Evaluation of Sperm Production, Testicular Measurements and Post-Thaw Sperm Quality in Tori and Estonian Breed Stallions

Ants Kavak
Department of Obstetrics and Gynaecology
Uppsala

Licentiate thesis
Swedish University of Agricultural Sciences
Uppsala 2004

The present thesis is a partial fulfilment of the requirements for a Licentiate Degree in Veterinary Medicine (Vet. Med. Lic.), at the Swedish University of Agricultural Sciences (SLU), in the field of Reproduction

ISBN 91-576-6600-8

Tryck: SLU Service/Repro, Uppsala 2004

To Merit and Hanna Kristin
To my mother and father

Thank you to all “sperm donors”: Ailur, Aisberg, Herakles, Hertsog, Hiirik,
Lakmus, Leigar, Ufo, Aku, Ando, Elder, Elkar, Rosett, Tukker, Viks and Vigur.

Abstract

Kavak, A. 2004. *Evaluation of sperm production, testicular measurements and post-thaw sperm quality in Tori and Estonian breed stallions*. Licentiate dissertation.
ISBN: 91-576-

The present study was aimed to evaluate daily sperm output, testicular measurements and post-thaw sperm quality of Tori and Estonian breed stallions.

In the first study, daily sperm output (DSO) and testicular measurements were evaluated. One ejaculate was collected daily for 10 subsequent days from 8 Tori (T) and 8 Estonian (E) breed stallions. The daily sperm output was calculated as the mean of the total sperm number (TSN) collected on days 8-10 in T stallions and on days 4-10 in E stallions. The DSO of T stallions was $12.9x10^9$ spermatozoa and of E stallions $4.5x10^9$ spermatozoa ($p<0.001$). All testicular measurements were significantly smaller in E than in T stallions ($P<0.001$). Testicular circumference was 45.4 cm and 35.4 cm in T and E stallions, respectively ($P<0.001$). The testicular circumference was correlated with DSO in both E ($P<0.05$) and T stallions ($P=0.071$).

In paper II, the sperm motility, membrane and acrosome integrity as well as the capacitation status of frozen-thawed spermatozoa from 7 T and 6 E stallions with CASA and flow cytometry (using a combination of the fluorophore stains SNARF, PI and FITC-PSA, and Merocyanine-540/Yo-Pro-1, respectively) were studied. Two ejaculates were cryopreserved from each stallion in 0.5 ml plastic straws according to the Hannover method. Two straws per ejaculate and per stallion were thawed at 37°C for 30 sec. Motility was analysed with CASA immediately after thawing, whereas for flow cytometry, spermatozoa were cleansed by 70:40 % Percoll discontinuous density gradient separation before being analysed for sperm viability, acrosome integrity (triple stain) and capacitation status (Merocyanine 540/Yo-Pro-1). Results are presented as least square means (LSmeans). The motility of frozen-thawed semen was 43.4 % for T and 42.3 % for E stallions ($P>0.05$). After Percoll separation, 79.3 % of the spermatozoa from T stallions had intact acrosomes and 1.7 % of them showed early signs of capacitation. The same parameters for E stallions were 84.5 % and 2.3 %, respectively. There were no statistically significant differences between breeds or ejaculates within breed for any evaluated parameter.

Keywords: testicular measurement, daily sperm output, motility, plasma membrane integrity, acrosome status, capacitation status, horse

Author's address: Ants Kavak, Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Centre for Reproductive Biology in Uppsala, SLU, P.O. Box 7039, SE-750 07 Uppsala, Sweden

Contents

Appendix

The thesis is based on the following papers, which are referred to in the text by their Roman numerals I and II:

I. Kavak, A., Lundeheim, N., Aidnik, M., Einarsson, S. 2003. Testicular measurements and daily sperm output of Tori and Estonian breed stallions. *Reproduction in Domestic Animals 38*: 167–169

II. Kavak, A., Johannisson, A., Lundeheim, N., Rodriguez-Martinez, H., Aidnik, M., Einarsson, S. 2003. Evaluation of cryopreserved stallion semen from Tori and Estonian breeds using CASA and flow cytometry. *Animal Reproduction Science 76*, 205–216

Introduction

The use of artificial insemination (AI) in equine breeding has increased during recent decades, becoming important worldwide. AI offers many advantages over natural mating, such as safety for both the mare and the stallion, reduced risk of infectious disease transmission, and transport inconvenience. Adequate short- and long-term preservation of stallion semen is a prerequisite for a successful AI procedure. Semen of good fertilising capacity is necessary for obtaining high fertility results. The large number of mares to be bred by one stallion, the shipment of fresh chilled semen and the freezing of semen all require large numbers of spermatozoa of good quality from the stallion.

The total horse population in Estonia is approximately 6,000. There are two main local horse breeds: Tori and Estonian horse. The Tori horse is a full-size horse with a mean height of 166 cm and the Estonian horse is between a miniature and a full-size horse with a mean height of 144 cm. Since the numbers of those two breeds are very small, especially the number of pure-bred stallions, long-term preservation of semen is of great importance for preservation and improvement of existing breeds. In 2002 a total of 86 Tori breed foals and 105 Estonian breed foals were born in Estonia.

The capacity of a stallion to produce a large quantity of high quality spermatozoa is a requisite for being used for AI. Evaluation of testicular size and daily sperm output (DSO) gives valuable information on the reproductive capacity of a stallion. Different regimens have been used for evaluation of DSO, such as daily collection of one ejaculate for 10 weeks (Gebauer *et al*., 1974; Sullivan & Pickett, 1975), collection of five ejaculates at 1h apart, two ejaculates at 1h apart every 4 days, and one ejaculate per day for at least 3 weeks (Amann *et al*., 1979). Gebauer *et al*. (1974) suggested, based on their own results, that the ejaculate collected on day 7 should be representative, whereas Love *et al*. (1991) recommended the mean value of the ejaculates collected on days 5-7 as the most representative for estimation of DSO. Testicular size and volume are direct measures of the amount of testicular parenchyma present, which in turn determines potential sperm production (Thompson *et al*., 1979). Recently, Stich *et al*. (2002) reported that differences in testicular measures must be considered when determining at which collection day DSO can be calculated. Thus, in stallions with smaller testicles DSO can be calculated as early as day 5 of collection, whereas in stallions with larger testicles calculation of DSO can take place on day 6 or 7 of collection. Testicular size is influenced by breed (stallion size) and age (Thompson *et al*., 1979; Pickett *et al*., 1987). Models for evaluation of breeding soundness for full-size stallions have been worked out by Kenney *et al*. (1983) and for miniature stallions by Paccamonti *et al*. (1999). Total scrotal width is considered to be the most commonly used predictor of DSO in stallions (Thompson *et al*., 1979). However, no investigation concerning any correlation between scrotum circumference and DSO in stallions has been published. For Estonian and Tori breed stallions, no information at all is available concerning testicular measurements and DSO.

There has been a great development since 1789, when Spallanzani reported that frog spermatozoa were able to fertilise eggs after freezing and thawing (Luyet & Gehenio, 1940). Despite this finding, it was not possible to start using cryopreserved semen in breeding programs until Polge, Smith & Parkes in 1949 accidentally discovered glycerol as a successful cryoprotectant for spermatozoa. Barker & Gandier reported the first pregnancy from frozen stallion semen in 1957. Freezing in propylene straws of different sizes and volumes, which are mainly used today, was first presented by Martin, Klug & Guntzel, (1979), Tischner (1979), and Palmer (1984). Semen for preservation is collected mainly by two types of artificial vaginas: open- and closed-end. With a closed-end artificial vagina the whole ejaculate, including the first fraction (pre-sperm-rich) and the post-sperm-rich fraction (gel-fraction) is collected, whereas with the open-end artificial vagina only the sperm-rich jets of the ejaculate are collected, excluding the rest of the ejaculate. Different extenders and protocols have been used for cryopreservation of stallion semen with acceptable results (*e.g.* Martin, Klug & Guntzel, 1979; Tischner, 1979; Palmer, 1984; Amann & Pickett, 1987). No evident differences in fertility results have been presented between using programmable freezing compared with freezing in the vapours above a liquid nitrogen surface. The most important step seems to be the thawing rate. Although the connection between freezing rate and the packaging method is well known, the choice of thawing method has to be carefully considered (Klug *et al*., 1992).

Independent of which freezing method is used, there are differences in freezability of semen among stallions. It has been estimated that 25% to 30% of the stallions produce semen that cryopreserves well, 25% to 50% of stallions produce semen that cryopreserves moderately well and 25% to 40% of stallions produce semen that cryopreserves poorly (Pickett & Amann, 1993). Only sparse information is available on the freezability of semen in the Tori and Estonian horse breeds.

Processing of semen such as freezing and thawing is detrimental to sperm functionality and usually results in the death of large numbers of spermatozoa. Selection of fresh semen of high quality for freezing is therefore necessary. For fertilisation of an oocyte with frozen-thawed semen there is obviously a need for a certain population of viable, motile, non-capacitated spermatozoa with intact acrosome. Several studies have aimed at finding correlations between different parameters of frozen-thawed semen and fertility in several species including stallions. Unfortunately, so far no single in vitro parameter has been identified that can predict fertility. Sperm motility is the most commonly used criterion for evaluating quality of cryopreserved semen. It has been found that computer-assisted sperm analysis (CASA) is adequate for clinical use in determining individual motility patterns (Amann, 1987; Jasko *et al*., 1988; Blach *et al*., 1989) but not for fertility. Even motility evaluation is inadequate for prediction of fertilising capacity of frozen-thawed stallion semen (Jasko *et al*., 1992; Heitland *et al*., 1995). With CASA an objective evaluation of sperm hyperactivation is possible (Jasko, Lein & Foote, 1991; Irvine, 1995), and this is highly correlated with sperm capacitation (Suarez, 1996). One of the aims of the present study was to investigate the motility and its pattern using CASA of frozen-thawed semen from Estonian and Tori breed stallions.

Different probes have been used to measure sperm viability and acrosome reaction in different species (Graham, Kunze & Hammerstedt, 1990; Garner & Johnson, 1995; Papaioannou *et al*., 1997). In stallions, assessment of sperm viability and acrosome reaction have been performed by using Propidium Iodide (PI) (Papaioannou *et al*., 1997), PI and SYBR-14 (Merkies *et al*., 2000; Boe-Hansen *et al*., 2001; Kirk *et al*., 2001), PI and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) (Cheng *et al*., 1996; Rathi *et al*., 2001). No study seems to have been made on stallion semen using triple fluorophore-staining (Carboxy SNARF-1, PI and FITC-PSA), which was reported to be successful for frozen-thawed dog semen (Peña, Johannisson & Linde-Forsberg, 1999).

There are several specific probes for evaluation of capacitation, such as merocyanine 540 (Harrison, Ashworth, & Miller, 1996), chlortetracycline (CTC) (Varner *et al*., 1987), combined CTC and Hoechst 33258 (Neild *et al*., 2003) and fluorescein-PSA and ethidium homodimer-1 staining (Meyers, 2001). Recently, a method for evaluation of the early capacitation changes in the membrane of spermatozoa using Merocyanine 540/Yo-Pro-1 staining was established for the stallion (Rathi *et al*., 2001). Using a method of that kind there is a good possibility to evaluate the capacitational status of frozen-thawed semen from Tori and Estonian breed stallions.

Development of flow cytometry has offered great advantages for evaluation of different frozen-thawed semen parameters stained with different fluorophores compared with the previously used fluorescent microscopy when only 100-200 spermatozoa per sample were assessed (Harrison & Vickers, 1990; Valcarel *et al*., 1994; Brinsko *et al*., 2000). Flow cytometry offers the added advantage of allowing simultaneous evaluation of sperm viability by inclusion of a membrane-impermeable DNA dye such as propidium iodide (Rathi *et al*., 2001). The combination of stains allows differentiation between non-activated, capacitated/acrosome intact and capacitated/acrosome reacted, viable spermatozoa. In addition, flow cytometry allows the very rapid and objective discrimination of large numbers of spermatozoa (*e.g*. 10,000 in a few seconds). The possibility to use flow cytometric equipment for evaluating membrane and acrosome integrity of stallion semen with triple staining (Carboxy SNARF-1, PI and FITC-PSA) should be investigated.

In the flow cytometric evaluation of dog semen it has been found necessary to eliminate egg yolk particles and cell debris from frozen-thawed semen by washing (Peña, Johannisson & Linde-Forsberg, 1999). For elimination of these particles from frozen-thawed stallion semen, Percoll density gradient centrifugation has been used (Landim-Alvarenga *et al*., 2001; Meyers, 2001). Leão *et al*. (2001) found that Percoll density gradient centrifugation is effective in selecting spermatozoa with good motility, thereby increasing the longevity of cryopreserved semen.

Aims of the study

The aims of the present study were to:

- investigate the DSO and testicular measurements of Tori and Estonian breed stallions
- determine if the functional parameters motility, membrane, and acrosome integrity of cryopreserved stallion spermatozoa could be evaluated using CASA and a triple combination of fluorophore staining and flow cytometry
- evaluate quality (motility, membrane, acrosome integrity and capacitation) of frozen semen from Tori and Estonian breed stallions.

Materials and methods

Animals

A total of 16 clinically healthy stallions [8 Tori breed (T) and 8 Estonian breed (E)] aged 4-22 years with proven fertility were investigated in study I and 13 (6 T and 7 E stallions) of those stallions aged 4-14 years were used in study II. The stallions were sexually rested and stabled in a normal daytime light regimen at least for one month before being used. The stallions were transported to the Veterinary Clinic of the Estonian Agricultural University.

Semen collection and processing

Semen was collected using the Missouri type of artificial vagina while the stallion was mounting a teaser mare in oestrus. The ejaculate was filtered through gauze to remove the gel fraction. The gel-free volume of semen was measured with a graduated cylinder and motility was immediately subjectively evaluated in a light microscope at a magnification of x400, equipped with a heating stage (38°C). Sperm concentration was assessed with a haemocytometer (Bürker´s or Chorjajev´s chamber).

In paper I, one ejaculate of semen was collected daily for 10 subsequent days from each stallion. For total number of spermatozoa (TSN) the volume of gel-free fraction was multiplied by the sperm concentration. The TSN recovered in each ejaculate was calculated without correction for spermatozoa lost into the artificial vagina or the gel fraction of ejaculate. One day after the last semen collection the width, height and length of each testis were measured using callipers and the circumference with a tape measure.

In paper II, two proven ejaculates from each stallion, collected on different occasions during September-January, were processed and cryopreserved in medium straws (0.5 ml) using the Hannover method (Busch, Löhle & Peter, 1991), and thereafter stored in liquid nitrogen at -196°C until analysis. The final sperm number per straw was approximately 250 x 10^6.

Semen evaluation

Post-thaw sperm preparations

One straw was thawed in a 37°C water bath for about 30 sec. Then 5 μL of thawed semen was taken for motility assessment. The rest of the thawed semen was layered onto 2 mL 70/40% Percoll discontinuous density gradient and centrifuged at 650xg for 10 min. The final pellet was resuspended with Tyrode medium (300μL/700μL) for further analyses.

Post–thaw motility

Post-thaw sperm motility was estimated by using the CASA system (Strömberg-Mika Cell Motion Analyser, Bad Feilnbach, Germany). The motility parameters obtained were: percent of total motile spermatozoa, linear motility, circular motility, and hyperactivated as well as kinematic characteristics such as straight line velocity (VSL), and curvilinear velocity (VCL).

Flow cytometry

In paper II, sperm plasma membrane integrity and acrosomal status was evaluated using a combination of fluorophores: SNARF, PI and FITC-PSA. Capacitation status in control (bicarbonate-free medium) and capacitating conditions (bicarbonate-containing medium) was assessed using dual fluorescent staining with Merocyanine 540/Yo-Pro-1.

Fluorescent stainings

Triple FITC-PSA/SNARF/PI staining

A 10mM solution of SNARF (Molecular Probes, Inc., Eugene, OR, USA) in DMSO was prepared just before use. The DMSO solution was diluted in PBS to a final concentration of 100 μM of SNARF. A 2.4 mM stock solution of propidium iodide (PI) (Sigma, St. Louis, MO, USA) in water and a 20 μM FITC labelled PSA (Sigma, St. Louis, MO, USA) in PBS were prepared. To 300 μL of diluted semen SNARF was added to give a final concentration of 25 μM, PI to 50 μM and FITC-PSA to give a final concentration of 0.1 μM of lectin, which meant a 0.39 μM concentration of FITC. The semen sample was incubated in the dark for 15 min at 34-37°C and analysed by flow cytometry.

Dual Merocyanine 540/Yo-Pro-1 staining

Stock solutions of a 1 mM Merocyanine 540 (Molecular Probes, Inc., Eugene, OR, USA) in DMSO, a 25 μM Yo-Pro-1 (Molecular Probes, Inc., Eugene, OR, USA) in DMSO, polyvinylalcohol 10 mg/mL and polyvinylpyrolidone 10 mg/mL were prepared. To 400 μL of diluted semen, Merocyanine 540 was added to give a final concentration of 2.7 μM, Yo-Pro-1 to 25 nM, polyvinylalcohol to 0.5 mg/mL and polyvinylpyrrolidone to give a final concentration 0.5 mg/mL and analysed by flow cytometry.

Flow cytometry analyses

Triple FITC-PSA/SNARF/PI staining

Flow cytometric analyses were performed on a Facstar Plus flow cytometer (Becton Dickinson, San Jose, CA, USA). The fluorophores were excited by a 200 mW argon ion laser operating at 488nm. Data from 100,000 events per sample were collected in list mode for Triple FITC-PSA/SNARF/PI staining. FITC fluorescence was collected in the FL1 sensor using a 530/30 nm band-pass filter,

SNARF in the FL2 sensor using a 575/30 bandpass filter and PI fluorescence was collected in the FL3 sensor through a 660/20 nm long pass filter. About 60,000 to 80,000 spermatozoa were analysed per run.

Dual Merocyanine 540/Yo-Pro-1staining

For Dual Merocyanine 540/Yo-Pro-1 staining 30,000 events per sample were collected in list mode. Yo-Pro-1 fluorescence was collected with a FL1 sensor using a 530/30 nm band-pass filter and the Merocyanine 540 fluorescence was collected with the FL2 sensor through a 575/30 bandpass filter.

Flow cytometric data were analysed using a Facstation computer and the program Cell Quest version 3.3 (Becton Dickinson, San Jose, CA, USA) using a gate in forward and side scatter to exclude any remaining debris and aggregates from the analysis.

Statistical analyses

Statistical analyses were performed using the SAS version 6 software (SAS Institute Inc., 1989). Student's *t*-test was used for testing testicular measurements between breeds and differences between left and right testicular measurements within breed. Monofactorial analysis of variance, using the GLM-procedure, was applied to the "concentration variables" (one observation per stallion) to test the difference between breeds. For all other variables, analysis of variance was performed using the MIXED-procedure. Spearman rank correlations between different variables were calculated within breed. To estimate the repeatability of variables, Spearman rank correlations were calculated between the two samples for each stallion.

Results

Testicular measurements and Daily Sperm Output (I)

Testicular measurements

The testicular measurements, including testicular circumference, were significantly larger in T stallions than in E stallions ($P < 0.001$). For E stallions, all measurements of the left testis were significantly larger than that of the right testis ($P < 0.05$), whereas no significant differences between left and right testis were found in T stallions.

Daily sperm output

In E stallions, the total sperm number (TSN) decreased significantly from day 1 to day 4 and in T stallions TSN decreased successively from day 1 to day 8, thereafter remaining constant. In T stallions, significant differences of varying degree were found between days from day 1 to day 4 and between every second day from day 4 to day 8. The DSO for T stallions was $12.9 \pm 0.8 \times 10^9$ and for E stallions $4.5 \pm 0.3 \times 10^9$ ($P < 0.001$). The correlation between testicular circumference and DSO for E stallions was $r = 0.72$ ($P < 0.05$) and for T stallions $r = 0.67$ ($P = 0.071$).

Effect of breed on post-thaw sperm characteristics of frozen-thawed semen (II)

Motility

The subjective evaluation of motility did not reveal any significant differences between breeds. The computerised motility evaluations (CASA) of frozen-thawed semen showed that there were no significant differences after post-thawing of semen for any evaluated parameter ($P > 0.05$). The motility of fresh semen decreased after freezing-thawing from $67.8 \pm 6.4\%$ to $43.4 \pm 4.7\%$ in T stallions, and from $67.5 \pm 7.5\%$ to $42.3 \pm 1.1\%$ in E stallions.

Plasma membrane integrity and acrosome status

Using triple staining, two sperm populations (SNARF-positive cells – live; PI-positive sperm cells – dead) were easily identified. The percentages of total viable, live spermatozoa post-thaw were $22.5 \pm 4.4\%$ in T stallions and $25.3 \pm 4.8\%$ in E stallions. Statistical analysis did not reveal any differences between breeds in percentages of viable spermatozoa ($P > 0.05$). In further investigations of live spermatozoa, $79.3 \pm 6.9\%$ were identified with intact acrosome in T stallions and

84.5 ± 7.5% in E stallions. The results did not reveal any significant differences between breeds ($P > 0.05$).

Capacitation status

The evaluation of capacitation status of frozen-thawed semen (Merocyanine 540/Yo-Pro-1 staining) in control (bicarbonate-free medium) and capacitating conditions showed no differences between breeds ($P > 0.05$). In control medium, the percentages of non-capacitated spermatozoa were 36.8 ± 9.1 and 41.9 ± 9.9 for T and E stallions, respectively, and after incubation in capacitating medium for 30 min, 14.4 ± 2.2 and 14.4 ± 2.3 for T and E stallions, respectively. After incubation in capacitating medium, the percentages of viable spermatozoa in control medium were 24.4 ± 3.9 and 27.6 ± 4.2 for T and E stallions, respectively ($P > 0.05$), and in capacitating medium after incubation for 30 min 15.0 ± 2.1 and 16.0 ± 2.2 for T and E stallions, respectively ($P > 0.05$).

Comparison between methods

Comparisons of percentages of viable spermatozoa between different methods were made. There were significant positive correlations between Merocyanine 540/Yo-Pro-1 staining in control media and after incubation in capacitating media at 30 min for both breeds (T stallion $r = 0.60$, $P < 0.05$; E stallion $r = 0.78$, $P < 0.01$).

General discussion

Data on the number of mated mares and number of foaling mares (born foals) of the stallions used in the present studies were collected. All these stallions had a foaling rate of more than 65% (mean 70.1%). Therefore it can be concluded that all stallions used in this experiment were of proven fertility.

In the evaluation of reproductive capacity of stallions it is of great importance to collect information both about testicular size and daily sperm output (DSO). Previous studies (Thompson *et al*., 1979; Pickett *et al*., 1987; Love *et al*., 1991) have shown that testicular volume, estimated by testicular measurements, correlates with sperm production. The present study (Paper I) demonstrated a significant correlation between testicular circumference and DSO in both Estonian and Tori breed stallions. This result supports earlier observations where smaller testicles yielded lower sperm output than bigger testicles. There are contradictory data on seasonal variation of total sperm number in ejaculates from stallions. Thus Magistrini, Chanteloube & Palmer (1987) found a seasonal influence on sperm concentration and volume of the ejaculate, but not on the total number of spermatozoa. Janett *et al*. (2003a, 2003b), on the other hand, found seasonal variations in both sperm concentration, ejaculate volume and total sperm number. Semen collections in the present studies were performed during September-January, i.e, outside the breeding season. Therefore, there might have been a slight seasonal influence on the total number of spermatozoa in the ejaculates collected and used in the present studies. However, only stallions with acceptable sperm quality (sperm morphology and sperm motility according to Kavak *et al*., 2004) even outside the breeding season were used. The decrease in total sperm number outside the breeding season is thought to be caused by changes in photoperiodicity. Several investigators (*e.g*. Hoffman & Landeck, 1999; Gerlach & Aurich, 2000) have shown that increasing daylength stimulates the testicular endocrine activity and secretion of accessory glands in the stallion.

The finding in the present investigation (Paper I) that DSO was reached sooner in stallions with smaller testicles (Estonian breed) than in stallions with bigger testicles (Tori breed) is in accordance with earlier observations by Gebauer *et al*. (1974), Sullivan & Pickett (1975) and Stich *et al*. (2002). The reason for this is most likely due to the size of the extragonadal sperm reserves, because younger stallions with smaller testicles have smaller extragonadal sperm reserves than older stallions with larger testicles (Amann *et al*., 1979).

Testicular size has earlier been investigated in both miniature stallions (Paccamonti *et al*., 1999) and full size stallions (Kenney *et al*., 1983). The present study (Paper I) clearly showed that there is a direct connection between stallion size and testicular size. Like several other investigators (Gebauer *et al*., 1974; Thompson *et al*., 1979; Paccamonti *et al*., 1999) we found differences in left-right testicular measures between breeds. No scientific explanation for the variation in size between left and right testicle is known.

Visual assessment of the percentage of motile spermatozoa is the most widely used evaluation method of both fresh and cryopreserved semen. Nevertheless,

variation between observers may occur due to the subjective nature of the work (Davis & Boyers, 1992). Consequently, finding a quick and reproducible method for semen assessment that might supplement or even replace the subjective visual motility evaluation is of great interest. The evaluation of motility of frozen thawed semen in the present study (Paper II) was therefore performed by CASA, which is a more objective method. The cryopreservation was performed during the non-breeding season because the stallions were used for natural mating during the breeding season. In a recent study, Janett *et al*. (2003a) found that the post-thaw motility of frozen stallion semen was better during the winter season than during the autumn or the spring. In the present study (Paper II), semen for freezing was collected during late autumn and winter, and the post-thaw sperm motility was at least as good as in previous studies (Jasko *et al*., 1992; Blottner *et al*., 2001; Janett *et al*., 2003a).

According to many reports (Amann & Pickett, 1987; Samper, Hellander & Crabo, 1991; Harkema & Boyle, 1992; Wöckener *et al*., 1992; Braun *et al*., 1994; Dobrinski, Thomas & Ball, 1995; Vidament *et al*., 1997) the process of cryopreservation has a negative influence on the morphological and functional integrity of stallion spermatozoa. Blottner *et al*. (2001) also found an increased proportion of spermatozoa with detached acrosomes after freezing/thawing, reducing the fertilising competence of the spermatozoa. For evaluation of the viability and acrosome status of frozen-thawed spermatozoa, triple staining with fluorophores in combination with flow cytometry, allowing simultaneous evaluation of multiple characteristics of the sample of stallion spermatozoa was used for the first time. In previous similar evaluations of dog semen, it was found that frozen-thawed samples have to be freed from egg yolk, debris cells and other particles before successful flow cytometric evaluations can be made (Peña, Johannisson & Linde-Forsberg, 1999). Different mechanical clean-up methods (filtration with glass wool, glass beads or polysaccharide beads, Percoll separations, centrifugation with different mediums) are available. In the present study, washing with TALP medium or Percoll separation were tested. Washing with TALP medium had a killing effect on stallion spermatozoa and therefore it was decided to use solely the Percoll density gradient centrifugation. The reason of the detrimental effect of washing with TALP is unknown. Percoll separation has been used previously and been found to be effective in selecting spermatozoa of different quality (Landim-Alvarenga *et al*., 2001; Leão *et al*., 2001; Meyers, 2001).

The purpose of this study was also to evaluate viability, acrosome and membrane integrity with fluorophore staining using flow cytometry. Based on successful evaluations of frozen-thawed dog spermatozoa in our laboratory (Peña, Johannisson, Linde-Forsberg, 1999) we studied viability and acrosome status of frozen-thawed stallion spermatozoa with triple (FITC-PSA/SNARF/ PI) staining, which has not been used before for evaluation of stallion semen. The viability of the frozen-thawed semen in the present study (Paper II) is lower than corresponding results of Janett *et al*. (2003a,b), but comparable with the results of Merkies *et al*. (2000). On account of triple staining being used for the first time to evaluate frozen-thawed stallion semen, there are no data available for comparison with our results. The freezing in the present study was performed outside the breeding season. However, no obvious seasonal differences in the freezability of

stallion spermatozoa have been found in earlier studies, whether measured with SYBR-14/PI (Janett *et al*., 2003a) or using eosin-nigrosin staining (Magistrini, Chanteloube & Palmer, 1987).

The evaluation of capacitation status of frozen-thawed spermatozoa in the present study was done with Merocyanine 540/Yo-Pro-1 staining. The observations were mainly directed at changes in the percentages of non-capacitated sperm populations. Decreases in the percentage of alive, non-capacitated spermatozoa were found after incubation in capacitating medium (Paper II). These data confirmed observations from earlier studies (Rathi *et al*., 2001), *i.e*., that frozen-thawed non-capacitated spermatozoa are viable and functional and suitable for undergoing fertilisation steps.

Blottner *et al*. (2001) showed that quality parameters of fresh spermatozoa as well as the degree of declined cellular integrity by preservation showed considerable differences between stallions. Merkies *et al*. (2000) also found differences in viability of frozen-thawed spermatozoa among stallions and ejaculates for various parameters measured. These results are in accordance with many reports on the generally limited freezability of stallion spermatozoa and that the stallion is the major factor influencing freezability. In our study, we could find neither differences between ejaculates nor between breeds. The number of stallions used of each breed was rather small, and also the high criteria set to select the semen for freezing may cancel out these differences among ejaculates. It seems that ejaculates with high sperm motility and high percentage of spermatozoa with normal morphology freeze better. Therefore, fresh semen of high quality represents an important criterion for selection of stallions intended to be used for freezing of semen and for artificial insemination. Janett *et al.* (2003a) concluded that variation exists not only between but also within stallions and, consequently, the best time for freezing semen must therefore be found for each individual stallion. Results of Neild *et al.* (2003) show that freezing-thawing of stallion semen induces cell damage and a relative increase in live capacitated/acrosome reacted sperm cells.

General conclusions

Based on the studies presented the following conclusions can be drawn:

- Testicular measurements and DSO are related to the body size of the stallion's breed, and testicular circumference is correlated with the DSO

- CASA and Triple combination of fluorophores using flow cytometry can be used for evaluation of motility, viability and acrosomal status of frozen-thawed stallion spermatozoa

- Semen from Tori and Estonian breed stallions, and frozen according to the Hannover method had acceptable post-thaw motility, acrosome status and capacitation status as evaluated with CASA and flow cytometry.

References

Amann, R.P. 1987. Computerized evaluation of stallion spermatozoa. *Proceedings of the 33rd annual convention of the American Association of Equine Practitioners 33*, 453 – 473.

Amann, R.P., Pickett, B.W. 1987. Principles of cryopreservation and review of cryopreservation of stallion spermatozoa *Journal of equine veterinary science 7*, 145-173

Amann, R.P., Thompson, D.L., Squires, E.L., Pickett, B.W. 1979. Effects of age and frequency of ejaculation on sperm production and extragonadal reserves in stallions. *Journal of reproduction & fertility Supplement 27*, 1-6.

Barker, C.A.V., Gandier, J.C.C. 1957. Pregnancy in a mare resulting from frozen epididymal spermatozoa. *Canadian journal of comparative medicine and veterinary science 21*, 4-51.

Blach, E.L., Amann, R.P., Bowen, R.A., Frantz, D. 1989. Changes in quality of stallion spermatozoa during cryopreservation: plasma membrane integrity and motion characteristics. *Theriogenology 31*, 283-298.

Blottner, S., Warnke, C., Tuchscherer, A., Heinen, V., Torner, H. 2001. Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. *Animal reproduction science 65*, 75-88.

Boe-Hansen, G., Christensen, P., Stryhn, H., Nielsen, J.M., Lehn-Jehsen, H. 2001. Using flow cytometry for assessment of the quality of frozen-thawed stallion spermatozoa. *Animal reproduction science 68*, 328-329.

Busch, W., Löhle, K., Peter, W. 1991. *Kunstliche Besamung bei Nutztieren*. Gustav Fischer Verlag, 738 p.

Braun, J., Sakai, M., Hochi, S., Oguri, N. 1994. Preservation of ejaculated and epididymal stallion spermatozoa by cooling and freezing. *Theriogenology 41*, 809-818.

Brinsko, S.P., Van Wagner, G.S., Graham, J.K., Squires, E.L., 2000. Motility, morphology and triple stain analysis of fresh, cooled and frozen-thawed stallion spermatozoa. *Journal of reproduction & fertility Supplement 56*, 111-120.

Cheng, F.P., Fazeli, A.R., Voorhout, W.F., Bevers, M.M., Colenbrander, B. 1996. Use of PNA (peanut agglutinin) to assess the acrosomal status and the zona pellucida induced acrosome reaction in stallion spermatozoa. *Journal of andrology 17*, 674-682.

Davis, R.O., Boyers, S.P. 1992. The role of digital image analysis in reproductive biology and medicine. *Archives of pathology & laboratory medicine 116*, 351-363.

Dobrinski, I., Thomas, P.G.A., Ball, B.A. 1995. Cryopreservation reduces the ability of equine spermatozoa to attach to oviductal epithelial cells and zonae pellucidae in vitro. *Journal of andrology 16*, 536-542.

Garner, D.L., Johnson, L.A. 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biology of reproduction 53*, 276-284.

Gebauer, M.R., Pickett, B.W., Voss, J.L., Swiestra, E.E. 1974. Reproductive physiology of the stallion. Daily sperm output and testicular measurements. *Journal of American veterinary medical association 165*, 711-713.

Gerlach, T., Aurich, J.E. 2000. Regulation of seasonal reproductive activity in the stallion, ram and hamster. *Animal reproduction science 58*, 197-213.

Graham, J.K. 1996. Cryopreservation of stallion spermatozoa. *The veterinary clinics of North America: Equine Practice* 131 – 147.

Graham, J.K., Kunze, E., Hammerstedt, R.H. 1990. Analysis of sperm cell viability, acrosomal integrity and mitochondrial function using flow cytometry. *Biology of reproduction 43*, 55-64.

Harkema, W., Boyle, M.S. 1992. Use of fluorescent stains to assess membrane integrity of equine spermatozoa. *Proceedings of the 12th International Congress on Animal Reproduction*, The Hague 3 1424-1426.

Harrison, R.A., Ashworth, P.J., Miller, N.G. 1996. Bicarbonate/CO_2 an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Molecular reproduction and development 45*, 378-391.

Harrison, R.A.P., Vickers, S.E. 1990. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *Journal of reproduction & fertility 88*, 343-352.

Heitland, A.V., Jasko, D.J., Graham, J.K., Squires, E.L., Amann, R.P., Pickett, B.W. 1995. Motility and fertility of stallion spermatozoa cooled and frozen in a modified skim milk extender containing egg yolk and liposome. *Biology of reproduction Monograph 1*, 753-759.

Hoffmann, B., Landeck, A. 1999. Testicular endocrine function, seasonality and semen quality of the stallion. *Animal reproduction science 57*, 89-98.

Irvine, S. 1995. Computer-assisted semen analysis systems: sperm motility assessment. *Humane reproduction 10(1),* 53-59.

Janett, F., Thun, R., Bettschen, S., Burger, D., Hassig, M. 2003a. Seasonal changes of semen quality and freezability in Franches-Montagnes stallions. *Animal reproduction science 77*, 213-221.

Janett, F., Thun, R., Niederer, K., Burger, D., Hässig, M. 2003b. Seasonal changes in semen quality and freezability in the Warmblood stallion. *Theriogenology 60*, 453-461.

Jasko, D.J., Lein, D.H., Foote, R.H. 1991. The repeatability and effect of season on seminal characteristics and computer-aided sperm analysis in stallion. *Theriogenology 35*, 317-327.

Jasko, D.J., Little, T.V., Smith, K., Lein, D.H., Foote, R.H. 1988. Objective analysis of stallion sperm motility. *Theriogenology 30*, 1159-1167.

Jasko, D.J., Little, T.V., Lein, D.H., Foote, R.H. 1992. Comparison of spermatozoal movement and semen characteristics with fertility in stallions: 64 cases (1987-1988). *Journal of American veterinary medical association 200*, 979-985.

Kavak, A., Lundeheim, N., Aidnik, M., Einarsson, S. 2004. Sperm morphology of Estonian and Tori breed stallions. *Acta veterinaria scandinavica* (accepted for publication)

Kenney, R.M., Hurtgen, J., Pierson, R., Whiterspoon, D., Simons, J., 1983. Theriogenology and the Equine. Part II. The Stallion, vol. 9. Society for Theriogenology, Hastings, NB, 1-100.

Kirk, E.S., Graham, J.K., Bruemmer, J.E., Squires, E.L. 2001. Evaluating frozen semen by flow cytometry. *Animal reproduction science 68*, 348-349.

Klug, E., Röbbelen, I., Kneissl, S., Sieme, H. 1992. Cryopreservation of stallion spermatozoa. *Acta veterinaria scandinavica Supplement 88*, 129-135.

Landim-Alvarenga, F.C., Alvarenga, M.A., Seidel, G.E., Squires, E.L., Graham, J.K. 2001. Penetration of zona-free hamster, bovine and equine oocytes by stallion and bull spermatozoa pretreated with equine follicular fluid, dilauroylphosphatidylcholine or calcium ionophore A23187. *Theriogenology 56*, 937-953.

Leão, K.M., Alvarenga, M.A., Landim-Alvarenga, F.C., Papa, F.O. 2001. Improvement of motility, longevity and viability of frozen stallion sperm selected by discontinuous Percoll density gradient. *Animal reproduction science 68*, 342-343.

Love, C.C., Garcia, M.C., Riera, F.R., Kenney, R.M. 1991. Evaluation of measures taken by ultrasonography and calipers to estimate testicular volume and predict daily sperm output in the stallion. *Journal of reproduction & fertility Supplement 9*, 99-105.

Luyet, B.J., Gehenio, P.M. 1940. The lower limit of vital temperatures in life and death at low temperatures. *Normandy, MO, Biodynamica*.

Magistrini, M., Chanteloube, P., Palmer, E. 1987. Influence of season and frequency of ejaculation on production of stallion semen for freezing. *Journal of reproduction & fertility Supplement 35*, 127-133.

Martin, J.C., Klug, E., Guntzel, A.R. 1979. Centrifugation of stallion semen and its storage in large volume straws. *Journal of reproduction & fertility Supplement 27*, 47-51.

Merkies, K., Chenier, T., Plante, C., Buhr, M.M. 2000. Assessment of stallion spermatozoa viability by flow cytometry and light microscope analysis. *Theriogenology 54*, 1215-1224.

Meyers, S.A. 2001. Equine sperm-oocyte interaction: the role of sperm surface hyaluronidase. *Animal reproduction science 68*, 291 – 303.

Neild, D.M., Gadella, B.M., Chaves, M.G., Miragaya, M.H., Colenbrander, B., Agüero, A. 2003. Membrane changes during the different stages of a freeze-thaw protocol for equine semen cryopreservation. *Theriogenology 59*, 1693-1705.

Paccamonti, D.L., Buiten, A.V., Parlevliet, J.M., Colenbrander, B. 1999. Reproductive parameters of miniature stallions. *Theriogenology 51*, 1343-1349.

Palmer, E. 1984. Factors affecting semen survival and fertility. *Proceedings of the 10th International Congress on Animal Reproduction and Artificial Insemination,* Illinois 3, 377-379.

Papaioannou, K.Z., Murphy, R.P., Monks, R.S., Hynes, N., Ryan, M.P., Boland, M.P., Roche, J.F. 1997. Assessment of viability and mitochondrial function of equine spermatozoa using double staining and flow cytometry. *Theriogenology 48*, 299-312.

Peña, A., Johannisson, A., Linde-Forsberg, C. 1999. Post-thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. *Theriogenology 52*, 965-980.

Pickett, B.W., Amann, R.P. 1993. Cryopreservation of stallion semen. In*: Equine Reproduction*. A.O. McKinnon and J.L. Voss, Lea&Febinger Philadelphia. 769-789.

Pickett, B.W., Voss, J.L., Bowen, R.A., Squires, E.L., McKinnon, A.O. 1987. Seminal characteristics and total scrotal width (T.S.W.) of normal and abnormal stallions. *Proceedings of the thirty-third annual convention of the American Association of Equine Practitioners 33*, 487-518.

Polge, C., Smith, A.U., Parkes, A.S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature 164*, 666.

Rathi, R., Colenbrander, B., Bevers, M.M., Gadella, B.M. 2001. Evaluation of in vitro capacitation of stallion spermatozoa. *Biology of reproduction 65*, 462-470.

Samper, J.C., Hellander, J.C., Crabo, B.G. 1991. Relationship between the fertility of fresh and frozen stallion semen and semen quality. *Journal of reproduction & fertility Supplement 44*, 107-114.

Statistical Analysis Systems Institute Inc., 1989. SAS/STAT *User's Guide, Version 6*, Fourth Edition, Volume 2, Cary, NC SAS Inc.

Stich, K., Brinsko, S., Thompson, J., Love, C., Miller, C., Blanchard, D., Varner, D. 2002. Stabilization of extragonadal sperm reserves in stallion. Application for determination of daily sperm output. *Theriogenology 58*, 397-400.

Suarez, S.S. 1996. Hyperactivated motility in sperm. *Journal of andrology 17*, 331-335.

Sullivan, J.J., Pickett, B.W. 1975. Influence of ejaculation frequency of stallions on characteristics of semen and output of spermatozoa. *Journal of reproduction & fertility Supplement 23*, 29-34.

Thompson, D.L., Pickett, B.W., Squires, E.L., Amann, R.P. 1979. Testicular measurements and reproductive characteristics in stallions. *Journal of reproduction & fertility Supplement 27*, 13-17.

Tischner, M. 1979. Evaluation of deep-frozen semen in stallions. *Journal of reproduction & fertility Supplement 27*, 53-59.

Valcarel, A., de la Heras, M.A., Perez, L.J., Moses, D.F., Baldassarre, H. 1994. Fluorescent staining as a method of assessing membrane damage and post-thaw survival of ram spermatozoa. *Theriogenology 41*, 483-489.

Varner, D.D., Ward, C.R., Storey, B.T., Kenney, R. M. 1987. Induction and characterization of acrosome reaction in equine spermatozoa. *American journal of veterinary research 48*, 287-296.

Vidament, M., Dupere, A.M., Julienne, P., Evain, A., Noue, P., Palmer, E. 1997. Equine frozen semen freezability and fertility field results. *Theriogenology 48*, 907-917.

Wöckener, A., Malmgren, L., Opden kamp, B., Boyle, M., Bader, H., Colenbrander, B., 1992. Freezing of stallion semen – Effects on sperm motility and morphology. *Proceedings of the 12th International Congress on Animal Reproduction*, The Hague 4, 1933-1935.

Acknowledgements

The studies were carried out at the Department of Obstetrics and Gynaecology in co-operation with the Department of Anatomy and Physiology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden and at the Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Estonian Agricultural University, Tartu, Estonia.

Financial support was provided by Swedish Institute, AGRIA and Estonian Scientific Foundation Grant no. 4111.

Travel grants were supported by Swedish Institute and Phare project ES 01.05.02

I wish to express my sincere appreciation to:

Professor **Hans Kindahl,** present Head of the Department of Obstetrics and Gynaecology, for allowing me to continue my work in this wonderful department. It has been great pleasure to work in such a fruitful atmosphere.

Professor **Stig Einarsson**, my scientific supervisor, for taking me under his supervision and introducing me into the world of science. Words will never be enough to express my gratitude. Thank you for continuous support, finding time for discussions and reading my manuscripts, for guidance and criticism. There will not be my papers without you. THANK YOU!!!

Professor **Heriberto Rodriguez-Martinez**, my co-supervisor, for guidance in the world of sperms not only at the beginning of my studies but also throughout it, as well as for his valuable suggestions and comments.

Dr. **Anders Johannisson**, my co-supervisor, for his help and wide knowledge of all fluorescent things, for his kindness and patience, and for being such a nice teacher.

Dr. **Nils Lundeheim**, my co-author, for introducing me to the world of numbers and continuous help in working with my "raw data" and giving them a scientific value. He maintained his attentiveness and patience despite my continuous e-mails.

Assoc. Prof. **Madis Aidnik,** my co-author and scientific advisor at the Estonian Agricultural University, for his continuous help with my research and steadfast encouragement.

Estonian Horsebreeders Society for support using the Tori and Estonian breed stallions in my studies.

Karin Selin Wretling, **Annika Rikberg** and **Åsa Jansson** for excellent laboratory work and for always being there and solving quickly any kind of problems I encountered.

Birgitta Berner for excellent and skilful secretarial help.

Co-ordinators of **New Visby programme** - Dr. **Ulf Magnusson** and Dr. **Toomas Tiirats** for finding financing for my travelling between Estonia and Sweden.

Nigel Rollison for linguistic revision of manuscripts.

Tom Jangby, **Bo Fred** and **Kjell-Ove Eklund** for kind and quick help in solving any kind of technical problem.

Mikael Eklund for excellent library service.

All the Estonians who have been here and sharing hard times for me away from home: Assoc. Prof. **Kalle Kask**, Dr. **Triin Hallap**, Dr. **Mihkel Jalakas,** Prof. **Ülle Jaakma**, and Dr. **Jevgeni Kurõkin**.

All the **Professors, Researchers, Postgraduate students**, past and present at the Department of Obstetrics and Gynaecology, for help in various ways and for making my work as pleasure.

My colleagues from the Veterinary Clinic at the Estonian Agricultural University: **Aivar, Olev, Rainer, Andzela, Gerli, Kadri, Kristi, Laura, Urve, Kalmer** and **Uve** for giving me leave-of-absence to be in Uppsala while keeping my job.

I also owe gratitude to:

My **parents**, for constant encouragement and endless love during my whole life. You have given me the strength of spirit to accomplish all the things that I started to do.

My dear **Merit** and **Hanna Kristin,** words can never express my debt to you. Thank you for your patience, commitment and love throughout the years.