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

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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

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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.

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 fluorescent values were recorded on a logarithmic scale. Obscuration bars were set for maximum sensitivity in order to obtain L-shaped forward light – scat-

ter/sideways light scatter distribution of sperm cells. Fluorescence of Yo-PRO 1 was detected on a 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 a flow rate of. 200 cells/s. Acquisitions were made using CellQuest Pro software (Becton Dickinson, San Jose, USA). Dot plots for offline analyses were drawn by WinMDI 2.8. Events accumulated in the lower left corner correspond to sample debris and were excluded from the analysis. 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 as 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 FCM 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 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:

$$PNRR= -136.4-0.72\times Pmot+1.397\times GMot+174.27\times LIN+9.407\times ALH+0.165\times LSM,$$

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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