermatozoa in FT semen. Thus, there is a high probability that spermatozoa with intact membranes before extension and cryopreservation maintain good motility after the FT procedure. Consequently, it is possible to apply an individual semen processing method to different semen batches to obtain an optimal quantity of semen doses while taking into account the proportion of spermatozoa with functionally intact membranes when diluting the semen.

Furthermore, positive correlations between the HOS-tests and sperm GMot and PMot, all measured in FT semen, were observed, which is similar to earlier findings (Neild et al., 1999; Mandal et al., 2003). With regard to the relationships between the spermatozoa with functionally intact membranes and other traits in FT semen, Januskauskas et al. (1996) have observed associations between ATP content and sperm membrane integrity, assessed using fluorophore probes. Zuge et al. (2008) have reported a high positive correlation between the proportion of sperm cells with high mitochondrial activity and that with intact membranes, determined using the HOS test.

The results of this study found that the age of bulls had a significant effect on the HOS-2, GMot, and PMot ($P < 0.05$), VCL, LIN, and ALH ($P < 0.001$) values at batch level, and VCL, LIN, and ALH ($P < 0.05$) values at bull level in FT semen. The better semen quality in mature bulls can be associated with an increase in testosterone levels which has an effect on the volume of the ejaculate, sperm concentration, and motility (Kastelic et al., 2001; Silva et al., 2008). An increase in testosterone levels in blood plasma is related to an increase in scrotal circumference (SC) (Andrade et al., 2008) which in turn is strongly correlated with the weight and age of bulls (Chacón-Calderón et al., 2002).

Evaluation of sperm motility characteristics has traditionally been the most frequently used semen quality test in the AI industry. In this study, as expected, a strong correlation was observed between the GMot and PMot of FT spermatozoa and NRR ($P < 0.01$), which confirms previous findings (Holt et al., 1997; Farell et al., 1998; Januskauskas et al., 2003; Hallap et al., 2006). Similarly to Revell and Mrode (1994) and Correa et al. (1997a), a significant positive correlation was found between the results of the HOS-1 test in FT semen and NRR (batch level, $P < 0.01$). The correlation coefficients were even higher between the results of the HOS-2, HOS-3, and NRR at both batch ($P < 0.001$) and bull ($P < 0.01$) level, which evidences that HOS-2 and HOS-3 are more suitable for semen quality testing. The HOS-2 test appears to be especially attractive for the AI industry as it is neither expensive nor time-consuming.

It was found earlier that the results of post-thaw hypo-osmotic swelling tests can be used to predict potential fertility of bovine semen samples used for IVF (Tartaglione and Ritta, 2004). According to Brito et al. (2003), the HOS test was the only plasmalemma evaluation method that significantly contributed to conventional sperm quality tests in predicting *in vitro* fertilization rate.

A high positive correlation was found between predicted and actual NRRs, where, in addition to the HOS tests and sperm kinematics, the age of the bulls was taken into consideration in developing the model. The highest correlation coefficient observed was between PNRRage and NRR for young bulls ($P < 0.001$). Attempts have previously been made to include the HOS test in the regression equation for fertility. Tartaglione and Ritta (2004) included an eosin-nigrosin supravital stain combined with the HOS test into a regression equation as a predictor of *in vitro* fertility of FT bull semen. Pérez-Llano et al. (2001) also included the HOS test in a regression equation along with other semen parameters such as morphology, motility, and acrosome integrity to predict the fertility of boar semen. Contrarily, also working with boars, Gadea and Matas (2000) found that the results of the HOS test were not significant when regression models for fertilization rate were evaluated. In the current study, using a regression equation with five parameters (HOS-1, ΔHOS-3, GMot, PMot, and LIN), the predicted NRRs were highly correlated with the actual NRRs.

It can be concluded that the HOS-1 test on fresh bull semen was correlated well with sperm motility in FT semen, which makes the test suitable for the prediction of post-thaw semen quality. Furthermore, the modification of the original HOS test, named HOS-2, was found to be an even better method for the assessment of the quality of FT semen samples, as it is quicker and less expensive. A combination of motility parameters and the results of the HOS-1 and HOS-3 tests is a good model for predicting the potential fertility of bull semen for the AI industry. Values of sperm membrane intact-

ness post-thaw assessed using HOS-2 and of sperm motility measurements were higher in mature bulls when compared to those in young bulls.

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Corresponding Author

Peeter Padrik, MSc., Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Department of Reproductive Biology, 1 Kreutzwaldi St, 51014 Tartu, Estonia
Tel. +37 24 890 681, fax +37 24 890 680, e-mail: must@estpak.ee

IV

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CONVENTIONAL LABORATORY TEST AND FLOW CYTOMETRY IN THE PROGNOSTIC TESTING OF BULL SEMEN FERTILITY

Peeter Padrik[1, 3], Triin Hallap[1], Aloyzas Januškauskas[2], Tanel Kaart[1], Tanel Bulitko[3], Ülle Jaakma[1]
[1]*Department of Reproductive Biology, Institute of Veterinary Medicine and Animal Sciences Estonian University of Life Sciences, Kreutzwaldi 62, 51014 Tartu, Estonia*
[2]*Lithuanian University of Health Sciences, Veterinary Academy; Tilžės Str. 18, LT-47181, Kaunas, Lithuania*
[3]*Animal Breeders Association of Estonia, Keava, Estonia*

Corresponding author: Peeter Padrik
e-mail: must@estpak.ee; Tel.: +372 731 34 66; Fax.: +372 731 37 06; Kreutzwaldi 62; 51014 Tartu Estonia

Abstract. We aimed to determine the relationships between the results of routine AI laboratory tests and flow cytometric analysis (FCM) of the quality of frozen-thawed (FT) bovine spermatozoa. The results were compared to the field fertility data. Forty five ejaculates from fifteen (14 to 86 Mo age) Estonian Holstein (EHF) dairy bulls were examined for motility (subjectively by light microscope and objectively using a computer assisted motility analyzer (CMA)), hypo-osmotic swelling tests (HOS-1, HOS-2, HOS-3), membrane lipid architecture status (Merocyanine 540 staining) and mitochondrial membrane potential (Mitotracker Deep Reed 633 staining). Stained spermatozoa were assessed by FCM. Significant positive correlations were observed between subjectively assessed motility variables (SubMot), general motile (GMot) and progressively motile (PMot) spermatozoa and sperm with stable membrane (LSM) ($P<0.001$). Strong positive correlations with non-return rates (NRR) were obtained for HOS-2, HOS-3, SubMot, PMot, GMot and curve line velocity (VCL) ($P<0.01$). The best predictive model PNRR (predictive non-return rates), according to the results of routine laboratory tests and FCM analysis, included seven parameters ($R^2=0.91$). The strongest positive correlation was found between PNRR and NRR on bull level ($r=0.96$; $P<0.001$) compared to that of batch level. Combinations of common AI laboratory tests (motility analysis, HOS) and FCM assays can be used for the prediction of the FT bull semen fertility.
Keywords: bull semen quality, flow cytometry, fertility prediction.

ĮPRASTINIŲ SPERMATOZOIDŲ KOKYBĖS VERTINIMO METODŲ IR TĖKMĖS CITOMETRIJOS METODO PALYGINAMASIS ĮVERTINIMAS, NUSTATANT BULIAUS SPERMOS APVAISINAMĄJĄ GALIĄ

Peeter Padrik[1, 3], Triin Hallap[1], Aloyzas Januškauskas[2], Tanel Kaart[1], Tanel Bulitko[3], Ülle Jaakma[1]
[1]*Reprodukcinės biologijos katedra, Veterinarinės medicinos ir gyvulininkystės institutas Estijos gyvybės mokslų universitetas, Kreutzwaldi 62, 51014 Tartu*
tel. +372 731 34 66; faks. +372 731 37 06; el. paštas: must@estpak.ee
[2]*Veterinarijos akademija, Lietuvos sveikatos mokslų universitetas; Tilžės g. 18, LT-47181, Kaunas, Lietuva*
[3]*Estijos gyvulių veisėjų asociacija, Keava, Estija*

Santrauka. Darbo tikslas – nustatyti ryšį tarp bulių kriokonservuotos spermos spermatozoidų kokybės tyrimų rezultatų, gautų atliekant įprastus laboratorinius testus ir analizuojant tėkmės citometrijos metodu (TCM). Rezultatus palyginome su apvaisinimo duomenimis, gautais lauko sąlygomis (NRR). 45-iuose ejakuliatuose, surinktuose iš penkiolikos (14–86 mėnesių) Estijos holšteinų pieninų bulių, ištirtas spermatozoidų judrumas (subjektyviai, naudojant šviesinį mikroskopą, ir objektyviai, naudojant kompiuterinį judrumo analizatorių), plazminės membranos rezistentiškumas, naudojant hipoosmotinius testus (HOS-1, HOS-2, HOS-3), membranos lipidų struktūrinė būklė (dažymas merocianinu) ir mitochondrijų membranų potencialas (dažymas naudojant „Mitotracker Deep Reed 633"). Nudažyti spermatozoidai įvertinti TCM. Pastebėta ženkli teigiama koreliacija tarp subjektyviai įvertintų judrumo verčių (SubMot), bendro judrumo (GMot), progresyvaus spermatozoidų judrumo (PMot) ir spermatozoidų su stabilia plazmine membrana (SSM) verčių ($p<0,001$). Aukšta teigiama koreliacija su NRR buvo būdinga HOS-2, HOS-3, SubMot, PMot, GMot ir spermatozoidų greičio vertei (VCL) ($p<0,01$). Įprastų laboratorinių testų ir TCM rezultatai parodė, kad geriausias prognostinis apvaisinimo rezultatų (PNRR) modelis apima septynis tiriamuosius rodiklius ($r^2=0,91$). Buliaus lygmeniu koreliacija tarp PNRR ir NRR buvo aukštesnė ($r=0,96$; $p<0,001$) už koreliaciją bulių grupės lygmeniu. Nustatant buliaus kriokonservuotos spermos apvaisinimo galią, galima derinti įprastus laboratorinius metodus (judrumo analizė, HOS) su TCM.
Raktažodžiai: bulių spermos kokybė, tėkmės citometrija, apvaisinimo nustatymas.

Introduction. Evaluation of frozen-thawed semen quality in the AI industry is often based on subjective evaluations by experienced professionals, and not guaranteed by objective methods. Although many AI

laboratories are nowadays equipped with computer–assisted sperm analyser (CASA), enabling objective measurement of motility, combination of multiple sperm attributes could explain more variation in fertility between the bulls than can variables of motility alone (Christensen et al. 1999). Using of fluorescent markers in combination with flow cytometry allows simultaneous assessment of several sperm parameters in thousands of sperm cells and therefore, has become an valuable tool in semen quality assessment (Gillan et al. 2005; Hua et al. 2006). Thus, the fast development of measurement techniques has provided AI industry with the variety of objective tests for the semen quality. The question is, however, if the traditional simple (and cheap) methods should be replaced by the more precise but expensive methods or could they rather be incorporated into the semen quality assessment simultaneously.

In our study, we focused on sperm plasma membrane integrity, membrane stability and mitochondrial activity along with the sperm motility characteristics. Sperm membrane integrity, which is essential for normal metabolism, capacitation and further acrosome reaction can be evaluated with several methods such as light or fluorescent microscopy combined with vital stains (Brito et al., 2003; Celeghini et al., 2008) or flow cytometry (Kasai et al., 2002 Kasimanickam et al., 2006). One of the simplest methods to evaluate the sperm plasma membrane integrity is subjection of sperm cells to the hypo-osmotic swelling test. The traditional hypo-osmotic swelling (HOS) test presented by Jeyendran (1984) enables the determination of the functional intactness of the sperm membranes as spermatozoa "swell" under hypo-osmotic conditions and the expansion of the membranes causes the tails to coil. Several authors have emphasized the suitability of the hypo-osmotic test for evaluation of the quality of fresh and frozen semen and fertilizing capacity of the spermatozoa of different farm animals (Tartaglione et al., 2004). In addition to absence of damage, the plasma membrane needs to be stable to avoid premature capacitation in the female reproduction tract (Hallap et al., 2006). One possibility for estimation of sperm plasma membrane stability is to use the hydrophobic dye Merocyanine 540 (M540) and flow cytometry to determine the level of scrambling of the phospholipids in the plasma membrane of spermatozoa (Harrison et al. 1996).

Estimation of subjective motility of spermatozoa under light microscope, and objective motility with CASA, gives a good overview of the quality of fresh and FT semen and these results often correlate well with female fertility (Rodrigues-Martinez, 1998). Assessment of ATP (adenosine triphosphate) content as an energy source for sperm, is an indirect possibility to evaluate the motility potential of spermatozoa. A positive correlation has been found between sperm motility and ATP content in FT semen (Söderquist et al., 1991). Measurement of mitochondrial membrane potential (organells where ATP is syntesized) using FCM has also been found to be useful as another parameter related to sperm motility (Volpe et al., 2009).

The aim of this study was to estimate the relationships between the results of conventional AI laboratory tests such as motility measurement and hypo-osmotic resistance and flow cytometrically estimated mitochondrial activity and plasma membrane stability. We also studied the relationships between the test results and field fertility after AI.

Materials and methods

Animals, semen collection and processing

Semen from fifteen (age from 14 to 86 months) Estonian Holstein (EHF) bulls 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, Germany), packed in 0.25 ml plastic straws each containing ~25-40×10^6 spermatozoa, and frozen using a manually regulated biological freezer. The frozen straws were stored in liquid nitrogen until tested or used for the insemination. Doses of 45 frozen-thawed semen batches were used to inseminate 3,475 cows and heifers (average 77 inseminations per batch and 231 inseminations per bull) by four AI technicians in four different herds according to the breeding program. Inseminations were performed routinely within one year on heifers and cows of different parity during all seasons of the year.

Non-return rates (NRRs) 60 days after AI were recorded for each semen batch and used for the analyses without correction for season, area, and parity. The fertility of an individual bull was calculated using non-return rates for all his semen batches and ranged from 37.5 to 71.5%. The fertility of semen batches ranged from 22.8 to 80.0%. Semen evaluation was performed immediately after the thawing. The semen from two straws of the same batch was thawed by immersion in water at +35°C for 20 seconds, pooled and used for testing. Following preservation, a post-thaw motility ≥50% was set as the threshold limit, and batches of semen that did not comply with this threshold were discarded from further use both for AI and for laboratory testing.

Hypo-osmotic swelling (HOS) test

The HOS-test was performed with three modifications. In HOS-1, the thawed semen straw was emptied into the test tube with 1ml hypoosmotic solution (150mOsm/kg; 7.35g sodium citrate and 13.51g fructose per litre of distilled water). After incubating at +37ºC for 60 min, 0.2 ml of eosin (0.99%, Pioneer Research Chemicals, Ltd. England) was added. Wet preparation (5 µL semen suspension per slide) was evaluated under the phase contrast microscope (x 1000) and the ratio of spermatozoa with swollen tails was expressed as a percentage from a mean of three replicates. One hundred spermatozoa were assessed in each replicate.

In HOS-2 test (Padrik 1999), the proportion of FT spermatozoa with swollen tails was determined in 0.2% and 0.4% NaCl solutions (osmotic pressure 66 and 130 mOsm/kg, respectively). After incubating spermatozoa in 1 ml of solution at room temperature (+20…22°C) for 2 minutes, 0.2 ml of eosin was added.

Wet preparations of each sample were evaluated under phase contrast microscope (×1000). One hundred

spermatozoa were assessed in each replicate and the ratio of spermatozoa with swollen tails was expressed as a percentage from a mean of three replicates. ΔHOS-2 was estimated by subtracting the ratio of the spermatozoa with intact membranes in 0.2 % NaCl solution from the similar value in 0.4% NaCl solution.

In HOS-3 test, described by Padrik and Jaakma (2000), three straws of frozen semen were thawed, emptied into a test tube with 3 ml of 2.9% sodium citrate solution (Tallinn Pharmacy Ltd.), mixed and incubated for six hours at 37°C. Thereafter, 100 µl of sperm suspension were pipetted into tubes containing 1 ml 0.2 and 0.4 % NaCl. After 2 minutes incubation at room temperature 0.2 ml of eosin was added into each NaCl solution and wet preparations were made. One hundred spermatozoa were assessed in each preparation and the ratio of the spermatozoa with swollen tails was given as a percentage from a mean of three replicates. ΔHOS-3 was estimated as described in HOS-2 test.

Sperm motility

Sperm motility characteristics were determined with a computer-assisted motility analyzer (Computer Assisted Cell Motion Analyzer (CMA), Sperm Vision, Minitüb GmbH&Co, Germany).

5µl samples were placed in Makler chamber (Sefi-Medical Instruments, Ltd.) where ~400 post-thaw spermatozoa were tracked and assessed (×400) at +38ºC. The following parameters were determined: percentages of general motile (GMot) and progressively motile (PMot) spermatozoa, curve line velocity (VCL, µm/sec), linearity (LIN, %) and amplitude of lateral head displacement (ALH, µm). Subjective sperm motility (Submot) was estimated under a phase contrast microscope (×400) equipped with a warm stage (+38ºC). The mean value from evaluations of four fields was recorded in % from total.

Sperm plasma membrane stability

The following working solutions were prepared: Merocyanine 540 (M-540; Molecular Probes, M24571, Leiden, The Netherlands) 1mM in dimethyl sulfoxide (DMSO); Yo-PRO 1 (Molecular Probes, Y3603) 25 µM in DMSO. Washed spermatozoa were stained with 25 nM Yo-PRO 1 and further incubated at 38°C for 9 min in the dark as previously described (Harrison et al.1996).

Thereafter 10 µL of a 40 µM solution of M-540 in SP-TALP was added to give a final M-540 concentration of 2.7 µM and vortexed for 10 s before analysis on a flow cytometer (FacsCalibur, Becton Dickinson, San Jose, USA). Data collection was started at 60 s after M-540 addition. Measurements were made with a flow cytometer, equipped with standard optical lasers as excitation sources. The M-540 and Yo-PRO 1 dyes were excited by an Argon ion 488 nm laser running at 15 mW. Forward and side scatter values were recorded on a linear scale; while florescent values were recorded on a logarithmic scale. Obscuration bars were set for maximum sensitivity in order to obtain L-shaped forward light – scatter/sideways light scatter distribution of sperm cells. Fluorescence of Yo-PRO 1 was detected on detector FL 1 (530/28 nm), while M-540 fluorescence was detected on detector FL 2 (585/2 nm). From each sample, a total of 10,000 events were measured with flow rate of approx. 200 cells/s. Acquisitions were made using CellQuest Pro software (Becton Dickinson, San Jose, USA). Dot plots for offline analyses were drawn by WinMDI, version 2.8. Events accumulated in the lower left corner correspond to sample debris and were excluded from the analysis by gating. On FL 1/FL 2 (Yo-PRO 1/M-540) dot plots regions were set to differentiate viable, stabile plasma membrane LSM (Yo-PRO 1 negative and M-540 negative); viable, scrambled plasma membrane (Yo-PRO 1 negative and M-540 positive); and dead (Yo-PRO 1 positive) events.

Sperm mitochondrial activity

The staining protocol was identical to that described by Hallap et al. (2005). The measurements were made using a FacsCalibur flow cytometer (Becton Dickinson, San Jose, USA). The SYBR-14 dye was excited by a 15 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 by a 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 made using the CellQuest Pro software (Becton Dickinson, San Jose, USA).

Non-sperm events were gated out based on SYBR-14 fluorescence (DNA content). The FC was used at a 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. On SYBR-14 (FL 1/FL 2) dot plots, regions were drawn around the SYBR-14-positive cluster, and these events were classified as spermatozoa. In SYBR-14/ MitoTracker Deep Red dot plots sperm cells with low MTDR-L) and high (MTDR-H) Deep Red fluorescence were specified.

Statistical Analyses

The statistical analyses based on two datasets composed on batch level (analyses of 45 semen batches), and bull level (analyses the data of 15 bulls). In the last case the values for each bull were calculated by pooling the data of the different batches. The results were expressed as means ± S.D.

The Pearson correlation test was used to calculate the correlations between different sperm parameters in fresh and FT semen and between the sperm parameters measured and field fertility (60-days NRR). The analysis of variance with SAS software (version 9.1.3; SAS Institute Inc., Cary, NC) was used to assess the influence of different sperm quality characteristics and bulls' age on the fertility and to find the predictive model for non-return rates. On batch level analyses the effect of repeated measurements of the bulls was considered.

Results

Relationships between conventional AI laboratory tests, flow cytometric analysis and *in vivo* fertility

In general, sperm motility parameters estimated either

subjectively or by CASA and the results of the hypo-osmotic tests correlated to percentage of viable sperm with stable membrane (LSM) and sperm with high mitochondrial activity (MTDR-H) (Table 1 and 2). The strongest correlations were found between PMot and LSM on batch level (P<0.001) (Table 1) and between PMot and (MTDR-H) on bull level (P<0.001) (Table 2).

Several conventional and flow cytometric parameters were positively related to fertility: ΔHOS-2 (P<0.001), ΔHOS-3 (P<0.05), SubMot (P<0.01), GMot (P<0.001), PMot (P<0.01), VCL (P<0.001), ALH (P<0.001), and MTDR-H (P<0.05) on bull level and the same parameters plus LSM (all P<0.001) on batch level.

Table 1. **Correlation between laboratory tests and flow-cytometric analysis of bull semen and 60-days NRR** (batch level)

Parameters	Batches (n=45)				
	means±S.D.	Range	LSM	MTDR-H	NRR
			r	r	r
HOS-1 (%)	33.9±9.7	17…50	0.24	0.21	0.15
ΔHOS-2 (%)	5.4±8.9	–12 …+25	0.44**	0.38**	0.63***
ΔHOS-3 (%)	–1.1±9.2	–11…+12	0.44**	0.30*	0.65***
SubMot (%)	57.8±9.9	35.0…75.0	0.67***	0.62***	0.69***
GMot (%)	75.6±10.6	49.0…87.5	0.66***	0.59***	0.68***
PMot (%)	59.3±12.0	27.8…73.6	0.72***	0.67***	0.65***
VCL (μm/sec)	91.4±9.8	75.0…115.5	0.33*	0.38*	0.61***
LIN	0.47±0.04	0.42…0.60	-0.28	-0.24	-0.52
ALH(μm)	2.9±0.3	2.2…3.5	0.13	0.14	0.57***
LSM (%)	55.7±15.7	16.8…80.3	-	0.81***	0.52***
MTDR-H (%)	74.4±18.6	24.7…92,7	0.81***	-	0.51***

SubMot- Subjective motility; GMot - general motility; PMot - progressively motile; VCL - curve line velocity; LIN - linearity; ALH - amplitude of lateral head displacement; LSM- Live stable membrane; MTDR-H - high mitochondrial activity;*P<0.05; **P<0.01; ***P<0.001

Table 2. **Correlation between laboratory tests and flow cytometric analysis of bull semen and 60-days NRR** (bull level)

Parameters	Bulls (n=15)				
	means±S.D.	range	LSM	MTDR-H	NRR
			r	r	r
HOS-1 (%)	34.9±6.1	22.0…44.5	0.41	0.25	-0.001
ΔHOS-2 (%)	6.2±5.7	–4.6…+14.0	0.42	0.44	0.73***
ΔHOS-3 (%)	–0.7±3.7	–6.8…+8.6	0.34	0.34	0.57*
SubMot (%)	58.1±7.2	39.0…66.3	0.71**	0.71**	0.71**
GMot (%)	76.6±7.5	55.7…83.6	0.63**	0.66**	0.72***
PMot (%)	60.8±8.7	34.1…68.7	0.73***	0.75***	0.63**
VCL (μm/sec)	94.4±8.6	80.4…106.2	0.21	0.27	0.74***
LIN	0.48±0.04	0.42…0.53	-0.06	-0.11	-0.69
ALH (μm)	2.9±0.3	2.5…3.2	-0.03	0.004	0.76***
LSM (%)	57.6±13.3	21.9…79.8	-	0.86***	0.36
MTDR-H (%)	77.2±14.3	29.6…86.9	0.86***	-	0.53*

SubMot- Subjective motility; GMot - general motility; PMot - progressively motile; VCL - curve line velocity; LIN - linearity; ALH - amplitude of lateral head displacement; LSM- Live stable membrane; MTDR-H - high mitochondrial activity;*P<0.05; **P<0.01; ***P<0.001.

The strongest positive correlation was found between ALH and NRR on bull level (P<0.001) and SubMot and NRR on batch level (P<0.001).

Relationships between predicted and observed non-return rates

The NRR prediction equation I (PNRR-I) included five parameters from conventional laboratory tests: HOS-2, SubMot, GMot, PMot and ALH.

Bull level:

PNRR-Ibull=26.78+0.70xΔHOS-2+0.46xSubmot+1.51xPMot-.30xGMot+25.52xALH

R^2=0.77; Adj. R^2=0.65

Batch level:

PNRR-Ibatch=4.43+0.35xΔHOS-2+0.39xSubmot+0.61xPMot-.58xGMot+12.06xALH

R^2=0.62; Adj. R^2=0.57

Another prediction equations, PNRR-II, were obtained by including seven parameters from both conventional laboratory tests and flow cytometric analysis.

Bull level:

PNRR-IIbull=-7.13+0.49×ΔHOS-2+0.22×Submot+0.77×PMot-1.65×GMot+33.23×ALH-0.21×LSM+0.58×MTDR-H;

R^2=0.91; Adj. R^2=0.83

Batch level:

PNRR-IIbatch=1.46+0.35×ΔHOS-2+0.31×Submot+0.51×PMot-0.57×GMot+13.96×ALH-0.07× LSM+0.06×MTDR-H;

R^2=0.63; Adj. R^2=0.56

These models were used to calculate the predicted fertility outcomes on bull and batch level. The highest positive correlation was found between PNRR-II and NRR on bull level (r=0.96; P<0.001). Positive correlation was also found between PNRR-I and NRR on batch level (r=0.84; P<0.001) and on bull level (r=0.87; P<0.001) and PNRR-II and NRR on batch level (r=0.78; P<0.001).

Discussion. The aim was to study the relationships between the results of conventional laboratory tests such as sperm motility measurement or HOS and flow cytometrically estimated mitochondrial activity and plasma membrane stability. We also studied the relationships between the test results and field fertility after AI and determined the best fertility predictive model as combination of these tests.

The study showed a positive correlation between the results of conventional laboratory tests (SubMot, PMot, GMot, VCL, ΔHOS-2, Δand HOS-3) and sperm quality variables LSM and MTDRR-H obtained by FCM analysis.

In our study, strong positive correlation was found between subjectively assessed motility (SubMot) and proportion of spermatozoa with stable plasma membrane (LSM) (r=0.71; P<0.01) on bull level, however good correlations between PMot and LSM and/or between PMot and MTDR-H (r=0.73 and r=0.75, respectively) must be considered even more important because of the lack of subjectivity.

The results of our study also showed significant positive correlation between percentage of cells with intact membrane (estimated by the simple HOS-tests) and MTDR-H (r=0.39; P<0.01). Similarly, Zuge et al. (2008) observed a significant positive correlation between sperm cells with full mitochondrial activity and percentage of cells with intact membrane assessed by the HOS test. Hallap et al. (2006) and Hua et al. (2006) reported a positive correlation between sperm motility and high mitochondrial activity and between percentage of cells with stable membranes and motility in fresh and FT semen. Some earlier studies (Januskauskas et al., 1996) described significant association between ATP content in FT semen and sperm motility. In our study, strong positive correlation was found between PMot and MTDR-H (r=0.75, P<0.001) which indirectly indicates importance of ATP energy produced by the intact mitochondria (Vishwanath et al., 1986; Rajender et al., 2010). The high positive correlation in our study was found between LSM and MTDR-H (r=0.86, P<0.001). These results confirm earlier findings that stable sperm plasma membrane is the prerequisite for mitochondrial functioning and that this in turn makes possible sperm motion (Kasai et al., 2002; Hua et al., 2006; Zuge et al., 2008).

Relations between the laboratory measurements of bull semen quality and their field fertility have been under discussion for many years. In our study, strong correlation was recorded between the SubMot, GMot, PMot, VCL and ALH of FT spermatozoa and NRR (r=0.63…0.76; P<0.01) on bull level, similarly to Zhang et al. (1998) and Januskauskas et al. (2003).

A significant positive correlation was also found between the results of different HOS tests in FT semen and NRR on batch level (r=0.15; P>0.05; r=0.63 and 0.65; P<0.001; for the HOS-1, HOS-2 and HOS-3, respectively). HOS-3 included 6 hours incubation of spermatozoa before performing a hypo-osmotic test and therefore, the results were directly dependent on sperm survival which is limiting factor also *in vivo* in the female reproductive tract. However, HOS-2 test gave similar results and from the practical point of view was more suitable as less time consuming. Usefulness of HOS tests in semen quality evaluation has been demonstrated earlier by Revell and Mrode (1994); Correa et al.(1997). It was found (Brito et al. 2003; Tartaglione and Ritta 2004) that results of hypoosmotic swelling test post-thaw could be used for prognosis of the potential fertility of bovine semen samples used for A.I.

In our study, a significant positive correlation was also obtained between the LSM, MTDR-H in FT semen and NRR on batch level (correspondingly r=0.51 and r=0.52; P<0.001) and between the MTDR-H and NRR on the bull level (r=0.53; P<0.05).

A positive correlation was found between several single parameters of common AI laboratory tests and NRR similar to the earlier findings (Correa et al. 1997; Zhang et al. 1998, Verbeckmoes et al. 2002). The single tests have not been sufficiently discriminative (Christensen et al. 1999) because each of them measures only a single attribute necessary for the fertilization. Therefore, it would be rational to combine different single parameters into a prediction model (Rodriguez-Martinez, 2006).

We tested two models, one of them (PNRR-I) was based on conventional laboratory tests only and another also included flow cytometrical measurements of sperm membrane stability and mitochondrial activity (PNRR-II). The highest positive correlation was found between PNRR-II and NRR on bull level (r=0.96; P<0.001). Similarly, motility parameters and membrane integrity of

FT spermatozoa were included into models proposed earlier by Januskauskas et al. (2003), Tartaglione and Ritta (2004), Phillips et al. (2004). They also have reported a strong correlation between predicted and actual non-return rates. Phillips et al. (2004) found that post-thaw sperm parameters such as morphologically normal sperms, the proportion of intact sperms, and cleavage of embryos, can be used to predict field fertility of dairy sires. In the study of Tartaglione and Ritta (2004) eosin-nigrosin supravital stain combined with the HOS-test has been included in a regression equation as predictors of *in vitro* fertility of FT bull semen.

In conclusion, the results of common laboratory tests (subjectively assessed motility and the parameters measured by the CASA, hypo-osmotic resistance tests) correlated well with flow cytometrically measured membrane stability and mitochondrial activity. Strong correlations were found between single semen quality parameters GMot, PMot, HOS-2, HOS-3 and NRR. Medium correlations were found between FCM analysis and NRR. However, a combination of conventional laboratory tests and flow cytometric analysis of membrane stability and mitochondrial activity in FT spermatozoa allowed better prediction of the potential fertility of bull semen in comparison to the conventional AI laboratory tests alone.

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Effect of insemination-related factors on pregnancy rate using sexed semen in Holstein heifers

J. Kurykin[1], T. Hallap[1], M. Jalakas[1], P. Padrik[1], T. Kaart[2], A. Johannisson[3], Ü. Jaakma[1]

[1]Department of Reproductive Biology, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia
[2]Department of Animal Genetics and Breeding, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia
[3]Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden

ABSTRACT: The objectives were to determine the effects of insemination with sexed semen at spontaneous and induced estrus, fixed-time insemination at synchronized estrus, the deposition site, estrous intensity, housing, age, body weight, and bull on the pregnancy rate in Holstein heifers, and to compare the quality traits of sexed sperm with those of unsexed semen. The study was conducted on 3206 heifers, housed in three free-stall barns and in four tie-stall facilities. After synchronization by two prostaglandin F2α (PGF2α) treatments 14 days apart, 281 heifers were inseminated conventionally and 118 intracornually with sexed semen, and 532 and 148 heifers, respectively, with unsexed semen 80–82 h after the second treatment. At spontaneous estrus, 1129 heifers were inseminated with sexed and 529 with unsexed semen, and at estrus induced by a single PGF2α treatment 185 heifers were inseminated with sexed and 284 with unsexed semen. Heifers were inseminated conventionally with sexed semen 12 h after detection of estrus, and with unsexed semen according to the a.m.–p.m rule. Sexed and unsexed semen doses from five bulls were evaluated for motility, morphology, membrane integrity, and chromatin stability. Overall pregnancy rate with sexed semen (41.7%) was 80.8% of that with unsexed semen (51.6%) and was lower than with unsexed semen irrespective of the type and intensity of estrus, and deposition site. Insemination at spontaneous estrus resulted in a higher pregnancy rate (53.4%) than at induced (41.9%) or synchronized (44.7%) estrus. Pregnancy rates did not differ after intracornual (44.9%) or conventional insemination (48.4%). Strong estrus resulted in higher pregnancy rate (by 14.4% points) compared to weak estrus. The type of housing, age, and weight of heifers had no effect irrespective of the type of semen. The total, progressive and linear motility, and membrane integrity were lower and proportions of immotile sperm greater, for sexed than for unsexed semen.

Keywords: Holstein; sex-sorted semen; estrus; sperm quality; intracornual insemination; fertility

INTRODUCTION

The invasive nature of flow cytometry exerts detrimental effects on the viability and quality of sex-sorted sperm resulting in lowered conception rates compared with unsexed semen. For that reason heifers are mainly used for insemination with sexed semen, as their fertility potential is higher than that of lactating cows (Schenk et al. 2009; DeJarnette et al. 2011).

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Apart from the small number of sperm per dose and reduced fertility of sex-sorted sperm, there are numerous factors which may affect the success of insemination. According to Hunter and Greve (1998), the decline in conception rates due to small sperm numbers could be avoided by fixed-time intracornual deposition at synchronized estrus, although other relevant studies on sexed semen have reported contradictory results (An et al. 2010). The sorting procedure and subsequent freezing-thawing were found to accelerate the acrosome reaction and capacitation, reducing the lifespan of sperms in the genital tract (Moce et al. 2006), thus timing of insemination with sexed semen relative to ovulation could be more critical compared to unsexed semen (Schenk et al. 2009). Insemination with sexed semen could be carried out 12 h later compared to the recommended insemination time when using unsexed semen (Schenk et al. 2009). The type of estrus (Abdel-Azim 2010) and an evidence and intensity of the estrous signs (DeJarnette et al. 2010) appeared to be related with the pregnancy rate when sexed semen was used. Different results have been reported concerning insemination with sexed semen in the studies that examined the interaction effect of the bull and the type of estrus on the pregnancy rate (Abdel-Azim 2010; Sa Filho et al. 2010). Considering the complex nature of the fertility of semen, estimation of the functional and structural traits of sex-sorted sperm could be useful when evaluating the fertility potential of sexed semen. Although potentially viable sperm are selected by detecting membrane integrity, flow-sorting may cause unrecognizable damage leading to decreased motility (Sartory et al. 2004). Membrane and chromatin integrity damage can be caused by the exposure of sperm to pressure, ultraviolet radiation, and Hoechst/laser interaction (Boe-Hansen et al. 2005; Garner 2006; Gosalvez et al. 2011).

Consequently, further information concerning the effect of the insemination-related factors and those related to the fertility of semen on pregnancy rate is required as it would facilitate the use of sexed semen for insemination in dairy herds.

The objectives of this study were to determine the effects of insemination with sexed semen at spontaneous and induced estrus, fixed-time insemination at synchronized estrus, the deposition site, estrous intensity, housing, age, body weight, and bull on the pregnancy rate in Holstein heifers, and to estimate the structural and functional quality traits of sex-sorted sperm in comparison with those of unsexed semen.

MATERIAL AND METHODS

Animals, semen, and inseminations. The study was conducted on 3206 heifers of the Holstein breed (mean age 478.5 ± 71.0 days, and mean weight 418.0 ± 40.4 kg) from seven dairy herds. The heifers were housed in three free-stall barns and in four individual tie-stall facilities. Commercial frozen semen doses from 10 Holstein bulls, containing flow-sorted 2.1×10^6 X-chromosome bearing sperm, or 15×10^6 unsorted sperm in 0.25 ml straws (Cogent Ltd., Chester, UK and Select Sires Inc., Plain City, USA) were used. Due to the genetic requirements of the on-farm breeding programs, the bulls were not balanced within and across farms. Only first service inseminations with a single dose of sexed or unsexed semen were used.

For insemination at synchronized estrus, 1079 heifers were treated twice with 25 mg of prostaglandin F2α (PGF2α) (Dinolytic®; Pharmacia N.V./S.A., Puurs, Belgium) 14 days apart. Intracornual and conventional inseminations were performed with sexed or unsexed semen, respectively, 80–82 h after the second PGF2α treatment. A total of 281 heifers were inseminated conventionally and 118 intracornually using sexed semen, while 532 heifers were inseminated conventionally and 148 intracornually with unsexed semen. At intracornual insemination, semen was deposited near the tip of the uterine horn, ipsilaterally to the ovary bearing the largest, presumed to be ovulatory follicle, identified by transrectal ultrasonography (HS-1500V, equipped with a 7.5 MHz linear-array transducer; Honda Electronics Co., Toyohashi City, Japan). Prior to insemination, the intensity of estrus was evaluated. The presence of mucous discharge, vulvar edema, hyperemia of vulvar mucosa, and relaxation of the cervix (ease to pass through with a catheter) were recorded. Estrus was considered "strong" if at least three of the signs were well expressed, and catheter passed easily through the cervix. In the absence of at least two of these signs, and if a difficulty passing through the cervix was encountered, the estrus was considered "weak". Evaluations of the estrus, ultrasonography as well as conventional and intracornual inseminations were performed by an experienced veterinarian. The inseminations were performed on all the seven farms, and eight bulls were used.

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A total of 2127 heifers which displayed estrus spontaneously (n = 1658) or following a single treatment with PGF2α (n = 469) were inseminated upon detection of estrus. Observations for the detection of estrus were performed four times a day by on-farm personnel. At spontaneous estrus, 1129 heifers were inseminated with sexed, and 529 with unsexed semen. At induced estrus, detected after a single treatment with PGF2α (Dinolytic®; Pharmacia N.V./S.A.), 185 heifers were inseminated with sexed, and 284 with unsexed semen. Insemination with sexed semen was performed 12 h after detection of spontaneous or induced estrus (Schenk et al. 2009), and unsexed semen was used according to the a.m.–p.m. rule. Prior to insemination, the intensity of estrus was evaluated as described above and recorded. Observations for the detection of estrus and inseminations were carried out by on-farm technicians. The deposition site of both types of semen was the uterine body at both spontaneous and induced estrus. The inseminations were performed on six farms using semen from eight bulls (six of the bulls overlapped with those used for insemination at synchronized estrus).

The pregnancy status of heifers was diagnosed by rectal palpation of the uterus 45–60 days after insemination.

Assessment of sperm quality. The sexed and unsexed semen doses of the same batches from the five bulls that produced a large difference in pregnancy rates for sexed semen (35.2–60.3%) were evaluated for motility, morphology, membrane integrity, and chromatin stability. Unless otherwise specified, all chemicals were purchased from Becton Dickinson (San Jose, USA) and Sigma Aldrich (St. Louis, USA).

The total, progressive, linear and non-linear motility, immotility, average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), wobble (WOB, %; VAP/VCL × 100), straight line velocity (VSL, μm/s), lateral head displacement (ALH, μm), and beat cross frequency (BCF, Hz) of sperm were assessed using computer assisted semen analysis (CASA, Sperm Vision; Minitüb GmbH & Co., Tiefenbach b. Landshut, Germany). Samples of 3 μl were placed in 20-μl deep disposable four-chamber Leja® slides (IMV, Maple Grove, USA), and about 400 sperm were tracked and assessed at 200× under a phase contrast microscope Olympus CX 31 (Olympus Corp., Tokyo, Japan).

The morphology of sperm was evaluated in air-dried smears stained with Spermac stain (Stain Enterprises Inc., Wellington, USA). A total of 100 sperm cells were counted on duplicate slides at 1000×. The frequencies of sperm with abnormal acrosomes, mid-pieces, head shapes, and coiled or missing tails were recorded as a percentage of the number of sperm evaluated. The membrane integrity was assessed by the hypoosmotic swelling test (HOST). After incubation of 20 μl of unsexed semen and 40 μl (due to the low concentration of sperm) of sexed semen with 0.5 ml of a 150 mOsm/kg hypoosmotic solution for 60 min, 100 sperm were assessed in two replicates by two operators at 1000×. The average percentage of sperm with swollen tails was calculated.

The susceptibility of DNA to acid-induced denaturation was measured using a method that is based on the ability of acridine orange (AO) to metachromatically shift from green (double-stranded DNA) to red (denaturized, single-stranded DNA) fluorescence (Evenson et al. 1980). Denaturation was expressed as a function of α_t ratio of red to red + green (total sperm DNA) fluorescence intensity. In the samples, α_t was calculated for each spermatozoon, and expressed as the percentage of sperm with high α_t values (excess of single-stranded DNA, or DNA fragmentation index (DFI)). Samples were diluted to $1–2 \times 10^6$/ml in TNE buffer (0.01M TRIS, 0.15M NaCl, 1mM EDTA, pH 7.4). After 60 s, 200 μl of semen was mixed with 400 μl of acid-detergent solution (0.15M NaCl, 0.08 N HCl, 0.17% Triton-X100, pH 1.2). After 30 s, the samples were stained with 1.2 ml of the AO solution (0.2M Na_2HPO_4, 1mM EDTA, 0.15M NaCl, 0.1M citric acid, 6 μg/ml AO, pH 6.0; Merck, Kebo Lab, Stockholm, Sweden). The flow-cytometry was started 3 min after the acidic treatment (FACSStar Plus; Becton Dickinson). AO was excited with an argon ion laser Innova 90 (Coherent, Santa Clara, USA) at 488 nm, and running at 200 mW. In association with double-stranded DNA, AO fluorescence is green (530 ± 30 nm, detected with the FL 1 detector), while it is red with single-stranded DNA (> 660 ± 20 nm, detected with the FL 3 detector). Data acquisition was performed by using the CellQuest 3.1 software (Becton Dickinson), and stopped after 10 000 events were recorded. The resulting list-mode files were processed using FCS Express Version 2 (De Novo Software, Thornhill, Canada) for calculation of DFI.

Statistical analysis. As the number of inseminations was different at different factor levels and combinations of levels, the multifactorial generalized linear model with logit link function was used instead of the simple univariate analysis to compare the pregnancy rates. The following factors were considered simultaneously: type of

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semen (sexed and unsexed), deposition site (uterine horn and uterine body), type of estrus (synchronized, spontaneous, and induced), intensity of estrus (strong and weak), bull, type of housing (tie- and free-stall), and the farm nested to housing type. The effect of inseminator was covered by the effect of the farm, while the effects of heifer age and body weight were not considered since the effect of these factors on pregnancy rate was not statistically significant ($P > 0.05$). To determine the interaction effects of the deposition site, type of estrus, intensity of estrus, donor bull, and farm by the type of sperm, the corresponding interaction effects were included in the model. As simultaneous estimation of all the interaction effects was impossible, a separate model was fitted for each interaction. The non-significant effects of deposition site and type of housing were excluded to guarantee the estimability of interactions. In addition to the logistic regression model parameters, the adjusted pregnancy rates and adjusted odds ratios were estimated. For all the factors studied, the base level was that with the lowest pregnancy rate. Modelling was performed using the GLIMMIX procedure of the SAS software (Statistical Analysis System, Version 9.1, 2006).

Semen quality analysis was performed using the data obtained from ten observations (five bulls, two treatments), the pairwise *t*-test was used to test the effect of treatment, and the relationships between sperm quality and pregnancy rate were estimated using the Pearson's correlation analysis. The results were considered to be significant at $P < 0.05$.

RESULTS

Factors affecting pregnancy rate in heifers. The adjusted pregnancy rate for heifers inseminated with sexed semen at spontaneous, PGF2α induced, and synchronized estrus (Table 1) was 9.9% points lower than that with unsexed semen (41.7% and 51.6%, $P < 0.001$, respectively) across the entire experiment.

The adjusted pregnancy rate at spontaneous estrus was 11.5% points higher than that at induced estrus ($P < 0.001$), and by 8.7% points higher compared to that at synchronized estrus ($P < 0.001$). The pregnancy rates obtained at synchronized and induced estrus did not differ ($P = 0.545$). At strong estrus, the adjusted pregnancy rate was by 14.4% points higher than that at weak estrus ($P < 0.001$).

Table 1. Estimates of simultaneously considered multivariate logistic model parameters, adjusted pregnancy rates (aPR), and adjusted odds ratios (aOR)

Effects[1]	No. of heifers	Estimate (± SE)	aPR (%)	aOR	*P*-value
Type of semen					
Unsexed	1493	0.399 ± 0.094	51.6	1.49	< 0.001
Sexed	1713	0	41.7	1	
Deposition site					
Uterine body	2940	0.139 ± 0.173	48.4	1.15	0.423
Uterine horn	266	0	44.9	1	
Type of estrus					
Spontaneous	1658	0.462 ± 0.144	53.4	1.59	< 0.001
Synchronized	1079	0.112 ± 0.186	44.7	1.12	0.545
Induced	469	0	41.9	1	
Intensity of estrus					
Strong	2074	0.584 ± 0.079	53.9	1.79	< 0.001
Weak	1132	0	39.5	1	
Housing					
Tie-stall	2300	0.150 ± 0.119	48.3	1.16	0.208
Free-stall	906	0	44.5	1	

[1]the number of inseminated heifers per farm varied between 59 and 1530, and the adjusted pregnancy rates among tie-stall farms ranged from 39.5 to 63.9% and among free-stall farms from 30.6 to 54.4% ($P < 0.001$); the number of inseminated heifers per bull varied between 100 and 684, and the adjusted pregnancy rates among bulls ranged from 32.1 to 64.0% ($P < 0.001$)

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Table 2. Quality traits of sperm in sexed and unsexed semen across bulls (CDt–HMs)

Traits	Sexed semen						Unsexed semen						*P*-value
	CDt	DDr	ECt	FDx	HMs	means ± SD	CDt	DDr	ECt	FDx	HMs	means ± SD	
Motility (%)													
Total	28.6	26.2	56.3	53.4	64.2	45.7 ± 17.2	42.1	48.5	63.1	79.1	85.4	63.6 ± 18.7	< 0.01
Progressive	17.1	13.9	49.3	41.6	58.2	36.0 ± 19.7	41.1	47.0	56.8	75.2	80.8	60.2 ± 17.3	< 0.01
Linear	11.4	7.6	36.6	34.6	47.8	27.6 ± 17.3	28.6	42.1	50.6	51.7	85.7	51.7 ± 21.1	< 0.01
Non-linear	0	1.5	2.8	4.4	1.5	2.0 ± 1.6	0	0	0	3.5	0	0.7 ± 1.5	< 0.05
Immotility	71.4	73.8	43.7	46.6	35.8	54.3 ± 17.2	57.9	51.5	36.9	20.1	14.6	36.2 ± 18.9	< 0.01
VAP (μm/s)	65.2	62.7	70.4	56.3	69.9	64.9 ± 5.8	55.8	75.0	52.3	43.1	52.1	55.7 ± 11.8	0.22
VCL (μm/s)	126.8	120.8	118.6	94.1	124.0	116.9 ± 13.1	116.0	134.8	84.6	76.2	88.0	99.9 ± 24.6	0.14
WOB (%)	48.0	56.0	62.0	56.0	59.0	56.2 ± 4.7	51.0	52.0	59.0	60.0	56.0	55.6 ± 3.6	0.74
VSL (μm/s)	53.6	48.5	55.9	47.1	60.1	53.0 ± 5.3	47.1	64.2	42.0	35.2	44.7	46.6 ± 10.8	0.33
ALH (μm)	2.8	2.8	2.9	2.6	2.6	2.7 ± 0.1	2.7	3.3	2.2	2.0	2.2	2.5 ± 0.5	0.29
BCF (Hz)	31.3	30.5	29.9	31.0	28.4	30.2 ± 1.2	30.4	31.8	29.7	28.7	29.7	30.0 ± 1.1	0.81
Morphology (%)													
Acrosomal abnormality	2	1	0	0	0	0.6 ± 0.9	2	2	1	2	1	1.6 ± 0.5	< 0.05
Abnormal mid-pieces	0	1	0	0	0	0.2 ± 0.4	0	7	4	1	7	3.6 ± 3.3	0.06
HOST (%)	7.5	13.0	8.5	22.0	17.5	13.7 ± 6.1	49.0	48.5	43.0	62.5	60.0	52.6 ± 8.3	< 0.001
DFI (%)	4.5	4.7	3.8	3.0	4.2	4.0 ± 0.7	2.1	2.5	4.9	3.9	3.7	3.4 ± 1.1	0.44

VAP = average path velocity, VCL = curvilinear velocity, WOB = wobble, VSL = straight line velocity, ALH = lateral head displacement, BCF = beat cross frequency, HOST = hypo-osmotic swelling test, DFI = DNA fragmentation index

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No statistically significant difference in pregnancy rate was found due to deposition site ($P = 0.423$). Also, none of the interaction effects between semen and the deposition site, type of estrus, and intensity of estrus were statistically significant ($P = 0.243$, $P = 0.819$, and $P = 0.979$, respectively), which indicates that the difference in pregnancy rate between sexed and unsexed semen did not depend on other insemination-related factors.

The adjusted pregnancy rates varied between 30.6 and 63.9% among farms ($P < 0.001$). However, there was no statistically significant difference between tie-stall and free-stall farms ($P = 0.208$). Furthermore, the interaction effects between farm and type of semen were not statistically significant ($P = 0.133$), although there were two farms where the adjusted pregnancy rate with sexed semen was even higher than that with unsexed semen.

The adjusted pregnancy rates ranged from 32.1 to 64.0% ($P < 0.001$) among bulls, however the interaction effect of the bull and the type of semen on pregnancy rate was not statistically significant ($P = 0.828$). Other interaction effects between bulls and insemination-related factors were not statistically significant ($P > 0.05$), albeit the type of estrus and bull tended to interact ($P = 0.067$).

The mean age and body weight of heifers that conceived with sexed semen (482.9 days and 415.0 kg) did not differ from those of heifers that did not conceive (470.1 days and 414.4 kg), which was also the case with unsexed semen (483.4 days and 419.9 kg vs 471.8 days and 431.2 kg, respectively; all $P > 0.05$, t-test).

Semen quality traits and the relationship between semen quality and pregnancy rate. Table 2 outlines the quality traits of semen of the five bulls studied. The total, progressive, and linear motility of sexed sperm were lower ($P < 0.01$, all values) than those of unsexed semen, and greater proportions of non-linear and immotile sperm were observed ($P < 0.05$ and $P < 0.01$, respectively). The total motility of sexed sperm tended to correlate positively with pregnancy rate ($r = 0.82$, $P = 0.09$). The proportion of sperm with intact membrane was lower in sexed semen compared to unsexed semen ($P < 0.001$). Chromatin stability tests revealed no differences between sexed and unsexed sperm ($P > 0.05$).

DISCUSSION

The pregnancy rate for sexed semen from this trial is comparable to that previously reported for inseminations at spontaneous (Cerchiaro et al. 2007; Frijters et al. 2009) and synchronized estrus (Seidel and Schenk, 2008; An et al. 2010), but higher than that reported by Bodmer et al. (2005) and Mallory et al. (2013). Intracornual deposition of sexed semen did not prevent decline in pregnancies. This is in agreement with the findings by Seidel and Schenk (2008), whereas An et al. (2010) reported similar pregnancy rates after intracornual deposition of sexed (52.8%) and unsexed semen (59.6%). The sample sizes in that study were possibly too small ($n = 36$ and 47, respectively) to reach significance. Despite the intracornual deposition of sexed semen, the proportion of unfertilized ova in embryo donors was higher than in unsexed controls (Sartory et al. 2004; Peippo et al. 2009). The percentage of transferable embryos from the total embryos and ova recovered was 45% using sexed semen and intracornual deposition, whereas it was 70% using conventional semen, suggesting that sperm numbers were more important than insemination site (Kaimio et al. 2013).

The type of estrus affected pregnancy rates. Despite synchronization of estrus with PGF2α, the interval between the onset of estrus and ovulation can vary from 28 to 61 h depending on the maturity of the dominant follicle (Saumande and Humblot 2005). A combination of variable times of ovulation after synchronized estrus with reduced sexed sperm motility and viability may compromise chances of conception. Induction of estrus by a single PGF2α treatment at a random stage in the estrous cycle may initiate ovulation of immature follicles, and cause higher incidence of fertilization failure (Dorsey et al. 2011). The higher pregnancy rate that was observed for inseminations at spontaneous estrus, compared to that at induced or synchronized estrus, was probably due to the larger number of ovulations that occurred within the time appropriate for sexed sperm. The tendency towards interaction between the bull and the type of estrus is in accordance with the decreased conception rates related to the use of sexed semen for synchronized services, compared to non-synchronized services (Abdel-Azim 2010). With regards to sperm longevity, individual bulls can maintain high fertility rates over a broad range of time relative to ovulation (Dorsey et al. 2011).

Regardless of the type of semen and the type of estrus, pregnancy rate was by 14.4% points higher

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at the presence of strong estrous signs, compared to the absence of signs, whereas Seidel et al. (1999) did not find this tendency when using sexed semen. Mallory et al. (2013) reported pregnancy rate with sexed semen in heifers that displayed estrus after synchronization by 20% points higher compared to those that did not express apparent signs. Unlike strong estrus, weak estrus has been associated with elevated progesterone levels (Schopper et al. 1993) that exert a suppressive effect on the amplitude and the frequency of LH pulses in the pre-ovulatory period (Savio et al. 1993). A shortened duration of estrus in heifers with weak signs in connection with improper timing of insemination can be related to fertilization failures (Yoshida and Nakao 2005).

Results from the studies on the use of conventional (Donovan et al. 2003) and sexed (Cerchiaro et al. 2007) semen indicate that neither age nor weight influence pregnancy rate in heifers. Brickell et al. (2009) reported a 30 kg higher weight of heifers that did not conceive after being served, compared to those that conceived, but no effect of age was found. The failure to conceive has been associated with high growth rates during the rearing period, which may result in physiological immaturity at the first service. The differences in pregnancy rates between farms were greater than those due to the type of semen, housing, and estrous intensity. Abdel-Azim (2010) found that the herds that contributed most to variation in fertility showed less variations between bulls than between technicians. Improper semen handling and inability to maintain sperm viability throughout the insemination process may result in a low conception rate, which can be expressed as "herd effect" (DeJarnette et al. 2011).

Compared to unsorted sperm, the motility traits of sex-sorted sperm were lower across the bulls along with the increase in nonlinearity and immotility. Sperm motility and pregnancy rate that showed a substantial variation between bulls indicate that the response to sorting is individual-specific. Reduced mitochondrial activity was found in oocytes exposed to the dye and laser light (Smith 1993), which could be linked to decreased motility in sex-sorted sperm, as mitochondria produce adenosine triphosphate as a source of energy (Mannella 2000). High dilution rates of semen causing a fall in the concentration of protective seminal lipids and proteins (Maxwell et al. 1997) may lead to greater nonlinearity and immotility. Lipids and phospholipids that are in the form of polyunsaturated fatty acids in the membrane are susceptible to oxidation by reactive oxygen species. Oxidative stress damages membranes and decreases motility in frozen-thawed sperm (Chatterjee and Gagnon 2001). It may occur to a greater extent in sexed semen due to the high susceptibility of sex-sorted sperm to cryopreservation stressors (Garner 2006). A combination of these factors in conjunction with the small semen doses that are currently commonly used, may lead to a sub-optimal functional sperm concentration for some individuals. Frijters et al. (2009) reported the effect of a low dose and sorting on the decline in the 56-day non-return rate for four bulls, and that of just sorting for two bulls.

Hyperactivation is a movement pattern of sperm that is characterized by higher VCL, ALH, and simultaneously lower linearity and WOB (Mortimer 2000). As hyperactivation coincides with sperm capacitation, it can be used to evaluate the capacitational status in the sperm undergoing flow-sorting and freeze-thawing. No signs of hyperactivation were found in the current study except a decline in sexed semen linearity. Several authors postulate that acrosomal responsiveness and hyperactivation processes are not completely tied to each other (Suarez 2008), and that sorting-induced alterations do not necessarily indicate the capacitated status of sperm (Bucci et al. 2012). However, capacitation-like changes in the sperm of some bulls due to sorting and freezing-thawing, reducing fertilizing lifespan (Moce et al. 2006), cannot be excluded.

The damage to the acrosome and the membrane integrity of sperm was found to occur due to mechanical stress during sorting and subsequent freezing-thawing (Sartory et al. 2004; Garner 2006). Across the bulls, the proportion of sperm with damaged membranes was ~39% points higher in sex-selected semen. Carvalho et al. (2010) documented ~20% points more sperm with damaged membranes in sexed samples than in unsexed samples. The differences in pressure and the susceptibility of sperm to cryopreservation-induced damage (Garner 2006; De Graaf et al. 2009) may account for the differences in the frequency of the alterations.

Morphological examination showed quite low differences in the values, with no category exceeding 4%. Since sperm abnormalities and chromatin

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integrity are considered to be interrelated (Enciso et al. 2011), low values for DFI were expected. In the current study, the average value of DFI did not exceed 5%, which seems to be common for bulls of various breeds (Hallap et al. 2005; Christensen et al. 2011). The damage to chromatin integrity has been associated with the exposure of sperm to ultraviolet radiation (Boe-Hansen et al. 2005) and the Hoechst/laser interaction (Gosalvez et al. 2011). Although the average DFI % did not differ between two semen types, increase in the proportion of sperm with DNA fragmentation was reported in three and reduction in two bulls. Reduction in the sperm number with damaged DNA in individual bulls has been reported (Blondin et al. 2009; Gosalvez et al. 2011), suggesting a beneficial effect on semen quality. Gosalvez et al. (2011) documented that two bulls out of ten were more efficient and consistent in resisting to sperm DNA fragmentation, irrespective of the ejaculate, while the others demonstrated higher levels of damage. This can be associated with the advantage of sexed over unsexed semen in one bull (CFDx), that exhibited reduced numbers of sperm with damaged DNA. Despite the similar improvement in another bull (AECt), the fertility of sexed semen from AECt was lower than that of unsexed semen.

CONCLUSION

The pregnancy rates in heifers inseminated with sexed semen were influenced by the bull as well as the type and intensity of estrus. The effect of the deposition site was not significant. Pregnancy rates differed between farms irrespective of the type of estrus, but no effects of tie-stall and free-stall housing, heifer age or body weight were found. As regards sexed semen, insemination 12 h after detection of spontaneous estrus appeared to be more efficient compared to insemination at estrus induced by a single PGF2α treatment, or that at a fixed-time after synchronization by two PGF2α treatments. Evaluation of estrus intensity could be used to maximize the number of pregnancies, especially in regard to inseminations at induced estrus, or those at a fixed time in synchronized estrus. There is no need to deposit sexed semen near the fertilization site as similar efficiency can be achieved by conventional insemination.

Inferior quality of sexed semen compared to unsexed semen can be associated with a lower proportion of progressive and linear motility, higher rate of immotility, and impaired membrane integrity. Assessment of these traits could be indicative of the fertility potential of sexed semen.

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Corresponding Author

Triin Hallap, Ph.D., research fellow, Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Department of Reproductive Biology, 62 Kreutzwaldi St., 51014, Tartu, Estonia
Phone: +372 55 615 149, e-mail: triin.hallap@emu.ee

CURRICULUM VITAE

First name:	Peeter
Surname:	Padrik
Date of birth:	December 18, 1961
E-mail:	ppadrik@gmail.com
Address:	Animal Breeders' Association of Estonia (ABAE), Keava 79005, Rapla County, Estonia
Education:	2018 external PhD student
	2002-2009 PhD student
	2002 Master`s Degree, "Sperm morphology and membrane hypoosmotic resistance in Estonian Holstein dairy bulls"; supervisors Prof. Ülle Jaakma and Prof. Olev Saveli, Estonian Agricultural University
	1990 Estonian Agricultural Academy
Institution and position held:	2003- AI laboratory manager, member of the Board, ABAE
	1999-2003 AI laboratory manager, ABAE
	1999-1998 AI laboratory technologist, ABAE
	1999-2017 Spermatologist (workload 0.1), Department of Reproductive Biology, Institute of Veterinary Medicine and Animal Sciences
Dissertations supervised:	„Influence of bull breed to fresh and frozen-thawed semen quality". Liis Landing, Maaülikool 2011.
	"Application of cryopreservation techniques for atlantic salmon (*Salmo Salar* L) milt in RMK Põlula fish rearing center". Raimo Palusalu, Maaülikool 2017.
Research projects:	Identification of sex-specific markers in reproductive veterinary medicine (Bovine Sperm Sexing, BOSS).

ELULUGU

Nimi:	Peeter Padrik
Sünniaeg:	18.12.1961
E-post:	ppadrik@gmail.com
Aadress:	Eesti Tõuloomakasvatajat Ühistu, 79005 Keava, Rapla maakond
Töökoht ja amet:	Laboratooriumi juhataja, juhatuse liige
Haridustee:	2018 doktorantuur, ekstern 2002–2009 doktorantuur, EPMÜ 2000–2002 magistriõpe EPMÜ 1985–1990 Põllumajandusakadeemia zooinseneriteaduskonna zootehnika eriala *cum laude,* zooinseneri kvalifikatsioon 1969–1980 Tartu 3. Keskkool
Teenistuskäik:	2003– Eesti Tõuloomakasvatajate Ühistu (ETKÜ) laboratooriumi juhataja ja juhatuse liige 1999–2003 ETKÜ laboratooriumi juhataja 1999–1998 ETKÜ laboratooriumi peatehnoloog 1999–2017 EPMÜ loomaarstiteaduskonna sigimisbioloogia osakonna spermatoloog (0,1 koormusega)
Teaduskraad:	Põllumajandusteaduste magistri kraad loomakasvatuse erialal 2002, juhendaja Ülle Jaakma, Olev Saveli. Magistritöö „Eesti holsteini tõugu sugupullide sperma morfoloogia ja spermide osmoresistentsus“.
Juhendamisel kaitstud väitekirjad:	„Lihatõugu noorpullide sperma kvaliteet, seda mõjutavad tegurid ning seos sperma sügavkülmutamiskindlusega”, Liis Landing, Maaülikool 2011. „Atlandi lõhe (*Salmo Salar* L.) niisa sügavkülmutamise tehnoloogia rakendamine RMK Põlula kalakasvatuskeskuses”, Raimo Pajusalu, Maaülikool 2017.
Uurimisprojektid:	Pulli seemnerakkude soospetsiifiliste markerite identifitseerimine reproduktiivveterinaarias (Bovine Sperm Sexing, BOSS).

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Jaakma, Ü., **Padrik**, **P**., Kurykin, J., Aidnik, M., Jalakas, M., Laidvee, U., Majas, L., Waldmann, A. 2002. Sperm motility, hypo-osmotic resistance and fertility after freezing of low doses of bull semen. *Abstract. In: Proceedings of the 18th scientific meeting of the European Embryo Transfer Association.* Rolduc, The Netherlands, 6-7 September 2002, 128.

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Jaakma, Ü., **Padrik**, **P**. 2000. Relationships between frozen-thawed sperm motility, membrane osmotic resistance and fertility in Estonian Holstein bulls. *14th International Congress on Animal Reproduction, Abstracts*, Vol. 2, Stockholm, 2-6 July, 2000.

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Padrik, **P**., Jaakma, Ü., Bulitko, T., Hallap, T. 2012. Innovaatiliste tehnoloogiate rakendamine sugupulli sügavkülmutatud/sulatatud sperma kvaliteedi tõstmiseks. *Tõuloomakasvatus* 2, 14–17.

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Landing, L., Viinalass, H., **Padrik**, **P**. 2010. Limusiini tõugu noorpullide värske sperma ja spermide kvaliteedi dünaamika ning sügavkülmutamiskindlus. *Tõuloomakasvatus* 2. 19–21.

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Landing, L., Viinalass, H., **Padrik**, **P**. 2010. Herefordi tõugu noorpullide sperma kvaliteedi dünaamika. *Tõuloomakasvatus* 1, 25–27.

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Padrik, **P**. 2001**.** Pullisperma kvaliteedist. *Tõuloomakasvatus* 1, 18-19.

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VIIS VIIMAST KAITSMIST

KERSTI VENNIK
MEASUREMENTS AND SIMULATIONS OF RUT DEPTH DUE TO SINGLE AND MULTIPLE PASSES OF MILITARY VEHICLES ON TYPICAL ESTONIAN SOILS AND NATURAL RECOVERY OF RUTS
MILITAARSÕIDUKITE ÜLESÕITUDE TULEMUSENA KUJUNENUD ROOPASÜGAVUSE MÕÕTMINE JA MODELLEERIMINE EESTI MULDADEL NING ROOBASTE LOODUSLIK TAASTUMINE
Professor **Endla Reintam**, **Thomas Keller**, PhD (Rootsi Põllumajandusülikool), **Peeter Kukk**, PhD (Tartu Ülikool)
19. juuni 2019

KARIN NURME
ENCODING OF ENVIRONMENTAL HEAT BY THE SENSORY TRIAD OF INSECTS ANTENNAL THERMO- AND HYGRORECEPTOR NEURONS
KÕRGETE VÄLISTEMPERATUURIDE SENSOORNE KODEERIMINE PUTUKATE ANTENNAALSETE TERMO- JA HÜGRONEURONITE TRIAADI POOLT
Vanemteadur **Enno Merivee**, teadur **Anne Must**, dotsent **Ivar Sibul**
19. juuni 2019

KAIE METSAOTS
HOLISTIC DEVELOPMENT OF THE OIL SHALE REGION AS AN INDUSTRIAL HERITAGE, RECREATIONAL, SPORTS AND TOURISM DISTRICT
PÕLEVKIVIREGIOONI TERVIKLIK ARENDAMINE TÖÖSTUSPÄRANDI-, PUHKE-, SPORDI- JA TURISMIPIIRKONNANA
Professor **Kalev Sepp**
27. juuni 2019

GUNNAR MOROZOV
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SÜSINIKU- JA LÄMMASTIKUVOOD ERINEVATE KASVUKOHTADE KAASIKUTES JA HALL-LEPIKUTES
Professor **Veiko Uri**
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RISTO RAIMETS
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SÜNTEETILISTE JA BIOLOOGILISTE PESTITSIIDIDE MÕJUD MEEMESILASTELE JA KIMALASTELE
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