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Theriogenology 60 (2003) 453–461
Theriogenology

# Seasonal changes in semen quality and freezability in the Warmblood stallion

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Received 20 November 2001; accepted 23 October 2002

**Abstract**

The objective of this study was to investigate seasonal changes in stallion semen quality and to determine the best time for semen cryopreservation. Experiments were performed using 10 Warmblood stallions from the National Stud Farm in Avenches (Switzerland). Ejaculates were collected and frozen every other week during 1 year from January to December 1999. Volume, concentration, and motility, and the number of morphologically normal sperm and sperm with major defects (abnormal heads, acrosome defects, nuclear vacuoles, proximal droplets, abnormal midpieces) were evaluated. For all frozen-thawed semen samples motility as well as viability (SYBR-14/PI) was tested, and the hypoosmotic swelling test (HOS) was performed. To analyze seasonal differences 4 periods of 3 months each were defined: autumn (September, October, November), winter (December, January, February), spring (March, April, May) and summer (June, July, August). During the 1 year experiment all semen quality parameters showed a clear seasonal pattern. The volume, total sperm count and motility in fresh semen were significantly higher ($P < 0.05$) in summer than in winter, while sperm concentration was significantly lower in summer compared to the other seasons. Regarding morphology, normal sperm was significantly lower ($P < 0.05$) in summer than at any other time of the year and higher values ($P < 0.05$) were found for major defects in summer than in spring and autumn. In frozen-thawed semen motility was significantly ($P < 0.05$) improved in autumn when compared to spring and summer. Viability was lowest in summer and differed significantly ($P < 0.05$) from other seasons. The HOS test revealed significantly more ($P < 0.05$) membrane damaged spermatozoa in winter than in spring, summer and autumn. Our results demonstrate that in our climatic conditions clear seasonal differences occur in semen quality of fresh and frozen-thawed semen and that cryopreservation of stallion semen should preferably be performed in autumn.


*Keywords:* Stallion; Season; Semen quality; Sperm morphology; Cryopreservation

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0093-691X/03/$ – see front matter © 2003 Published by Elsevier Science Inc.
doi:10.1016/S0093-691X(03)00046-3

## 1. Introduction

The use of artificial insemination (AI) in equine breeding has become increasingly popular in the horse industry, offering many advantages over natural service. Some of the reasons for this include the choice of a great number of stallions, safety for both the mare and the stallion, reduced risk of infectious disease transmission, and transport inconvenience. In addition, pregnancy rates have been shown to be equal or even higher after AI with fresh or chilled semen compared to natural mating [1]. Lower fertility is known to occur in mares inseminated with frozen semen [1]. To improve reproductive efficiency when using cryopreserved semen, attention should be given to factors such as the stallion, the quality and handling of the semen, and age as well as reproductive history and management of the mare [2]. Since the horse is a seasonal breeder with increased sexual activity during long days, annual changes in day length may also influence male gonadal activity. In the Northern hemisphere, the physiological breeding season lasts from April to October [3] and during this time sperm production [4–6] and endocrine testicular function [4,7–11] are clearly increased. Semen collection for cryopreservation, however, is usually performed during the nonbreeding season when sexual activity is low. Because only poor and conflicting information is available about annual changes in semen characteristics [4,12–14], especially sperm morphology [15,16] and semen freezability [13], the objective of this study was to investigate seasonal changes of semen quality and freezability and to determine the most suitable time for semen cryopreservation.

## 2. Materials and methods

Experiments were performed using 10 healthy Warmblood stallions, between 5 and 19 years of age, from the National Stud Farm. The animals were fed hay, oats and pellets supplemented with minerals. All animals were exercised daily for at least 1 h. Before starting the experiment the stallions were trained to mount a phantom and extragonadal sperm reserves were minimized by daily semen collection for 5 days. Semen was collected, evaluated and cryopreserved every other week during 1 year from January to December 1999. During the breeding season (March to July) the stallions were also used for AI, thus preventing a standardized ejaculatory frequency.

### *2.1. Climatic conditions*

Climatic conditions were recorded by the meteorological station in Payerne (46°49′N, 06°57′E), the area in which the stallions were located. Parameters relevant for our experiment were the mean ambient temperature, mean relative humidity, total sunshine and day length.

### *2.2. Semen processing and examination*

After semen collection and removal of the gel fraction (in-line gel filter), the volume (graduated cylinder) and concentration (spectrophotometer) were estimated. Progressive

motility was assessed with a Mika Motion Analyzer (Cell Motion Analyzer SM-CMA-1074, MTM, Montreux, Switzerland) on fresh diluted semen using the threshold values defined according to guidelines from the Institute of Reproductive Medicine, Veterinary School of Hannover, Germany [17]. For morphological examination five drops of fresh semen were fixed in 2 ml Hancock solution and smears prepared according to the method of Hancock [18]. For classification of sperm cells with major and minor defects [19,20] at least 200 spermatozoa were evaluated using phase contrast microscopy (Olympus BX50, UplanFl 100×/1.30). The gel-free semen was diluted with centrifugation medium (INRA 82-Hepes + 2% v:v egg yolk) to a concentration of $100 \times 10^6$ spermatozoa/ml and centrifuged at $1000 \times g$ for 2 min. After centrifugation, the supernatant was removed and the sperm pellet resuspended in freezing medium (75 ml lactose solution 11% wt./vol., 20 ml egg yolk, 5 ml glycerol) to a final concentration of $300 \times 10^6$ spermatozoa/ml. Thereafter, semen was cooled to 4 °C during at least 20 min, packaged into 0.5 ml straws and frozen in an automatic freezer (Minidigitcool 700 ZB 290, IMV, Aigle, France) at a cooling rate of 60 °C/min from +4 to −100 °C and 30 °C/min from −100 to −140 °C. The straws were then stored in liquid nitrogen until examination. From each ejaculate three straws were thawed (37 °C, 30 s), pooled and diluted with 3 ml centrifugation medium to determine motility, viability and hypoosmotic swelling (HOS). Viability testing was performed by dual DNA staining (LIVE/DEAD® Sperm Viability Kit, Molecular Probes Europe, Leiden, NL) using SYBR-14 in combination with propidium iodide (PI). The SYBR-14 (component A) was diluted with anhydrous dimethyl sulfoxide (DMSO) 1:10 (SYBR-14 working solution), while PI (component B) was used at its original concentration. 2 μl of the SYBR-14 working solution was added to 1 ml of diluted thawed semen. After incubation for 10 min at 37 °C, 5 μl of component B were added. 5 min later, 5 μl of stained semen were placed on a slide, covered with a coverslip (24 mm × 24 mm) and examined using fluorescence microscopy (Olympus BX50, UPlanApo 40×/0.85, FITC filter U-MWIB, high pressure Hg-lamp). Different sequences of fluorescence stained spermatozoa were monitored by connecting a video camera (SANYO VCC-2972, Basel, Switzerland) to the microscope. At least 500 sperm cells were counted on the screen and the percentage of green fluorescing sperm was defined as viable [21]. The functional membrane integrity of frozen-thawed semen was performed with the HOS test. 100 μl of thawed semen were added to 1 ml of a fructose–sodium citrate hypoosmotic solution (100 mOsm/l) and incubated at 37 °C for 30 min. At least 200 spermatozoa were observed at magnification 400× and classified by the presence or absence of a swollen tail (curled/coiled principal or end piece). The percentage of HOS-positive spermatozoa (number of spermatozoa with swollen tails per total number of spermatozoa × 100) was recorded for each sample [22,23].

### 2.3. Statistical analysis

All data were analyzed using StatView 5.0 software program (SAS Institute, Wangen, Switzerland). A multivariate analysis of variance (ANOVA) was carried out to analyze the effects of stallion and date of semen collection. To determine seasonal changes in semen quality parameters and climatic conditions, 4 periods of 3 months each were defined as autumn (September, October, November), winter (December, January, February), spring (March, April, May) and summer (June, July, August). Seasonal means of semen

characteristics were compared with Fisher's post hoc test. Correlation between different parameters in frozen-thawed semen was also calculated. Values were considered to be statistically significant at $P < 0.05$.

## 3. Results

The results from ANOVA (Table 1) demonstrate that the date of semen collection significantly ($P < 0.05$) influenced all quality parameters except volume, sperm concentration and total sperm. The influence of the stallion on semen quality was highly significant ($P < 0.0001$) for all parameters examined.

Seasonal differences for various semen quality parameters are shown in Table 2. During the 1 year experiment all parameters showed a distinct seasonal pattern. The volume, total sperm and motility in fresh semen were significantly higher ($P < 0.05$) in summer than in

Table 1
Effect of stallion ($n = 10$) and date of semen collection ($n = 26$) on quality parameters of ejaculates obtained every other week during 1 year

| Parameter | Stallion, $P$ | Date, $P$ |
|---|---|---|
| Volume (ml) | <0.0001 | 0.5805 |
| Concentration ($\times 10^6$/ml) | <0.0001 | 0.2552 |
| Total sperm ($\times 10^9$) | <0.0001 | 0.6187 |
| Motility fresh semen (%) | <0.0001 | 0.0005 |
| Normal spermatozoa (%) | <0.0001 | 0.0005 |
| Major defects (%) | <0.0001 | <0.0001 |
| Acrosome defects (%) | <0.0001 | 0.0069 |
| Nuclear vacuoles (%) | <0.0001 | <0.0001 |
| Motility thawed semen (%) | <0.0001 | <0.0001 |
| Viability (%) | <0.0001 | 0.0250 |
| HOS-positive (%) | <0.0001 | 0.0002 |

Table 2
Means (±S.E.M.) of semen characteristics from ejaculates of 10 Warmblood stallions collected every other week during autumn, winter, spring and summer

| Parameter | Autumn | Winter | Spring | Summer |
|---|---|---|---|---|
| Volume (ml) | $36.3 \pm 2.0^{a}$ | $33.9 \pm 2.0^{a}$ | $36.8 \pm 1.8^{a,b}$ | $41.2 \pm 1.8^{b}$ |
| Concentration ($\times 10^6$/ml) | $323.1 \pm 9.7^{a}$ | $305.2 \pm 9.6^{a,b}$ | $293.8 \pm 8.6^{b}$ | $270.0 \pm 8.6^{c}$ |
| Total sperm ($\times 10^9$) | $10.5 \pm 0.4^{a,b}$ | $9.5 \pm 0.4^{a}$ | $10.1 \pm 0.4^{a,b}$ | $10.6 \pm 0.4^{b}$ |
| Motility fresh semen (%) | $74.1 \pm 1.3^{a,b}$ | $70.2 \pm 1.3^{c}$ | $72.0 \pm 1.2^{a,c}$ | $75.9 \pm 1.2^{b}$ |
| Normal spermatozoa (%) | $29.4 \pm 1.0^{a}$ | $28.9 \pm 1.0^{a}$ | $29.9 \pm 0.9^{a}$ | $26.1 \pm 0.9^{b}$ |
| Major defects (%) | $58.9 \pm 1.0^{a}$ | $61.0 \pm 1.0^{a,b}$ | $59.3 \pm 0.9^{a}$ | $63.4 \pm 0.9^{b}$ |
| Acrosome defects (%) | $6.6 \pm 0.3^{a}$ | $6.4 \pm 0.3^{a}$ | $5.3 \pm 0.3^{b}$ | $6.4 \pm 0.3^{a}$ |
| Nuclear vacuoles (%) | $9.3 \pm 0.6^{a}$ | $8.7 \pm 0.6^{a,b}$ | $7.5 \pm 0.5^{b}$ | $9.8 \pm 0.5^{a}$ |
| Motility thawed semen (%) | $43.3 \pm 1.6^{a}$ | $42.4 \pm 1.5^{a,c}$ | $35.7 \pm 1.4^{b}$ | $39.9 \pm 1.4^{c}$ |
| Viability (%) | $58.9 \pm 1.0^{a}$ | $58.3 \pm 1.0^{a}$ | $58.1 \pm 0.9^{a}$ | $55.0 \pm 0.9^{b}$ |
| HOS-positive (%) | $45.8 \pm 1.4^{a}$ | $42.2 \pm 1.4^{b}$ | $49.0 \pm 1.2^{a}$ | $48.9 \pm 1.2^{a}$ |

Means with different superscripts (a, b, c) are significantly different ($P < 0.05$).

Table 3
Correlation between semen quality parameters in frozen-thawed semen

| | Viability | Motility |
|---|---|---|
| Motility | $r = 0.56^{*}$ | |
| HOS-positive | $r = 0.31^{*}$ | $r = 0.31^{*}$ |

* Significant ($P < 0.05$).

Table 4
Climatic data for seasons during the experiment

| Parameter | Autumn (September to November) | Winter (December to February) | Spring (March to May) | Summer (June to August) |
|---|---|---|---|---|
| Mean temperature (°C) | 9.4 | 1.2 | 9.8 | 17.7 |
| Mean relative humidity (%) | 85.0 | 81.3 | 76.7 | 74.3 |
| Total sunshine (h) | 301.1 | 200.8 | 439.3 | 678.1 |
| Minimal day length (h, min) | 09, 08 | 08, 32 | 11, 04 | 13, 53 |
| Maximal day length (h, min) | 13, 22 | 10, 41 | 15, 52 | 15, 36 |

winter, while sperm concentration was significantly lower ($P < 0.05$) in summer compared to other seasons. Regarding morphology, the percentage of normal sperm was significantly lower ($P < 0.05$) in summer than at any other time of the year and higher ($P < 0.05$) values were found for major defects in summer than in spring and autumn. In frozen-thawed semen samples motility was significantly ($P < 0.05$) improved in autumn when compared to spring and summer. Viability assessed by staining (SYBR-14/PI) was lowest in summer and differed significantly ($P < 0.05$) from other seasons. The HOS test revealed significantly ($P < 0.05$) more membrane damaged spermatozoa in winter than during spring, summer or autumn.

When examining interrelationships between parameters in frozen-thawed semen (Table 3), significant ($P < 0.05$) correlations were found between motility and viability ($r = 0.56$), and between HOS-positive and viability ($r = 0.31$), as well as motility ($r = 0.31$).

The mean ambient temperature, mean relative humidity, and total sunshine as well as day length during autumn, winter, spring and summer are shown in Table 4. The annual changes in all three parameters are characteristic of middle European climate conditions. The highest (17.7 °C) mean seasonal temperature was recorded during the summer months and the lowest (1.2 °C) in winter. Mean relative humidity during the four seasons fluctuated between 74.3% in summer and 85.0% during winter. Total sunshine was highest (878.1 h) in summer (maximal day length 15 h 52 min in June) and lowest (200.8 h) in winter (minimal day length 8 h 32 min in December).

## 4. Discussion

Seasonal changes of sperm morphology and semen freezability are not well documented in the stallion. Most authors are mainly dealing with routine fresh semen parameters such

as volume, concentration and motility [4–6] as well as endocrine changes [4–11]. In addition, only a small number of animals was studied and reduced semen collections during the breeding and nonbreeding season were performed which do not represent true annual changes. Especially when focussing on sperm abnormalities no published data about morphological changes during a whole year could be found. Regarding seasonal variation of semen freezability only one study [13] using three horses and three ponies has been conducted. Findings of our study in which we evaluated and cryopreserved a total of 260 ejaculates collected from 10 Warmblood stallions during a whole year demonstrate significant seasonal differences for all semen quality parameters investigated. Looking at cryopreservation the best time for semen freezing was in autumn when major sperm defects were lowest and motility as well as viability of thawed semen were highest. In our stallions, a high total sperm number was observed during summer and autumn which agrees with some findings [12,14], but differing from one report [13] in which a clear seasonal pattern was not found. Regarding sperm concentration, we observed highest values in autumn and lowest in summer which agrees with one report [13], but contrasts to others [12,14], in which high and low concentrations were found in August and December, respectively. The gel-free volume in our study was highest in summer and lowest in winter, although highest values were also seen in spring [13], July [12] or August [14], and lowest in March [12] or December [14]. These conflicting data may most likely be the result of differences in semen collection frequency, horse breed and latitude. Maximal values of total sperm and gel-free volume during summer as seen in the present work may be explained by an increased activity of male reproductive organs during long days [4–6,8,11].

Another important parameter for breeding soundness evaluation is the percentage of progressively motile spermatozoa [24], which has been shown to be related to stallion fertility [25]. In our work progressive motility in fresh semen was significantly higher in summer and autumn than in winter. Lowest motility during winter has also been reported by others [13,14], and in one investigation [12] no influence of season on motility was found. Possible reasons for the different findings are seasonal fluctuations in seminal plasma compounds [26], as well as changes in plasma volume shown to have a negative effect on sperm motility [27].

Morphological examination of spermatozoa in fresh semen revealed that the percentage of normal spermatozoa was lowest in summer, when major defects were significantly higher compared to spring and autumn. Results from routine obligatory breeding soundness examinations in the Netherlands [15] showed a higher incidence of abnormal spermatozoa during the nonbreeding season (September to February) than in the breeding season (March to August). These findings are in contrast to those reported only recently [16], where higher values of morphological intact spermatozoa were found during the nonbreeding season in 24 ejaculates of 4 stallions evaluated in May and December. However, results from the latter two studies do not represent seasonal changes because repeated semen collections were not performed during a whole year. In this context it must be remembered that intra- and inter-stallion variation in sperm morphology do occur [28] and in our study we could also demonstrate significant differences within the breeding season. A negative relation between the percentage of sperm with major defects and fertility was demonstrated earlier [20,28], supporting the view that sperm morphology must be considered an important criterion in breeding soundness examination. However, provided

the total number of normal spermatozoa ejaculated or inseminated is high enough, cells with defects of the acrosome or the tail (compensible defects) should be tolerable at a higher level than are those with nuclear defects (non-compensible defects), since these cells would not attach to ova, thus allowing other normal spermatozoa to fertilize the ovum [29].

In frozen-thawed semen motility, as well as sperm membrane integrity using SYBR-14/PI, and the HOS test, were applied. All these parameters examined showed a clear seasonal pattern. Highest values for progressive motility were found in autumn and winter and lowest in spring. Surprisingly, motility of frozen-thawed semen collected in summer and winter was similar, although motility in fresh semen was significantly higher in summer than in winter. This unexpected behavior has been previously noted [13]. Similar to our motility results viability (SYBR-14/PI) in frozen-thawed semen was lower in ejaculates collected in summer than in other seasons. The high number of dead spermatozoa after the freezing-thawing process in summer most likely indicates that seasonally related changes in seminal plasma compounds may affect semen preservation [26,27].

Hypoosmotic swelling is a test for evaluation of sperm membrane function [22] and has been applied in fresh [23,30–33] and frozen-thawed stallion semen [23,32]. The percentage of HOS-positive spermatozoa was lowest in winter and correlated significantly with the motility and viability of thawed semen. The hypoosmotic solution used in our investigation contained fructose–sodium citrate with an osmolarity of 100 mOsm. Using this solution, mean seasonal values ranged from 42% (winter) to 49% (spring) and were clearly higher compared to other reports [23,32] using lactose with an osmolarity of 50 and 150 mOsmol/l. Because hypoosmotic solutions and freezing protocols were not standardized, these results cannot be compared. Since membrane function is essential for fertilization and a negative correlation ($r = -0.40$) was found between tail swelling and number of services per pregnancy [33], the HOS test may provide additional information in routine equine semen analysis.

In addition to the seasonal changes observed in our study, a highly significant inter-stallion variation in semen characteristics was present. A possible breed effect [34,35] can be excluded, because only Warmblood stallions were used. Due to the large variation in semen quality, it is imperative that individual stallion testing occurs.

Under our climatic and management conditions semen collected in autumn showed the highest sperm concentration as well as the lowest number of major defects, and for frozen-thawed semen the best values of motility and viability were observed. Therefore, we suggest the months of September, October and November are most suitable for cryopreservation of stallion semen.

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