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## Change in Morphology of Spermatozoa from Dismount Semen during the Breeding Season in Thoroughbred Stallions in Japan

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*To clarify the physiological changes of sperm morphology in active Thoroughbred stallions during the breeding season, we examined the dismount semen collected from the penile urethra immediately after service. The spermatozoa were analyzed for relationships between the morphology and the stallion's age or the number of services. Seasonal variation was apparent in the rate of the sperm tail abnormalities, spermatozoa with cytoplasmic droplets, appearance of medusa cells, and sperm head length. Area and width of the sperm head correlated negatively with age ( $P<0.05$ ). The rate of appearance of medusa cells and the length of the sperm head were positively related to the number of services ( $P<0.05$ ), and the aspect ratio was negatively related ( $P<0.01$ ).*

**Key words:** breeding season, spermatozoa morphology, Thoroughbred stallion

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At popular stud farms, Thoroughbred stallions with proven racetrack performance and favorable genetic attributes may service several broodmares a day during the breeding season. The quality of the sperm delivered throughout the breeding season, however, has not been fully evaluated.

The morphology of equine spermatozoa has been reported to reflect the quality [6–8, 14, 18–20]. Jasko *et al.* reported that the rate of spermatozoa with normal morphology correlated positively with the rate of fertility [11]. Especially, the increase of sperm with abnormal head morphology has been reported to be related to reduction of pregnancy rate at estrus [2, 4, 11]. Further, the sperm head has been reported to be larger in the semen taken from subfertile stallions than in that from fertile stallions [5, 9]. The converse has also been reported [12]. Differences in sperm morphology have been reported between the four seasons of the year [7, 16], but little is known about the changes in sperm morphology throughout the breeding season itself. In Thoroughbred stallions in Japan, we previously found that the morphology of the

sperm head varies during the breeding season [14].

In general, service frequency and the stallion's age influence the percentages of spermatozoa having cytoplasmic droplets or other abnormalities [1, 6, 7, 18]. Semen quality (gel-free volume, sperm concentration, total sperm count, sperm abnormalities) has been reported to be the poorest in stallions under 3 years and over 11 years of age [6]. In an early study of Thoroughbred stallions that ejaculated five times an hour, however, in spite of decreased volume of total semen, the ejaculates showed no variation in sperm concentration or motility [19]. More recently, length of the interval between services has been shown to change the semen quality [7]. These reports, however, were based on studies performed under temporary conditions and extreme experimental design. Possible relationships between the morphology of spermatozoa and the number of services or the age of the stallion remain undetermined.

Thoroughbred stallion must perform natural service because the rules of horseracing prohibit artificial insemination. Consequently, the whole ejaculate of the active Thoroughbred stallions is rarely collected for evaluation during the breeding season. An understanding of the possible variations in

spermatozoa throughout the season is important, however, for estimating the quality of the sperm and scheduling the services of individual stallions. Gravance *et al.* have reported that the dimensions of the sperm head in dismount semen do not differ from those of the complete ejaculate, and those authors concluded that dismount semen is representative of complete ejaculate [8]. This suggests that using dismount semen would allow collection of semen samples throughout the breeding season. Examination of spermatozoa morphology in dismount semen may elucidate the physiological changes of the sperm during the heavily booked breeding season.

The purpose of this study was to clarify the physiological changes in sperm morphology in dismount semen of Thoroughbred stallions servicing daily during the breeding season. To identify the factors affecting such changes, we analyzed the morphology of the spermatozoa in relation to the stallion's age and the number of services the stallion that performed throughout two consecutive breeding seasons.

The study included 16 active Thoroughbred stallions in Hokkaido in 2001 and 14 in 2002. Eleven stallions were used in both years. The stallions ranged from 4 to 15 years of age in 2001 (mean  $\pm$  SD,  $8.9 \pm 3.2$ ) and from 4 to 12 years in 2002 ( $8.1 \pm 2.5$ ), and the number of services in stallions ranged from 69 to 412 ( $215 \pm 104$ ) from February to July in 2001 and from 68 to 363 ( $241 \pm 102$ ) from February to July in 2002. Samples were collected from the penile urethra immediately after services every week during the breeding season, from February to the beginning of July, on a stud farm in Hokkaido, Japan. Semen was smeared on the glass slide, then air-dried and kept at room temperature until fixing with 100% ethanol and staining with hematoxylin and eosin stain, and each slide preparation was completed with mounting medium and cover glass.

Abnormalities in the sperm head and tail; spermatozoa with cytoplasmic droplets; the appearance of medusa cells in the semen; and the area, length, width and aspect ratio (width/length) of the sperm head were investigated according to Koyago *et al.* [14]. Medusa cells were judged positive if two or more were found in 100 spermatozoa.

To determine when a change occurred during the breeding season, the services of each stallion that the time to begin and finish service was different were grouped into three periods: (1) early, (2) middle, and (3) late. The first 5 weeks from the start of service in

each stallion were designated the early period, the middle 5 weeks constituted the middle period, and the late 5 weeks before the service ended constituted the late period. In 2001, the mean numbers of services per week was  $5.2 \pm 5.0$  (early),  $15.3 \pm 6.6$  (middle), and  $7.2 \pm 4.8$  (late); and in 2002, the mean number of services per week was  $5.3 \pm 4.8$  (early),  $16.9 \pm 7.3$  (middle), and  $9.5 \pm 5.6$  (late).

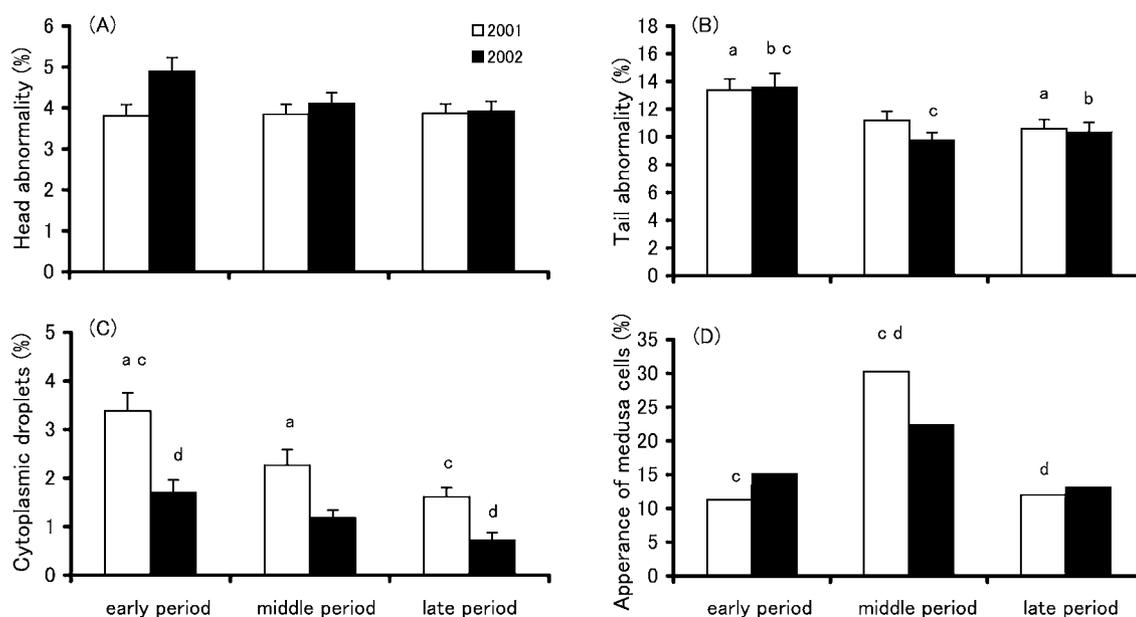
Statistical importance was assessed by one-way analysis of variance (ANOVA), averages were compared with Tukey-Kramer's post hoc test, and relationships were determined with the Pearson's correlation coefficient test. The presence and absence of medusa cells were compared by  $\chi^2$  test.  $P < 0.05$  was considered significant.

Sperm tail abnormalities tended to be high in February both years ( $15.8 \pm 8.1\%$  in 2001;  $18.3 \pm 9.6\%$  in 2002) and to decrease by March ( $12.2 \pm 5.8\%$  in 2001;  $12.4 \pm 6.0\%$  in 2002). No medusa cells appeared in the semen at the beginning of the breeding season but began to appear in March, and the number peaked in April, then tapered to July both years.

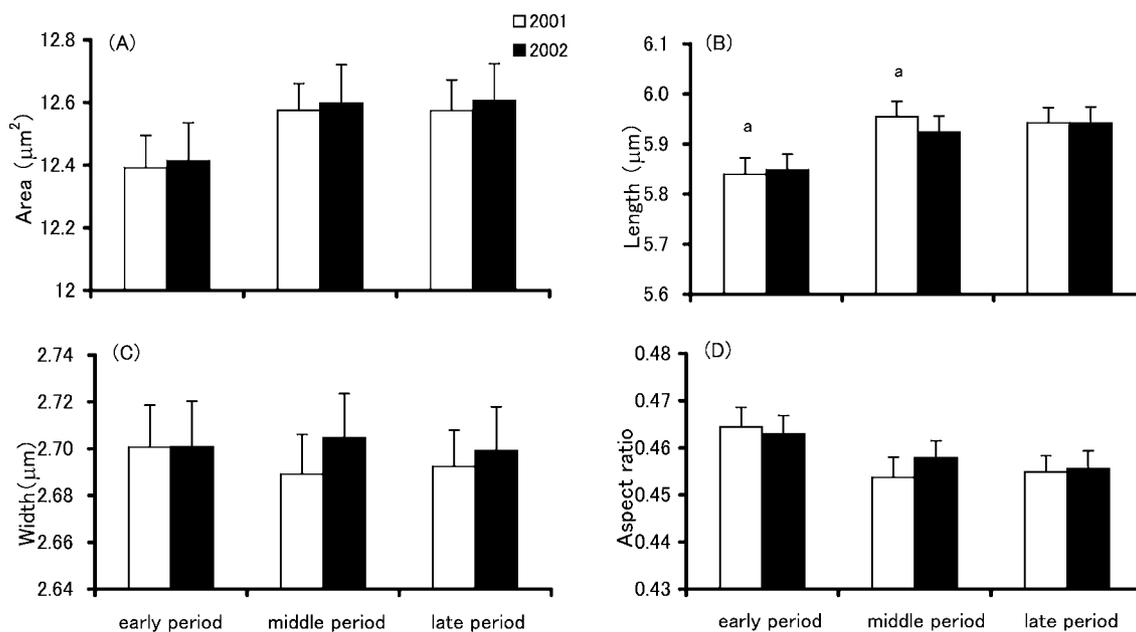
In sperm head abnormalities, no difference was noted among the three periods (Fig. 1A). The percentage of sperm tail abnormalities, however, was significantly higher in the early period than in the late period of 2001 ( $P < 0.05$ ) and higher in the early period than either the middle ( $P < 0.01$ ) or late ( $P < 0.05$ ) periods of 2002 (Fig. 1B). In 2001, the rate of spermatozoa with cytoplasmic droplets was significantly greater in the early breeding period than in the middle ( $P < 0.05$ ) and late ( $P < 0.01$ ) periods and was also greater than in the late period of 2002 ( $P < 0.01$ , Fig. 1C). However, the rate of medusa cells was significantly higher in the middle period than in the early and late periods of 2001 ( $P < 0.01$ , Fig. 1D).

Area and width of the sperm head showed no difference among the three periods (Fig. 2A, C). In 2001, the length of the sperm head increased significantly during the middle period in comparison with that of the early period ( $P < 0.05$ ); and in 2002, the length of the sperm head in both the middle and late periods tended to be greater than in the early period 2002 (Fig. 2B). In both years the aspect ratio in the middle and late periods tended to be lower than that in the early period (Fig. 2D).

Neither the number of services nor age of the stallion showed any correlation with sperm head and tail abnormalities or with spermatozoa with cytoplasmic droplets (Table 1). The number of services, however, had a positive correlation with the appearance of



**Fig. 1.** Sperm characteristics in Thoroughbred stallions during the beginning, middle, and end of the breeding season. Percentage of abnormalities in the sperm head (A) and sperm tail (B), spermatozoa with cytoplasmic droplets (C) and appearance of medusa cells in the semen (D) in 2001 and 2002. Early period was the first 5 weeks, middle period was the middle 5 weeks and late period was the last 5 weeks of the breeding season. Values are expressed as the means  $\pm$  SEM. The same letters (a–d) indicate significant difference (a, b:  $P < 0.05$ ; c, d:  $P < 0.01$ ).



**Fig. 2.** Dimension of sperm head in Thoroughbred stallions during the beginning, middle, and end of the breeding season. Area (A), length (B), width (C) and aspect ratio (D) of sperm head in 2001 and 2002. Early period was the first 5 weeks, middle period was the middle 5 weeks and late period was the last 5 weeks of the breeding season. Values are expressed as the means  $\pm$  SEM. The same letter (a) indicates significant difference ( $P < 0.05$ ).

**Table 1.** Correlation between each feature of the sperm and the number of services or age of the stallion

Sperm Features	Number of Services	Age of Stallion
Head abnormality	0.20	0.07
Tail abnormality	0.03	-0.11
Cytoplasmic droplets	-0.03	-0.04
Medusa cells	0.52**	0.05
Head area	0.20	-0.45*
Head length	0.53**	-0.26
Head width	-0.18	-0.37*
Aspect ratio <sup>a</sup>	-0.48**	-0.12

\*\* P<0.01, \*P<0.05, <sup>a</sup>: Width / Length.

medusa cells and with the length of the sperm head, and a negative correlation with the aspect ratio (P<0.01). The stallion's age had a significant negative correlation with the area and width of the sperm head (P<0.05).

Overall, in Thoroughbred racing stallions engaged in heavily booked servicing of brood mares during the breeding season in Japan, this study documents three major findings. First, the area and width of the sperm head becomes smaller with the increasing age of the stallions. Second, the length of the sperm head and the appearance rate of medusa cells increases with the increasing number of service. Third, the aspect ratio decreases with the increasing number of services.

The sperm head abnormalities showed no relation to either the age of the stallion or the number of services. Furthermore, no relation was indicated between the sperm head abnormalities and the early, middle, or late period of the breeding season. This result conflict with a report that sperm head abnormality was minimal in the summer in Australia (from December through February in the Southern Hemisphere) [7]. Our study (from February to the beginning of July in the Northern Hemisphere) did not include the height of Hokkaido summer, i.e., the period from mid-July through August, because the breeding season ends before that. The Australian study [7] raises the question whether adjusting the official breeding season in Hokkaido to include the summer would be productive or counterproductive. As the sperm head plays a vital role in reproduction, further study is warranted on possible seasonal influence. In our study, sperm tail abnormality was more prevalent in the early breeding period than in the middle and late periods. Accordingly, abnormal spermatozoa have been reported to be more prevalent during the non-breeding season than during the

breeding season [7, 20]. Whether this phenomenon is related to the season is not known.

In our study, the rate of spermatozoa with cytoplasmic droplets was higher in the early breeding period than in the middle and late periods. Dowsett and Knott, however, reported that the droplets appeared less frequently during summer or during a period of high frequency service allowing a mere 1-to-6-hr interval between services [7]. In stallions with low concentrations of peripheral testosterone, GnRH immunization increases the cytoplasmic droplets in the spermatozoa [15]. In boars, fructose has stimulated the shedding of cytoplasmic droplets originating from seminal vesicular fluid [10]. As the early period marks the transition from the non-breeding season to the opening of the breeding season, concentrations of LH, FSH and testosterone are low at that time [17]. The reason the droplets appeared on many spermatozoa in the early period of our study may be that the function of the accessory genital gland, particularly the seminal vesicle, was still insufficient, as in the off-season.

In the horse, the rate of medusa cells is generally greater than that in bulls, in which the ratio is about one per 10,000 spermatozoa [3]. In the present study, the rate of medusa cells increased significantly during the middle period and had a significantly positive correlation with the number of services. These results show that stallions with a large number of services throughout the breeding season are susceptible to a high rate of medusa cells appearing in the sperm. This principle is further supported by the fact that the rate was highest during the middle of the breeding period when the stallions were especially engaged in high-frequency services.

The area and width of the sperm head had a significantly negative correlation with the stallion's age. The area and width of the sperm head in individual stallions decreased in 72.7% (8 out of 11) of the stallions from 2001 to 2002 (data not shown). In boars older than 18 months, the area of the sperm head has been reported to be greater than that of boars under 14 months of age, although the length and width do not change [13]. The area of the sperm head of buffalo older than 109 months (-9 years) is smaller with the progress of age [12]. Length of sperm head significantly positively correlated with the number of service, and the aspect ratio was negatively. Therefore, the sperm head might become longer in the middle breeding season with a great number of services. In the early breeding season, the stallions had a small number

of services and could sufficiently afford the time to the spermatogenesis. In the middle breeding season, they got a great number of services and had to activate to the spermatogenesis. The process of the spermatogenesis might be smoothed by changing longer the length of sperm head when stallion has to serve at high frequent intervals. Given that the stallions provided more services during the middle period than the early period, a large number of services may cause a lengthening of the sperm head, thus resulting in lower aspect ratio mid-season.

In summary, this study of dismount semen of active Thoroughbred stallions during the breeding season indicates variation in the sperm morphology, particularly spermatozoa with cytoplasmic droplets, the appearance of medusa cells in the semen, and changes in the sperm head dimensions. These abnormalities increase with the age of the stallion and with the number of services performed. Taken together, the results of the study shed new light on the physiological changes in the spermatozoa throughout the breeding season in popular racehorses booked with large numbers of services per breeding season.

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