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CHARACTERISATION OF MOVEMENT PATTERN AND VELOCITIES OF STALLION SPERMATOZOA DEPENDING ON DONOR, SEASON AND CRYOPRESERVATION

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The aim of the study was to compare different types of movement pattern and velocities of stallion spermatozoa depending on cryopreservation during breeding and non-breeding season. Ejaculates were collected from four stallions during May (n = 24) and December (n = 24). Parameters of sperm movement were evaluated by computer-aided sperm analysis (CASA) system, and included percentages of motile spermatozoa, different patterns of motility, the velocity, linearity (LIN), amplitude of lateral head displacement (ALH) and beat-cross frequency (BCF). In winter the average percentages of motility were slightly higher compared to the breeding season in May (70.8 ± 12.7% vs. 66.8 ± 12.2%, respectively). Cryopreservation and thawing led to a significant decrease in the number of motile sperm to 11.3 ± 5.8% in May and 15.6 ± 7.0% in December. The pattern of motility was also changed. Detailed analysis by CASA demonstrated that cryopreservation resulted in a shift from the proportions of linear to more non-linear motile spermatozoa and to a significant increase of local motile and hyperactivated spermatozoa. Mean velocity of fresh motile spermatozoa differed between May and December (119.1 ± 43.9 vs. 164.4 ± 66.4 μm/sec, respectively; $P < 0.05$). Cryopreservation and thawing led to a slight increase of curvilinear velocity (VCL) and straight line velocity (VSL). The motility analysis has shown that the parameters BCF and ALH were highly correlated in stallion spermatozoa ($r = –0.67$; $P < 0.001$). The BCF of stallion spermatozoa was slightly reduced in the non-breeding season. Altogether, the influence of factors on the motility of stallion spermatozoa has the following rank order: cryopreservation ($P < 0.0001$) > stallion ($P < 0.001$) > season ($P < 0.05$).

Key words: Stallion spermatozoa, cryopreservation, CASA, motility, movement pattern, velocity

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Sperm motility is considered as an important determinant in stallion semen evaluation (Amelar et al., 1980; Galloway, 1987; Amann, 1989). At present, the most common method of determining stallion sperm motility is visual estimation, using the microscope. Because of deficiencies in both the accuracy and repeatability of results obtained by microscopic evaluation, development of a technique for objective assessment of sperm motility has been a continuous concern for workers involved in male fertility evaluation. The predominant problem in computerised semen analysis is whether the criteria for sperm size and brightness are sufficient to discriminate between leukocytes, epithelial cells and debris on the one hand and spermatozoa on the other. With the aid of the 'Strömberg-Mika' Cell Motion Analyser (SM-CMA) it is possible to detect spermatozoa not only by motility, size and contrast but also by sperm tails. The detection of tail structures in immotile cells as a further criterion, in addition to size, contrast and movement, enables the SM-CMA to distinguish between sperm cells and leukocytes, epithelial cells or debris.

Furthermore, cryopreservation of stallion semen is gaining importance. The efficiency of preservation concerning the percentage of motile and intact spermatozoa after thawing varies substantially, and is lower in horses compared to other farm animals. One of the prerequisites for successful preservation of stallion semen is a high initial quality, but this shows considerable differences between individual stallions. The distinct variation in freezability of stallion semen is one of the major factors determining the success of artificial insemination with frozen spermatozoa (Samper et al., 1991; Vidament et al., 1997). Furthermore, the normal equine breeding season begins in the spring and extends through mid-summer. During the breeding season, availability of stallion semen for cryopreservation is limited in practice. Consequently, stallion semen is usually collected and cryopreserved during the non-breeding season in autumn and winter. However, the best periods for management in cryopreservation and for high semen quality might not coincide, as the quality of semen may show seasonal fluctuations (Magistrini et al., 1987; Hoffmann and Landeck, 1999).

Evaluation of semen quality has to consider that the functional competence is multifactorially determined. The percentage of motile stallion spermatozoa after cryopreservation shows poor correlation with fertility (Grondahl et al., 1994; Boyle, 1996). However, the accurate estimation of the fraction of the sperm population exhibiting progressive motility and measurement of their velocities, their ALH, BCF and LIN should provide better information regarding the metabolic activity and fertilising capacity of sperm cells. Spermatozoa require sufficient progressive motility to overcome the barriers of the female reproductive tract to reach the site of fertilisation, and the pattern of sperm movement is associated with the functional competence in the fertilising process (Amelar et al., 1980; Pacey et al., 1995; Suarez, 1996; Yanagimachi, 1994; Bed-

ford, 1998). Therefore, the evaluation of sperm motility is an important parameter for the characterisation of semen quality.

The aim of the study was to compare various parameters of motility of fresh and cryopreserved spermatozoa from different stallions during breeding and non-breeding seasons. For each sample the percentage of motility, different pattern of sperm movement, VCL, VSL, ALH and LIN of spermatozoa were evaluated at 37 °C using a computer-aided sperm analysis system (SM-CMA, Strömberg Mika, Switzerland).

Materials and methods

Semen collection

The study included 12 ejaculates from each of four warm-blooded stallions. The stallions were in a regular semen production programme for AI. Ejaculates were collected twice per week during the breeding season (May 1998, n = 24) and during the non-breeding season (December 1998, n = 24). In D ecember the stallions were trained six weeks before starting the semen collection for the study, to avoid the collection of ancient spermatozoa owing to prolonged storage within the epididymis.

Fresh semen was collected from stallions with the aid of an artificial vagina, mixed with an equal volume of skim milk extender, centrifuged at 400 × g for 10 min, diluted with fresh skim milk extender and transported to the laboratory. Aliquots of 4 ml of this sperm suspension were centrifuged to separate the extender, and then either diluted in 4 ml TALP solution (Tyrode buffer with albumin, lactate and pyruvate) or in defined cryoextender.

Cryopreservation

Aliquots of this sperm suspension were resuspended with two cryoextenders in the ratio of 1:3. These cryoextenders included the commercially used 'Gent' extender (Minitüb, Tiefenbach, Germany) (G) and a combination of equal volumes of Triladyl® (Minitüb) and skim milk extender supplemented by 4% egg yolk and 2.5% glycerol (TS).

Diluted samples were filled in 0.5 ml straws and subsequently frozen in a computer-controlled freezer (Kryo 10-3.3; Messer Griesheim, Germany) with cooling rates of 0.3 °C/min to 5 °C, 10 °C/min to –15 °C and 25 °C/min to –150 °C. Then the straws were plunged in liquid nitrogen. This cooling regime is keeping in with the method described by Bader et al. (1992). The straws were thawed by agitation in a water bath of 38 °C for 30 sec. Thawed samples were diluted with TALP for subsequent evaluation in the same manner as unfrozen spermatozoa.

Evaluation of spermatozoa

Configuration of the CASA system. The motility was evaluated by the 'Strömberg-Mika' Cell Motion Analyser (SM-CMA, Strömberg Mika, Bad Feilnbach, Germany). The sample was equilibrated for 10 min at 37° C. A 6 µl aliquot was placed in a pre-warmed Mika chamber with a depth of 10 micrometers. To analyse a sufficient number of sperms in a diluted sample, 15 to 20 fields need to be acquired per analysis (Katz and Davis, 1987). At least 500 sperms were analysed for each sample.

The used settings of the motility analyser were as follows: number of frames per analysis: 32; minimal number of frames: 16; time of acquisition: 20 ms (50 Hz); minimum and maximum area of objects: 35 and 350 pixel; velocity limit for immotile objects: 10 µm/sec; velocity limit for locally mobile objects: 25 mm/sec; max. radius: 30 µm; min and max. area for the immobile objects: 20 and 100 pixel.

Parameters

The motility parameters obtained by the SM-CMA measurements were: curvilinear velocity (VCL; µm/sec), the velocity over the actual sperm track, which included all deviations of sperm head movement, straight line velocity (VSL; µm/sec), the velocity over the straight line distance between the beginning and the end of the sperm track, beat-cross frequency (BCF; Hz), the frequency with which the sperm crosses the smoothed path, amplitude of lateral head displacement (ALH; µm), the amplitude with which the sperms deviate from the averaged path and linearity (LIN; %), the straight-line distance divided by the incremental deviations along the actual path. Linearity was calculated by dividing the linear velocity by the curvilinear velocity and was expressed as a percentage.

Furthermore, a subdivision of progressive motile spermatozoa in linear, non-linear and hyperactivated spermatozoa is possible. For that purpose the path of every spermatozoon was investigated by drawing a comparison between the actual sperm track and the straight-line distance between the beginning and the end of movement. Hyperactivated spermatozoa are characterised by a VCL from above 80 µm/sec, LIN < 65% and ALH from at least 6.5 µm. A spermatozoon with straightness from ≥ 90% and velocity averaged path (VAP) > 25 µm/sec is categorised as linear. Distinguishing mark from non-linear movement is a straightness from lower than 90%.

Statistical analysis

For each variable, seasonal means ± standard errors of means (SEM) were calculated from the 4 individual seasonal means. All calculations were performed using the SAS 6.12 statistical software package. The CADMOD procedure was used to investigate the relationships between the dependent variables and the in-

dependent variables (main effects) of cryopreservation, extender for cryopreservation, stallion and season by analysis of variance (ANOVA). Spearman's rank order correlations were performed between the CASA motility parameters and other parameters before freezing and after thawing. The significance level was generally set to $P = 0.05$.

Results

Motility

A direct comparison between proportions of motile spermatozoa by CASA and by microscopic estimation in a parallel study demonstrated substantial agreement of results ($r = 0.97$; $P < 0.001$, Fig. 1). However, this parameter includes spermatozoa with different movement patterns. The detailed data of CASA showed that the categories of local, linear, non-linear and hyperactivated movement changed differently depending on the donor, season and preservation.

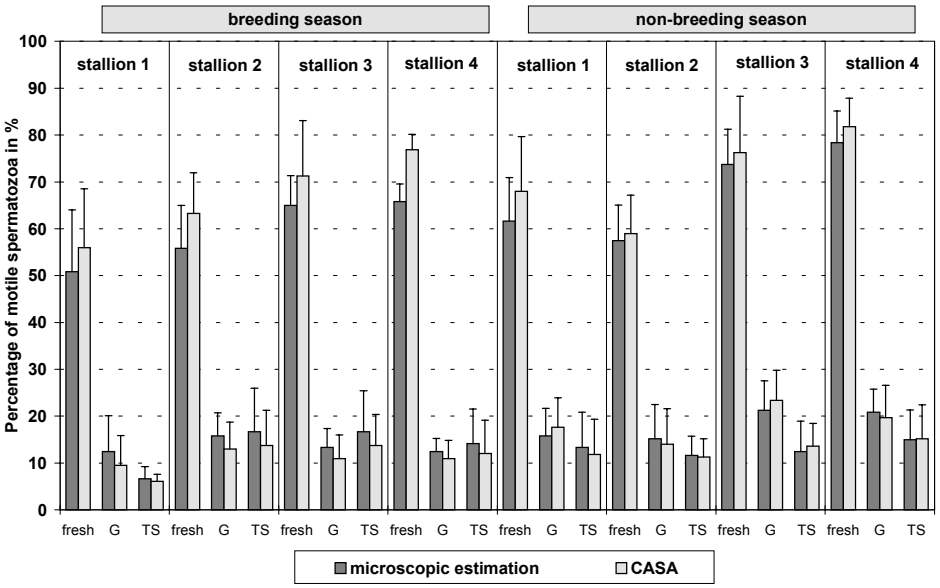

Fig. 1. Motility of stallion spermatozoa after microscopic estimation and CASA (G: Gent extender, TS: Triladyl/skim milk extender) during breeding season (May) and non-breeding season (December)

In winter the average percentage of motility was slightly higher versus breeding season in May: 70.8 ± 12.7% vs. 66.8 ± 12.2% (Fig. 2). Cryopreservation and thawing led to a significant decrease in the number of motile sperm ($P < 0.001$) to 11.3 ± 5.8% in May (range: 5 to 24%) and 15.6 ± 7.0% in December (range: 8 to 34%) (Fig. 3).

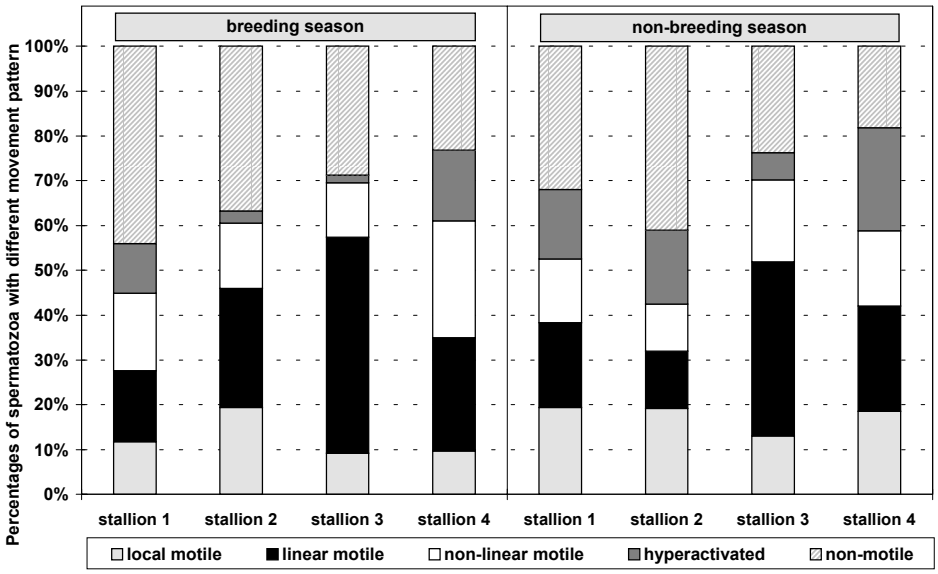

Fig. 2. Movement pattern of stallion spermatozoa before freezing during breeding season (May) and non-breeding season (December)

The influence of the type of extender used was only apparent in the non-breeding season. However, in contrast to the motility of frozen spermatozoa from May, spermatozoa of all stallions showed the best motility in December when treated with the extender G. The difference to the extender TS was significant ($P < 0.05$).

The percentage of motile spermatozoa also significantly depended on the donor of fresh semen ($P < 0.001$) as well as of cryopreserved semen ($P < 0.05$; Figs 2 and 3).

Movement pattern of stallion spermatozoa

Recovery of spermatozoa in December resulted in higher portions of local motile and hyperactivated spermatozoa. In winter the average percentages of local motile and hyperactivated spermatozoa ($17.9 \pm 6.5\%$ and $16.1 \pm 13.1\%$, respectively) were higher than during breeding season in May ($12.5 \pm 6.9\%$ and $7.8 \pm 7.1\%$, respectively) ($P < 0.05$). After freezing and thawing, the proportions of local motile and hyperactivated spermatozoa decreased to $0.5 \pm 0.3\%$ and $2.9 \pm 1.9\%$ in May and $2.5 \pm 2.0\%$ and $5.9 \pm 2.4\%$ in December (Fig. 3).

Furthermore, results of CASA demonstrated that cryopreservation resulted in a shift from the proportions of linear to more non-linear motile spermatozoa (Figs 2 and 3).

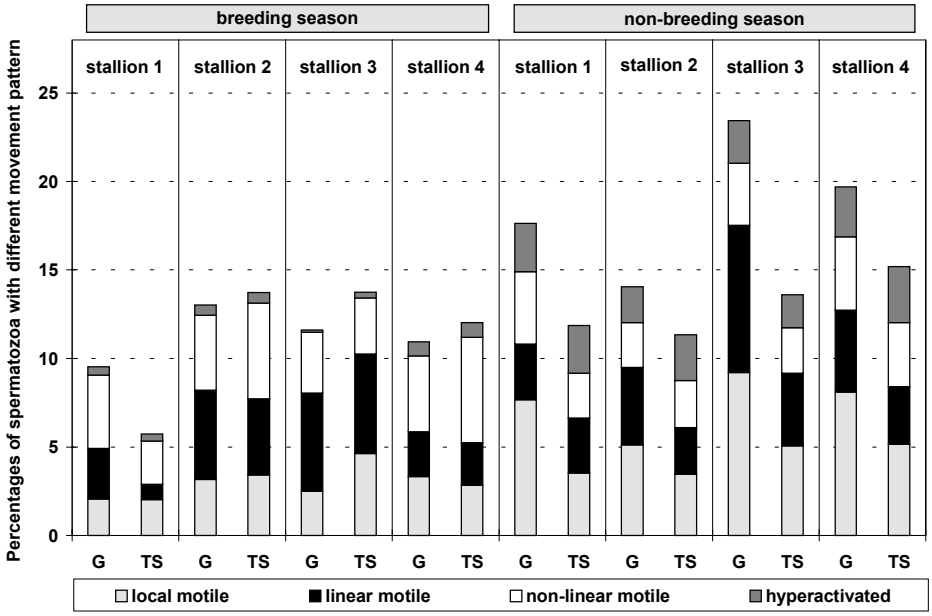

Fig. 3. Movement pattern of stallion spermatozoa after freezing/thawing (G: Gent extender, TS: Triladyl/skim milk extender) during breeding season (May) and non-breeding season (December)

Use of various extenders for cryopreservation had a minor effect on the sperm motility. After freezing in cryoextender G the percentage of linear motile spermatozoa was slightly higher than in extender TS (Fig. 3).

A significant influence of the donor on the pattern of sperm movement was observed. Figures 2 and 3 exhibit the differences in movement pattern of spermatozoa from various stallions. Semen from each stallion used is characterised by individual movement pattern of spermatozoa. Both in May and December stallion 3 reached the highest proportion of linear motile spermatozoa before freezing (48.3 ± 14.8% and 38.8 ± 12.3%, respectively, $P < 0.05$; Fig. 2) as well as after thawing (5.6 ± 3.7% and 6.2 ± 2.9; Fig. 3).

A significant correlation ($r = 0.69$; $P < 0.05$) was found between the percentage of linear motile spermatozoa before freezing and after thawing of the whole population (Fig. 4).

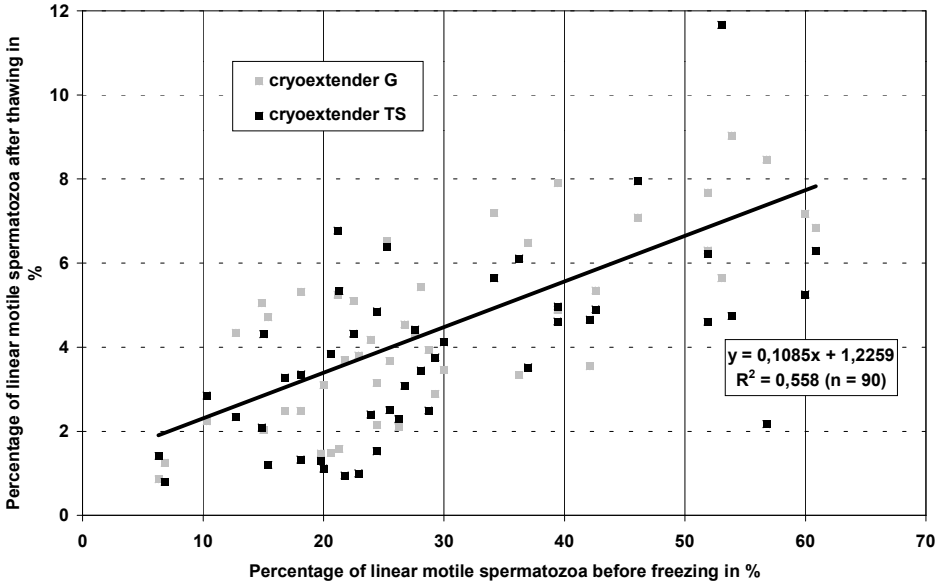

Fig. 4. Correlation between the percentages of linear motile spermatozoa before freezing and after cryopreservation

Velocity

Figure 5 shows that the mean VCL of fresh motile spermatozoa differed between May and December (119.1 ± 43.9 vs. 164.4 ± 66.4 µm/sec, respectively; $P < 0.05$). Cryopreservation and thawing of spermatozoa led to a slight increase of VCL and VSL. Mean VCL of motile cryopreserved spermatozoa differed also between May and December (151.88 ± 35.16 µm/sec vs. 175.24 ± 55.63 µm/sec, respectively, $P < 0.05$). The mean values of VCL and VSL from the spermatozoa of the four stallions are summarised in Table 1.

Main effects of extender and stallion on velocity of spermatozoa were significant for the parameter VCL ($P < 0.05$). Spermatozoa with different movement pattern differed in VCL and VSL. Hyperactivated spermatozoa from stallion 4 had the highest VCL (203.3 ± 59.9 µm/sec in May; 263.1 ± 68.6 µm/sec in December) after cryopreservation and thawing in extender TS.

In December, a significant increase of velocities (VCL and VSL) of linear motile and hyperactivated spermatozoa were established. VCL of linear motile spermatozoa increased from 101.7 ± 11.4 µm/sec in May to 148.3 ± 8.1 µm/sec in December and of hyperactivated spermatozoa from 196.1 ± 23.4 µm/sec in May to 243.3 ± 27.1 µm/sec in December ($P < 0.05$; Fig. 5).

Table 1

Mean velocities of fresh and frozen/thawed spermatozoa of individual semen donors (G: Gent extender, TS: Triladyl-skim milk extender) during breeding season (May) and non-breeding season (December) with forward motility in µm/sec (means ± SEM)

Donor	State of sperm	Breeding season		Non-breeding season	
		VCL	VSL	VCL	VSL
Stallion 1	fresh	133.5 ± 56.1	49.5 ± 20.1	169.9 ± 72.7	66.8 ± 31.1
	G	128.3 ± 60.2	55.3 ± 26.9	177.5 ± 87.7	83.0 ± 52.1
	TS	127.2 ± 51.9	48.1 ± 23.3	185.4 ± 87.3	81.3 ± 43.4
Stallion 2	fresh	106.4 ± 37.9	46.2 ± 22.9	151.9 ± 55.2	56.5 ± 32.1
	G	140.1 ± 65.0	69.7 ± 44.7	162.8 ± 89.0	76.3 ± 55.2
	TS	129.7 ± 49.2	57.8 ± 25.7	173.1 ± 83.7	77.4 ± 50.3
Stallion 3	fresh	104.7 ± 35.6	60.0 ± 20.3	163.7 ± 64.9	82.2 ± 34.8
	G	102.5 ± 46.9	62.6 ± 27.2	160.7 ± 78.9	95.1 ± 53.5
	TS	104.5 ± 53.4	64.2 ± 28.1	182.5 ± 92.6	97.1 ± 47.9
Stallion 4	fresh	131.8 ± 46.2	55.0 ± 19.8	171.9 ± 72.7	69.7 ± 33.9
	G	128.3 ± 53.7	53.7 ± 30.5	151.9 ± 75.5	74.8 ± 49.1
	TS	131.2 ± 53.0	50.7 ± 26.9	186.3 ± 84.2	84.5 ± 49.9

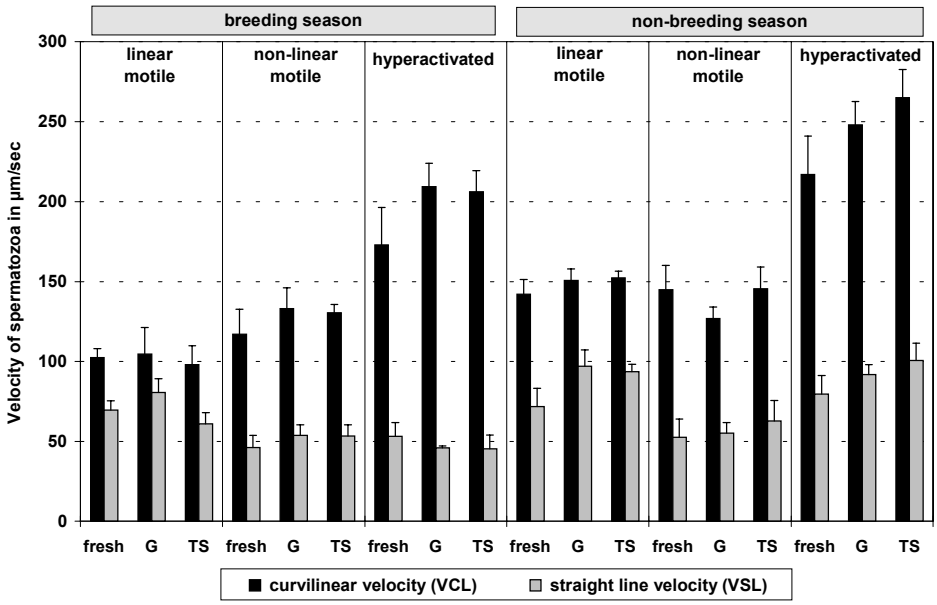

Fig. 5. Velocity of stallion spermatozoa before (fresh) and after cryopreservation (G: Gent extender, TS: Triladyl/skim milk extender) during breeding season (May) and non-breeding season (December) depending upon the movement pattern

Beat-cross frequency (BCF) and amplitude of lateral head displacement (ALH)

Figure 6 shows the negative correlation between amplitude of lateral head displacement and beat-cross frequency ($r = -0.55$; $P < 0.0001$). The resulting mean values of ALH and BCF are demonstrated in Fig. 7 for the spermatozoa categorised as linear motile, non-linear motile and hyperactivated spermatozoa. The parameters showed distinct differences between the seasons and between the different categorised spermatozoa.

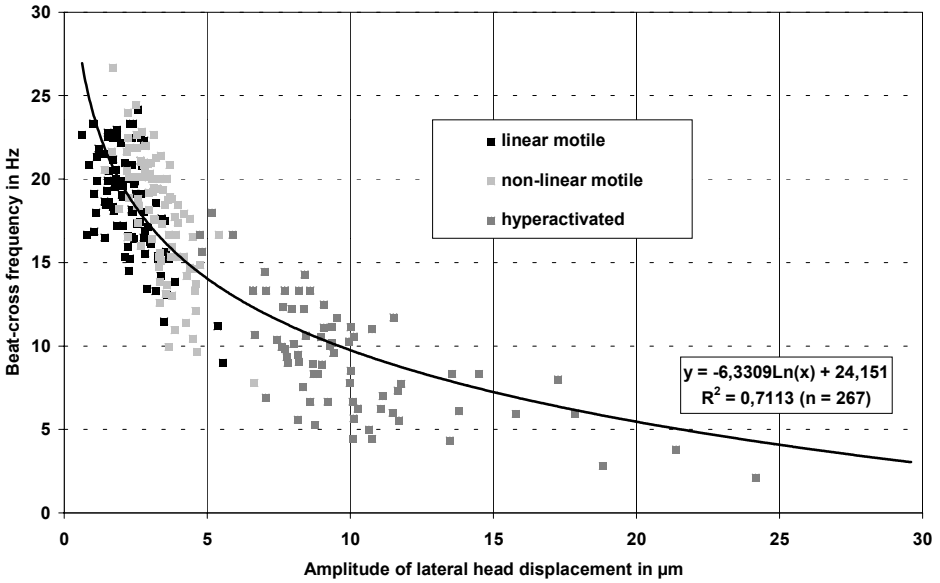

Fig. 6. Correlation between beat-cross frequency (BCF) and amplitude of lateral head displacement (ALH)

The mean value of BCF from stallion spermatozoa with linear movement pattern decreased in the non-breeding season (14.4 ± 2.0 Hz vs. 18.5 ± 2.2 Hz in May, $P < 0.05$), whereas that of ALH increased (3.9 ± 0.7µm vs. 2.3 ± 0.6 µm in May). This situation exists also for the group of non-linear spermatozoa. Hyperactivated spermatozoa are uninfluenced in their BCF, but their ALH is increased in the non-breeding season.

After cryopreservation and thawing we observed, irrespective of the different sperm groups, a slight overall rise of BCF from 12.5 ± 3.4 Hz before freezing to 14.8 ± 4.4 Hz after thawing. At the same time, the parameter ALH decreased from 6.7 ± 3.6 µm to 5.5 ± 3.7 µm.

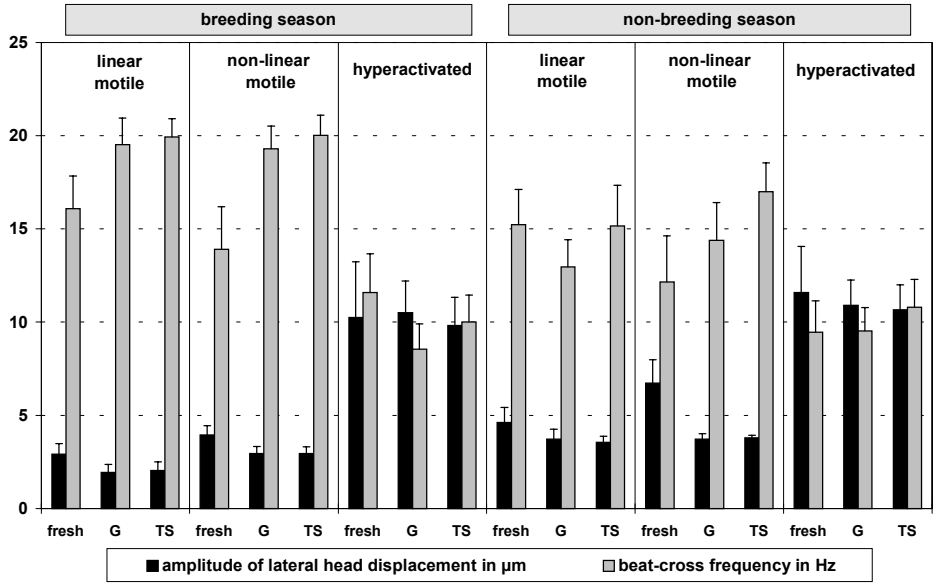

Fig. 7. Amplitude of lateral head displacement and beat-cross frequency of spermatozoa before (fresh) and after cryopreservation (G: Gent extender, TS: Triladyl/skim milk extender) during breeding season (May) and non-breeding season (December) depending upon the movement pattern

Linearity (LIN)

The degree of linearity (percentage) of linear moving spermatozoa was 53 ± 8.3% and showed no significant seasonal change. In contrast, we observed an increase of linearity after freezing and thawing to values of 62 to 68% ($P < 0.05$).

Hyperactivated spermatozoa showed the lowest degree of linearity ($P < 0.05$). Only in this group was the linearity influenced by season. In the non-breeding season an increase of linearity was observed (25 ± 4.9% in May vs. 36 ± 2.4% in December, $P < 0.05$).

Discussion

The direct comparison between percentages of motile spermatozoa estimated by CASA and by microscopic evaluation in a parallel study (Blottner et al., 2001) demonstrated substantial agreement. However, the computer-aided sperm analysis (CASA) used in this study is more objective and provides more detailed information. The obtained data show that several parameters of movement pattern are differently affected by each of the factors studied: cryopreservation, stallion and season. These results suggest that differentiated motion

analysis of stallion spermatozoa by CASA allows a better characterisation with regard to the functional aspects of motility.

The type of movement pattern seems to be relevant for fertilising competence. Gaddum-Rosse (1981) observed that only spermatozoa with linear progressive motility were able to penetrate the uterotubal junction of rats kept in organ culture, while immature sperms that swam in circles did not.

A straight swimming mode of progression, a small amplitude of lateral head displacement and a good progressive swimming speed (> 80 μm/sec) are the aspects of sperm movement *in vitro* that are the most effective in distinguishing between normal and infertile men and all appeared to be important qualities for fertilising capacity of the spermatozoa (Aitken et al., 1982). Only the method of CASA allows to distinguish exactly between linear, non-linear and hyperactivated spermatozoa.

The cryopreservation process has the most significant influence on the functional integrity of spermatozoa. This includes morphological and functional changes of stallion spermatozoa, which was shown in a parallel study (Blottner et al., 2001) and is in accordance with many reports (Amann and Pickett, 1987; Samper et al., 1991; Harkema and Boyle, 1992; Wöckener et al., 1992; Braun et al., 1994; Dobrinski et al., 1995; Vidament et al., 1997). The results of the present study show a distinct decline especially in the number of motile spermatozoa after freezing and thawing, as is expected. Analysis of sperm movement by CASA demonstrates that cryopreservation resulted in a shift from the proportions of linear to more non-linear motile and hyperactivated spermatozoa. These findings and the higher velocity of spermatozoa after cryopreservation could be a consequence of artificial, capacitation-like changes after freezing and thawing.

In our experiments we included two types of cryoextenders. In consideration of all examined parameters, the comparison of both media suggest that the composition of the extender has only little effect on the motility of stallion spermatozoa. In studies conducted by Heitland et al. (1996) VCL of stallion spermatozoa was highest when spermatozoa were frozen with skim milk egg yolk extender. This extender largely conforms to cryoextender G used in this study.

The second important factor influencing motility of spermatozoa is semen donor identity. The four stallions were not pre-selected either for high ejaculate quality or for freezability. The proportions of motile spermatozoa as well as the functional important motility categories (linearity, velocity, hyperactivation) of fresh and cryopreserved spermatozoa showed considerable individual differences between stallions. The included semen donors were characterised by a stallion-specific sperm movement pattern independent of season and cryopreservation. The differences between stallions were statistically significant. This corresponds with other cellular parameters examined in a parallel study (Blottner et al., 2001). These results are in accordance with several reports about the limited freezability of stal-

lion spermatozoa and about the donor being the major factor influencing freezability (Tischner, 1979; Samper et al., 1991; Harkema and Boyle, 1992).

The seasonal period of semen collection and preservation has also a substantial effect on the motility of stallion spermatozoa. Total proportions of motile spermatozoa as well as their velocities in fresh semen were different between May and December with higher values during the non-breeding season. However, the ratio of linear motile and hyperactivated spermatozoa is changed in fresh as well as in frozen semen in favour of hyperactivation in December. The cryopreservation of sperm during non-breeding season results in higher percentage of motile spermatozoa, but the proportions of linear and non linear spermatozoa are similar in both seasonal periods.

Magistrini et al. (1987) obtained the best freezability in winter evaluated by motility of frozen-thawed spermatozoa. One reason for the difference in the sperm movement characteristics and freezability could arise from seasonal differences in the composition and hormonal content of the seminal plasma (Johnson, 1991). Another possible cause is the seasonal difference in the efficiency of spermatogenesis.

Altogether, the post-thaw motility of frozen spermatozoa in ejaculates from non-breeding season was rather higher than in ejaculates from breeding season. This is in accordance with the earlier reported results regarding the morphological integrity of spermatozoa in comparison between both seasons (Blottner et al., 2001). These findings are of interest for practical work of cryopreservation for artificial insemination in breeding stations. They confirm the opportunity to preserve stallion semen in breeding season as well as in non-breeding season.

In conclusion, the influence of factors on different parameters of motility from stallion spermatozoa has the following rank order: cryopreservation ($P < 0.0001$) > stallion ($P < 0.001$) > season ($P < 0.05$).

Computer-aided sperm analysis, especially the estimation of the portion of linear motile spermatozoa, may be of importance in screening the suitability of stallion semen for cryopreservation and subsequent fertilising capacity. The utilisation of CASA for a better prediction of stallion fertility will now depend upon establishing the relationship between the motility parameters and the fertilising potential *in vivo* of the spermatozoa after cryopreservation in further studies.

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