Acta vet. scand. 2004, *45,* 11-18.

Sperm morphology in Estonian and Tori Breed Stallions

By *A. Kavak[1,3], N. Lundeheim[2], M. Aidnik[1], S.Einarsson[3]*

[1]Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Estonian Agricultural University, Tartu, Estonia, [2]Department of Animal Breeding and Genetics, Faculty of Agriculture, Landscape Planning and Horticulture, and [3]Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, CRU, Uppsala, Sweden.

Kavak A, Lundeheim N, Aidnik M, Einarsson S: Sperm morphology in Estonian and Tori Breed stallions. Acta vet. scand. 2004, 45, 11-18. – The standard procedure for assessing the breeding potential of a stallion includes the parameter total number of spermatozoa classified as morphologically normal. This study investigated sperm morphology of fresh semen in randomly chosen Estonian (E, n=8) and Tori (T, n=7) breed stallions with proven fertility. Two ejaculates were examined from each stallion. An aliquot from each ejaculate was fixed in 1 mL formol-saline immediately after collection and examined with phase-contrast microscope at a magnification 1000x for all types of morphological abnormalities. Furthermore smears were prepared and stained according to Williams (carbolfuchsin-eosin) for a more detailed examination of the sperm heads with light microscope at a magnification 1000x. Analysis of variance was applied to the data, and results are presented as LSmeans (±SE). One T stallion that had a disturbance in the spermatogenesis and one 22-year-old E stallion were not included in the analyses. The T stallions had on average 57.5±4.1% and the E-stallions 74.4±3.8% morphologically normal spermatozoa (p=0.012). In 4 of 7 T stallions and 7 of 8 E stallions both ejaculates had >50% morphologically normal spermatozoa. There was a significant difference between breeds in mean percentage of proximal droplets (17.3± 2.7% and 2.9±2.5% for T and E stallions, respectively; p=0.003).

semen; sperm morphology; horse.

Introduction

One criterion used to assess the breeding potential of a stallion is the total number of spermatozoa classified as morphologically normal (*Kenney et al.* 1983). Attempts to correlate the percentage of morphologically normal or abnormal spermatozoa with fertility have given conflicting results. Thus *Bielanski & Kaczmarski* (1979), *Bielanski et al.* (1982), *Hurtgen & Johnson* (1982), *Jasko et al.* (1990), *Hellander et al.* (1991) reported that sperm morphology is related with fertility to various degrees, while others (*Voss et al.* 1981, *Dowsett & Pattie* 1982) did not find any relationship between sperm morphology and fertility. Several investigators found a considerable inter-stallion (*Pattie & Dowsett* 1982, *Rousset et al.* 1987, *Love et al.* 2000) and intra-stallion (*Rousset et al.* 1987, *Jasko et al.* 1991, *Love et al.* 2000) variation in semen quality.

The standard evaluation of sperm morphology is performed with phase and/or light microscopy (*Kenney et al.* 1983). Computer-assisted methods have also been used (*Ball & Mohammed* 1995, *Casey et al.* 1997). However, available computer-assisted methods can only evaluate the sperm head, not count morpholog-

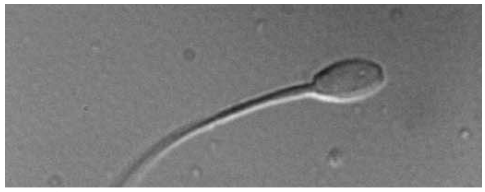
ical abnormalities of mid-pieces, tails and acrosomes.
The aim of the present study was to investigate the sperm morphology in fresh semen of Tori and Estonian breed stallions.

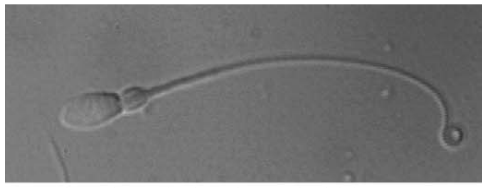
Materials and methods
The investigation was performed on 15 clinically healthy, randomly chosen stallions of 2 different breeds (7 Tori (T) and 8 Estonian (E) breed stallions) with proven fertility aged between 4-15 years (one E stallion 22 years). The stallions were transported to, and housed at, the Veterinary Clinic of Estonian Agricultural University.

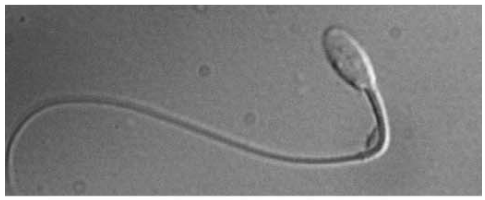
Semen collection and processing
Semen was collected during the non-breeding season (October - January). One ejaculate of semen was collected daily for 10 subsequent days from each stallion. Semen was collected using a Missouri type artificial vagina while the stallion was mounting a teased mare in oestrus. The ejaculate was filtered through gauze to remove the gel fraction. The semen was immediately put into an incubator at +34 °C, and all manipulations were conducted using warmed glassware. Two ejaculates, one among the first 5 ejaculates and one among the last 3 ejaculates, were chosen for evaluation of sperm morphology. An aliquot from the ejaculates was fixed in 1 mL formol-saline immediately after collection. One drop of semen was also placed on each of 3-4 glass slides, and smears were prepared and air-dried.

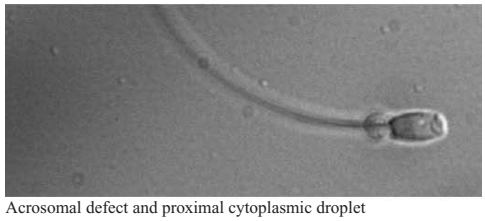
Morphological examination of spermatozoa
Morphological abnormalities of spermatozoa were studied in wet preparations made from the formol-saline fixed samples (*Hancock* 1957) under a phase-contrast microscope at a magnification of 1000x. Altogether 200 spermatozoa were counted in each preparation and all different abnormalities (see below) in each spermatozoon were recorded. The abnormalities were classified according to a system developed by *Bane* (1961).
For a more detailed examination of the sperm heads, smears were prepared as described above, stained with carbolfuchsin-eosin according to the method described by *Williams* (1920) and modified by *Lagerlöf* (1934). Five hundred spermatozoa were counted in each smear at a magnification of 1000x in a light microscope. The head abnormalities were classified according to *Lagerlöf* (1934). If the percentage of head abnormalities recorded in the spermatozoa stained with carbolfluchsin-eosin deviated from the percentage recorded in the formol-salin fixed samples, the former was used in the calculations.
The morphological abnormalities were counted as a percentage of the total number of counted spermatozoa. Morphological categories used in this study were: I. Abnormal heads (including pear shaped, narrow at the base, abnormal contour, undeveloped, loose abnormal head, narrow, big, little-normal, short-broad), II. Loose heads (including both those with normal and abnormal head morphology), III. Acrosome defects, IV. Proximal cytoplasmic droplets, V. Abnormal midpieces, VI. Abnormal tails (including double folded, single bent and coiled tails under the head) (see also Fig. 1a , 1b).
Presence of spermatogenetic cells or debris of spermatogenetic cells was recorded in smears stained according to a modification of a method originally described by *Papanicolaou* (1942) in a light microscope at a magnification of 250x.

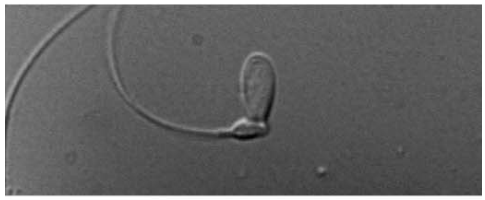
Statistical analyses
Statistical analyses were performed using SAS Version 8 software (SAS Institute Inc., Cary, NC, USA). Analysis of variance was performed using the MIXED-procedure according to a statistical model including the fixed effect of breed


Normal spermatozoa


Proximal cytoplasmic droplet and terminal tail defect


Distal cytoplasmic droplet and narrow head


Acrosomal defect and proximal cytoplasmic droplet


Abnormal midpiece

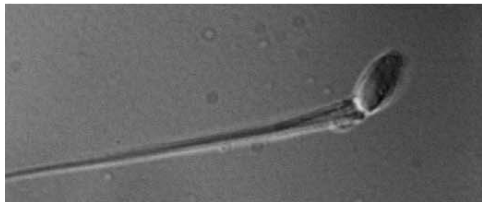
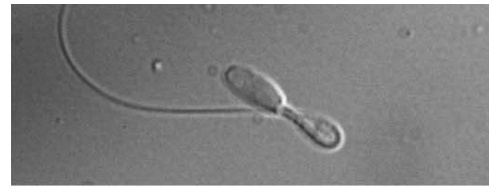
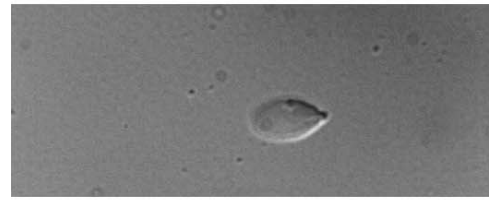
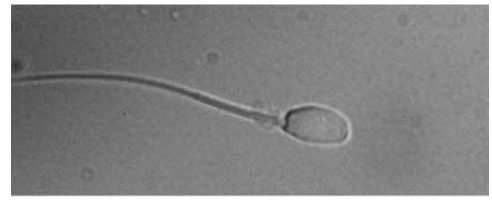

Double midpiece and proximal cytoplasmic droplet

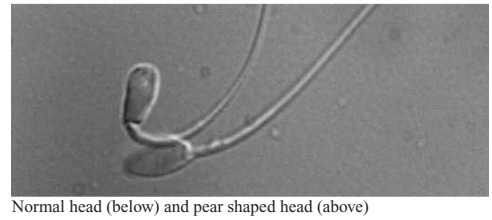
Figure 1a. Some sperm abormalities in stallion.


Single bent tail and narrow head


Coiled tail below the head


Tail stump defect and giant head


Abaxial tail


Normal head (below) and pear shaped head (above)

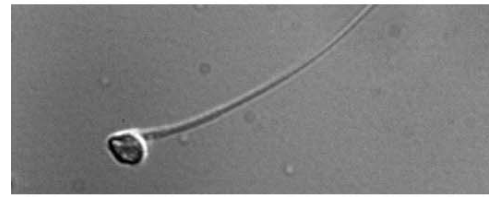

Underdeveloped head

Figure 1b. Some sperm abormalities in stallion.

Table 1. LSmean ± SE and range of percentage of different sperm abnormalities in the ejaculates of 6 Tori and 7 Estonian breed stallions.

Sperm morphology	Tori breed		Estonian breed	
	LSmean ± SE	Range	LSmean ± SE	Range
Proximal droplets %	$17.3^{a} \pm 2.7$	0.5 - 34.0	$2.9^{b} \pm 2.5$	0.0 - 10.0
Loose heads %	$2.3^{a} \pm 0.9$	0.0 - 10.5	$2.3^{a} \pm 0.9$	0.0 - 8.0
Acrosome defects %	$0.6^{a} \pm 0.4$	0.0 - 9.0	$1.6^{a} \pm 0.4$	0.0 - 4.5
Midpiece defects %	$5.1^{a} \pm 1.2$	0.0 - 11.5	$2.4^{a} \pm 1.1$	1.0 - 3.5
Abnormal tails %	$6.6^{a} \pm 1.4$	3.5 - 13.5	$6.4^{a} \pm 1.3$	2.5 - 17.0
Abnormal heads %	$12.6^{a} \pm 1.7$	8.4 - 20.0	$13.9^{a} \pm 1.5$	6.2 - 21.4
Normal %	$57.5^{a} \pm 4.1$	43.7 - 74.1	$74.4^{b} \pm 3.8$	55.1 - 88.8

LSmean values within row with different superscript letters are significantly different ($p < 0.05$)

(2), collection number (2) and the interaction between breed and collection number. The statistical model also included the random effect of stallion nested within breed (6 Tori; 7 Estonian).

Results

One of the T stallions had signs of a moderate disturbance in the spermatogenesis (high percentage of morphologically abnormal spermatozoa and presence of spermatogenetic cells in both ejaculates) and one E stallion was 22 years old. Their results were not included in the analysis of variance, but are presented separately. The LS means ± SE and the ranges of morphological categories in the ejaculates of the remaining 6 T and 7 E stallions are presented in Table 1. There were significant differences in the mean values between breeds in percentages of proximal droplets ($p=0.003$) and of morphologically normal spermatozoa ($p=0.012$). The LSmean ± SE of percentage proximal droplets were 17.3 ± 2.7% in T stallions and 2.9 ± 2.5% in E stallions and the percentage of normal spermatozoa 57.5 ± 4.1% and 74.4 ± 3.8% in T and E stallions, respectively. The percentages of abnormal sperm heads were 12.6 ± 1.7% and 13.9 ± 1.5% in T stallions and in E stallions, respectively. The T stallion with a disturbance in the spermatogenesis had 23.7 ± 10.8% normal spermatozoa, and the 22-year-old E stallion had 49.1 ± 8.2% normal spermatozoa.

There was a significant interaction between breed and collection for percentage of abnormal heads ($p<0.01$). Differences in least squares means show that for E stallions, this abnormality decreased with time (by 2.6% units) whereas for T stallions this abnormality increased with time (by 2.8% units).

For both the percentage of abnormal tails and the percentage of normal spermatozoa there were significant interactions between breed and collection time ($p<0.05$). Differences in least squares means show that for E stallions, percentage of abnormal tails decreased with time by 4.7% units, whereas for T stallions it increased with time by 1% unit. For the percentage of normal spermatozoa, the corresponding differences were: E stallions: -5.3% units; T stallions:-0.7% units.

In 4 out of 7 T stallions and 7 out of 8 E stallions both ejaculates contained >50% morphologically normal spermatozoa. Six T stallions and all E stallions had >50% morphologically normal spermatozoa in at least one of 2 examined ejaculates.

Discussion

The results of the present study gave information about the sperm morphology in ejaculates of randomly chosen Tori and Estonian breed stallions with proven fertility. A morphological examination of the spermatozoa is widely used in the evaluation procedure of semen in many mammalian species, including stallion. Various stains have been used for stallion spermatozoa (*Voss et al.* 1981, *Hurtgen & Johnson* 1982, *Malmgren* 1997). *Hurtgen & Johnson* (1982) reported that some staining techniques might induce morphologic changes in the spermatozoa. In the present study, smears were made from raw semen and stained according to the method described by *Williams* (1920) and modified by *Lagerlöf* (1934). This method is well established and is outstanding for evaluation of sperm head abnormalities in light microscope. Morphological abnormalities of the acrosome, the midpiece and the tail as well as presence of proximal cytoplasmic droplets were studied in unstained wet preparations made from formol-saline fixed samples under a phasecontrast microscope. The advantage of this method is that sperm morphology remains intact, which is not always the case when staining techniques are used (see above). The disadvantage associated with the buffered-formol saline method is that sperm head abnormalities can be difficult to evaluate in wet preparations. This disadvantage was compensated for by checking the occurrence of sperm head abnormalities in both stained smears and wet preparations (see above).

There were significant differences in one or 2 morphological parameters between the 2 ejaculates exmined from a stallion. This indicates the need of a morphological examination of at least 2 ejaculates (not the first one collected) for evaluation of the morphological quality of the semen.

The mean percentages of proximal cytoplasmic droplets were 17.3% and 2.9% in T and E stallions respectively (p=0.002). Similar large differences between breeds within studies and between stallion population from different countries have earlier been reported. Thus *Dowsett & Pattie* (1982) and *Jasko et al.* (1990) found mean percentages of 13.1% and 15.5% proximal cytoplasmic droplets, while *Voss et al.* (1981) reported 0.5%-1.4% of proximal cytoplasmic droplets. The reason for this wide variation is not known. *Dowsett & Pattie* (1982) recommended a careful interpretation of percentages of this defect in relation to fertility, because stallions appear to differ from other species in which excess numbers of cytoplasmic droplets are considered to be indicative of immature spermatozoa and deleterious to fertility (e.g. in bulls, *Söderquist et al*. 1991, *Amann et al.* 2000).

The mean percentages of abnormal sperm heads did not differ between the 2 breeds (12.6% and 13.9% for T- and E-stallions, respectively). In previous studies (*Voss et al.* 1981, *Dowsett & Pattie* 1982, *Jasko et al.* 1990) the mean percentages of head abnormalities varied between 6.4%-21.5%. The present investigation of stallions with proven fertility showed higher mean percentages of head abnormalities than reported for normal stallions (9%), but lower than reported for stallions with testicular degeneration (17%) (*Pickett* 1993). One T-stallion in the present study had a semen picture of both ejaculates indicating a current testicular degeneration (>30% morphologically abnormal sperm heads plus spermatogenetic cells in the ejaculate). This stallion had given acceptable foaling rate after natural mating during the previous breeding season. At the time of examination this stallion must have suffered from testicular degeneration and was therefore excluded from the mean values of the sperm morphology of randomly sampled stallions.

The mean percentages of loose sperm heads in

the present study were approximately at the same level as reported previously by *Voss et al.* (1981), *Bielanski et al.* (1982) and *Jasko et al.* (1990).

The percentages of abnormal midpieces of the spermatozoa from T and E stallions (5.1% and 2.4%, respectively) were comparable with corresponding results of *Jasko et al.* (1990) who reported a frequency of 7.4%. *Voss et al.* (1981) on the other hand reported a mean of 25.3% of midpiece defects in ejaculated semen from 3 stallions, which seems extremely high for normal stallions. No explanation for the high level is given in their report.

Abnormal tails were found in 6.6% and 6.4% of the spermatozoa in T and E stallions respectively. These results are comparable with earlier investigations done by *Dowsett & Pattie* (1982) (10.9 %), *Jasko et al.* (1990) (2.4%) and *Voss et al.* (1981) (4.0%-5.5%)

The overall percentages of morphologically normal spermatozoa were 57.5% and 74.4% for T and E stallions respectively ($p < 0.05$). These mean values correspond to earlier findings by *Jasko et al.* (1990) (52.5%), *Pattie & Dowsett* (1982) (60.8%), but lower than findings by *Bielanski et al.* (1982) (85%) who presented sperm morphology of stallions with high fertility, and with at least 60% motile spermatozoa in their ejaculates.

In the Netherlands it has been recommended that minimal values of semen quality of young (3 years old) stallions for registration in the studbook is a mean total number of progressive motile morphologically normal spermatozoa of $2x10^9$ and a mean of 50% for motility and 50% of morphology of 2 ejaculates collected at one h interval (*Parlevliet et al.* 1994). The mean number of spermatozoa per ejaculate of the 3-year-old Dutch Warmblood stallions was $11.3 \pm 7.1x10^9$ spermatozoa. In the present study 4/7 T stallions fulfilled the Dutch criteria. Of E stallions 6/8 had more than 50% morphologically normal spermatozoa and at least 50% motile spermatozoa in both ejaculates. However, not all of these E stallions had at least $2x10^9$ morphologically normal and motile spermatozoa per ejaculate, because the size of the Estonian horse is much smaller than the Tori horse. In a previous study, *Kavak et al.* (2003) showed that the daily sperm output (DSO) was $12.9 \pm 0.8x 10^9$ for T stallions compared with $4.5 \pm 0.3x10^9$ for E stallions ($p < 0.001$). Therefore 6 out of 7 E stallions (the 22 year old stallion did not fulfill these criteria) must be considered to have an acceptable production of morphologically normal and motile spermatozoa.

Conclusion

The T stallions had 57.5% ± 4.1% and the E-stallions 74.4% ± 3.8% morphologically normal spermatozoa ($p=0.026$). In T stallions 4 of 7 stallions had more than 50% of morphologically normal spermatozoa in both ejaculates; one ejaculate was under the limit in 2 stallions and both ejaculates in one stallion (testicular degeneration). In E stallions 7 of 8 stallions had more than 50% of morphologically normal spermatozoa in both ejaculates, and all 8 in at least one of 2 ejaculates.

Acknowledgement

The authors are grateful to Mrs. K. Selin-Wretling and Mrs. A. Rikberg for the excellent technical assistance. This study was financially supported by Swedish Institute, AGRIA, Estonian Scientific Foundation Grant no. 4111.

References

Amann RP, Seidel Jr GE, Mortimer RG: Fertilizing potential in vitro of semen from young beef bulls containing a high or low percentage of sperm with a proximal droplet. Theriogenology 2000, *54,* 1499-1515.

Ball BA, Mohammed HO: Morphometry of stallion spermatozoa by computer-assisted image analysis. Theriogenology 1995, *44,* 367-377.

Bane A: Acrosomal abnormality associated with sterility in boar. Proc. 4th Int. Congr. Anim. Reprod. & A.I., The Hague IV 1961, 810-817.

Bielanski W, Dudek E, Bittmar A, Kosiniak K: Some characteristics of common abnormal forms of spermatozoa in highly fertile stallions. J. Reprod. Fert. 1982, Suppl. *32,* 21-26

Bielanski W, Kaczmarski F: Morphology of spermatozoa in semen from stallions of normal fertility. J. Reprod. Fert. 1979, Suppl. *27,* 39-45

Casey PJ, Gravance CG, Davis RO, Chabot DD, Liu IKM: Morphometric differences in sperm head dimensions of fertile and subfertile stallions. Theriogenology 1997, *47,* 575-582.

Dowsett KF, Pattie WA: Characteristics and fertility of stallion semen. J. Reprod. Fert. 1982, Suppl. *32,* 1-8

Hancock JL: The morphology of boar spermatozoa. J. R. Microsc. Soc. 1957, *76,* 84-97.

Hellander JC, Samper JC, Crabo BG: Fertility of a stallion with low sperm motility and a high incidence of an unusual sperm tail defect. Vet. Rec. *1991,* 128, 449-451.

Hurtgen JP, Johnson LA: Fertility of stallions with abnormalities of the sperm acrosome. J. Reprod. Fert. 1982, Suppl. *32,* 15-20.

Jasko DJ, Lein DH, Foote RH: Determination of the relationship between sperm morphologic classifications and fertility in stallions: 66 cases (1987-1988). J. Amer. Vet. Med. Assoc. 1990, *197,* 389-394.

Jasko DJ, Lein DH, Foote RH: The repeatability and the effect of season on seminal characteristics and computer-aided sperm analysis in the stallion. Theriogenology 1991, *35,* 317-327.

Kavak A, Lundeheim N, Aidnik M, Einarsson S: Testicular measurements and daily sperm output of Tori and Estonian breed stallions. Reprod. Dom. Anim. 2003, *38,* 167-169.

Kenney RM, Hurtgen J, Pierson R, Withersponn D, Simons J: Manual for clinical fertility evaluation of the stallion. J. Soc. Theriogenology 1983, *9,* 3-100.

Lagerlöf N: Morphologische Untersuchungen über Veränderungen im Spermabild und in den Hoden bei Bullen mit vermindeter oder aufgehobener Fertilität. (Researches concerning morphologic changes in the semen picture and in the testicles of sterile and subfertile bulls.) Acta Path. Microbiolog. Scand. 1934, Suppl. 19, Thesis, Stockholm.

Love CC, Varner DD, Thompson JA: Intra- and inter-stallion variation in sperm morphology and their relationship with fertility. J. Reprod. Fert. 2000, Suppl. *56,* 93-100.

Malmgren L: Assessing the quality of raw semen: a review. Theriogenology. 1997, *48,* 523-530.

Papanicolaou GN: A new procedure for staining vaginal smears. Science 1942, *95,* 438-439.

Parlevliet JM, Kemp B, Colenbrander B: Reproductive characteristics and semen quality in maiden Dutch Warmblood stallions. J. Reprod. Fert. 1994, *101,* 183-187.

Pattie WA, Dowsett KF: The repeatability of seminal characteristics of stallions. J. Reprod. Fert. 1982, Suppl. *32,* 9-13.

Rousset H. Chanteloube PH, Magistrini M, Palmer E: Assessment of fertility and semen evaluations of stallions. J. Reprod. Fert. 1987, Suppl. *35,* 25-31.

SAS Institute Inc.: SAS/STAT Users Guide, Version 8, Cary, NC 1989.

Söderquist L, Janson L, Larsson K, Einarsson S: Sperm morphology and fertility in A.I. bulls. J. Vet. Med. 1991, Av., *38,* 534-543.

Voss JL, Pickett B., Squires E: Stallion spermatozoal morphology and motility and their relationship to fertility. J. Amer. Vet. Med. Assoc. 1981, *178,* 287-289.

Williams WW: Technique of collecting semen for laboratory examination with a review of several diseased bulls. Cornell Vet. 1920, *10,* 87-94.

Sammanfattning

Spermiemorfologi hos hingstar av estländsk och tori ras.

Bedömningen av en hingsts avelsvärde omfattar bland annat spermasamling och beräkning av totalantalet morfologiskt normala spermier i ejakulatet. Denna studie omfattar spermiemorfologisk undersökning av 2 ejakulat från slumpmässigt utvalda fertila hingstar av estländsk (E, n=8) och tori (T, n=7) ras. Omedelbart efter spermasamlingen fixerades en liten mängd sperma i en mL fysiologisk formolsalinlösning för bedömning av samtliga spermiemorfologiska avvikelser i faskontrastmikroskop (1000x). Dessutom gjordes utstryk på objektglas, som färgades enligt Williams metod (carbolfuchsin-eosin), för en mera detaljerad ljusmikroskopisk undersökning av spermiehuvudet (1000x). Den statistiska bearbetningen av erhållna data omfattade variansanalys och resultaten redovisas som LSmeans (±SE). En T-

hingst, som visade sig ha en störning i spermiogenesen och en 22 år gammal E-hingst inkluderades inte i de statistiska bearbetningarna. T-hingstarna hade i medeltal 57,5±4,1% och E- hingstarna 74,4±3,8% morfologiskt normala spermier (p=0,012). Hos 4 av 7 T-hingstar och 7 av 8 E-hingstar innehöll båda ejakulaten >50% morfologiskt normala spermier. Det var en signifikant rasskillnad avseende procentandelen spermier med proximal cytoplasmadroppe (17,3±2,7% och 2,9±2,5% hos T- respektive E-hingstar; p=0,003).

(Received June 26, 2003; accepted october 14, 2003).

Reprints may be obtained from A. Kavak, Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Estonian Agricultural University, Kreutzwaldi 62, 51014, Tartu, Estonia. E-mail: ants.kavak@og.slu.se, tel: 372 7 422 482, fax: 372 7 422 259.