Journal of Reproduction and Development, Vol. 60, No 2, 2014

—Original Article—

Relationships Between the Appearances and Changes of Estrous Signs and the Estradiol-17β Peak, Luteinizing Hormone Surge and Ovulation During the Periovulatory Period in Lactating Dairy Cows Kept in Tie-stalls

Toshiaki SUMIYOSHI[1, 2], Tomomi TANAKA[1, 2] and Hideo KAMOMAE[1, 2]

[1)]*Laboratory of Veterinary Reproduction, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan*
[2)]*United Graduate School of Veterinary Science, Gifu University, Gifu 501-1193, Japan*

Abstract. Lactating Holstein-Friesian cows kept in tie-stall barn were used as subjects in this study. Rectal examination, ultrasonography and blood sampling were conducted every other day and then daily after the day on which diameter of the corpus luteum decreased. After the luteal diameter decreased for 2 consecutive days, rectal and ultrasound examinations, blood sampling, and observation of estrous signs were conducted at 6-h intervals. Most of the estrous signs became obvious with the increase in estradiol-17β (E_2) and became most remarkable 24 to 30 hours before ovulation, at which point the E_2 peak and luteinizing hormone (LH) surge were achieved, and then weakened which progression to ovulation. The correlation between the intensity of four estrous signs (hyperemia and swelling of the intravaginal part of the uterus, opening of the external uterine orifice and viscosity of the cervical mucus) and the plasma E_2 concentration was higher than that of three estrous signs (swelling of the vulva, contraction of the uterus, diameter of uterine horn) and the plasma E_2 concentration. The relaxation of the intravaginal part of the uterus showed a unique change compared with the other estrous signs, and it became most obvious 6, 12 and 18 h before ovulation; this obviously relaxed period was consistent with the generally accepted theoretical optimal time for artificial insemination (AI), i.e., 6 to 24 h after initiation of estrus. These results suggest that observation of estrous signs by vaginoscopic examination gave useful information for detection of the optimal timing of AI in the periovulatory period in lactating dairy cows kept in a tie-stall barn.

Key words: Dairy cows, Estradiol-17β peak, Estrous signs, LH surge, Ovulation

(**J. Reprod. Dev. 60:** 106–114, 2014)

The milk productivity of the dairy cow has been increased rapidly over the past half century through genetic improvement and development of nutritional management. In Japan, the average 305-day milk production per cow was 5,826 kg in 1975, and this was increased to 7,798 kg by 1999 and 9,225 kg by 2011 [1]. On the other hand, the reproductivity of dairy cows has been decreasing throughout the world [2–4]. In Japan, the average calving interval of a dairy cow was 403 days in 1975, and by 2011, the interval had lengthened to 438 days [1]. The causes and factors of this decrease in reproductivity are considered not only to be abnormalities of the genital tract such as the uterus and ovary but also the periparturient diseases of lameness, mastitis, nutrition, milk yield, stress and so on. It has recently been reported that one of the main factors is that estrus and estrous signs in dairy cows have weakened and the duration of estrus in dairy cows has shortened [5]. These indistinct estrus and estrous signs and the shortened duration of estrus may cause the proper timing of artificial insemination (AI), to be missed, resulting in poor reproductivity.

In general, standing to be mounted, which is called standing estrus, is the most reliable indicator of estrus [3, 6–9]. It is important to observe standing estrus to determine a suitable time for insemination. The guide generally applied for the timing of AI is also based on the observation of standing estrus [10]. Several methods such as a heatmount detector, tail paint and chin-ball have been used to facilitate the detection of standing estrus [7, 9, 11–13]. Moreover, there are some reports indicating a way to detect estrous signs linked with estrus in dairy cows. Many studies have been carried out on measuring the increase in the activity of cows around estrus by using a pedometer, and this is being used and developed in practice [13–20]. In addition some methods to predict estrus by monitoring internal changes such as body temperature [20–22], vaginal mucus pH [23], electrical resistance or conductivity of reproductive tissue and their secretions [7, 21, 24–30] have been reported. However, neither method has become generally applied.

Due to the difficulties in observing standing behavior in tie-stall barns, the optimal time for AI is judged generally based on other estrous signs such as restlessness, hyperemia and swelling of the vulva, mucus discharges from the vulva, follicle and corpus luteum (CL) dynamics of the ovary and contraction of the uterus by rectal examination. However, a reliable index to judge the optimal time for AI has not yet been established. In addition, the indicated weakened and indistinct estrous signs may make it difficult to perform AI at the optimal time in tie-stall barns. For cows kept in tie stalls, a method of measuring the increase in a cow's activity around estrus by using a pedometer is also being examined. For example, Sakaguchi *et al.* [16] investigated the increase in the activity of cows by using

Received: November 5, 2013
Accepted: December 5, 2013
Published online in J-STAGE: February 1, 2014

Correspondence: H Kamomae (e-mail: kamomae@cc.tuat.ac.jp)

a pedometry system attached to the neck, a front leg and a hind leg in dairy heifers housed in a tie-stall barn and reported that the efficiencies and accuracies of each pedometer were 92 and 34% for the neck, 78 and 78% for the front leg and 87 and 83% for the hind leg, respectively. However, there are few reports regarding the use of pedometers for dairy cows housed in tie-stall barns.

From such a background, it is important to fully understand the expression and characteristics of each estrous sign for dairy cows kept in tie-stall barns. However, the relations between the expression and elimination of these estrous signs and blood estrogen dynamics, the luteinizing hormone (LH) surge and ovulation are not clear. Yoshida *et al.* [5] reported that the duration of standing estrus was substantially shortened and that more than one-third of cows did not show standing estrus, in an investigation of the duration and expression of standing estrus and secondary estrous signs such as sexual activity and external genital change. In their report, however, the duration of secondary estrous signs before and after standing estrus was not reduced. In addition, temporal changes in each estrous signs from luteolysis to ovulation have not been well examined in relation to preovulatory hormonal profiles in any previous reports.

Therefore, the objectives of the present study were to investigate the temporal changes in estrous signs and plasma profiles of ovarian steroids and luteinizing hormone in relation to luteolysis and ovulation in dairy cows kept in tie-stall barns, especially, the relationships between the estrous signs and the estrogen peak, LH surge and ovulation.

Materials and Methods

Animals and management

This study was carried out between November 2009 and March 2012 at two tie-stall style farms located in Fujisawa, Kanagawa, Japan. Data were collected from ten Holstein-Friesian cows (7 at Farm A and 3 at Farm B). At the beginning of the study, the average (mean ± SD) age (year), parity (times) and time after parturition (day) of the cows were 3.8 ± 0.9, 2.3 ± 0.8 and 126.6 ± 53.0, respectively. Their average body condition score based on a five-point scale [31] was 2.6 ± 0.2. They were milked twice a day, and the average milk yield per lactation (305 days) was approximately 9,000 kg per cow at both farms. All of the cows were fed Total Mixed Ration (TMR) according to the Japanese feeding standards for dairy cattle (2006). These cows were more than 60 days postpartum and had no genital abnormality and normal estrous cycles before the study. AI was not practiced during this study. All experimental procedures were approved by the University Committee for the Use and Care of Animals of Tokyo University of Agriculture and Technology (No. 25-36).

Experimental procedure

Cows were examined daily before the study by rectal examination, and the experiment was conducted from the beginning (day 0: day of ovulation) to the end of the cycle (the subsequent ovulation). For analysis of uterine changes, follicular and luteal development and ovarian hormones, rectal examination, ultrasonography and blood sampling were conducted every other day and then daily after the day when the diameter of the CL decreased until the following ovulation. The second of two days on which the diameter of the CL decreased consecutively was regarded as the day of initiation of luteolysis. Beginning on the day of initiation of luteolysis, pedometer measurements, measurements of electrical resistance and pH of vaginal mucus, observation of vulval and vaginal estrous signs, rectal examination, ultrasonography and blood sampling were conducted at 6-h intervals until ovulation.

Pedometers

For examining the change in pedometric activity from initiation of luteolysis to ovulation, pedometers were attached to the right front leg just above the antebrachiocarpal joint. For attachment of a pedometer, a flexible bandage (3M^{TM} Vetrap^{TM}, Sumitomo 3M, Tokyo, Japan) was wound up to the proximal part of the antebrachiocarpal joint, and the pedometer was fixed to the superior border of the bandage with a clip. Two types of pedometers (Health Counter HJ-151, Omron Healthcare, Kyoto, Japan, and MANPO MK-365 Yamasa Tokei Keiki, Tokyo, Japan) for humans, which have different sensitivities, were used. Measurement of the step counts was carried out firstly in the examinations, and the displayed numerical value was recorded for accumulation of the number of steps.

Observation of estrous signs for the vulva and electrical measurement of vaginal mucus

First, hyperemia and swelling of the vulva and mucus discharges from the vulva were observed. Then measurement of electrical resistance of cervical mucus was examined. After cleaning and sterilizing the vulva, the electronic probe of a cervical mucus meter (Fujihira Industry, Tokyo, Japan) was inserted in the vagina deeply until it reached the bottom of the vagina right under the external uterine orifice, where cervical mucus was retained. The displayed numerical value was recorded.

Observation of estrous signs in the vagina (around the cervical uteri)

A Vaginoscope sterilized with antiseptic solution was inserted into the vagina to observe four estrous signs: hyperemia, swelling, relaxation of the intravaginal part of the uterus and opening of the external uterine orifice. After that, vaginal mucus around the external uterine orifice was collected aseptically by sterilized tampon, and the pH was measured with a pH meter (SK-632PH, Sato Keiryoki Mfg, Tokyo, Japan) immediately; the viscosity of the mucus was also measured.

Observation of the uterus and the ovary

After observation of estrous signs in the vulva, electrical measurement of vaginal mucus, vaginoscopic examination and measurement of the pH and viscosity of cervical mucus, the uterus and the ovaries were observed by rectal examination and ultrasonography with regard to changes in three items for the uterus: contraction of the uterus, diameters of the uterine horn by rectal examination and degree of accumulation of fluid secreted into the uterus by ultrasonography.

Ultrasonography

A B-mode ultrasound scanner (Tringa-V 50S, Esaote Pie Medical B.V., Maastricht, Netherlands) equipped with a 5.0-MHz linear array probe was used for ultrasound examination. The maximal areas of

Table 1. Scoring scale for observed estrous signs

Regions	Estrous signs	Scaling
Vulva	· Hyperemia	0 = no signs, 1 = moderate signs, 2 = obvious signs
	· Swelling	0 = no signs, 1 = moderate signs, 2 = obvious signs
	· Mucus discharge from vulva	0 = not present, 1 = present
Intravaginal part of the uterus	· Hyperemia	0 = no signs, 1 = moderate signs, 2 = obvious signs
	· Swelling	0 = no signs, 1 = moderate signs, 2 = obvious signs
	· External uterine orifice	0 = closing, 1 = opening, 2 = conspicuous opening
	· Relaxation	0 = no signs, 1 = moderate signs, 2 = obvious signs
	· Viscosity of the mucus	0 = starchy, 1 = sticky, 2 = watery
Uterus	· Contraction	0 = no signs, 1 = moderate signs, 2 = obvious signs
	· Diameter of uterine horn [a)]	0 = under 1.5 fingerbreadths, 1 = 1.5–2 fingerbreadths, 2 = 2 fingerbreadths and over
	· Accumulation of fluid secreted into uterus	0 = not present, 1 = present, 2 = present in large quantities

[a)] While relaxed.

all follicles and CLs that were greater than 6 mm in diameter were recorded based on three cross-sectional images. The diameters (mm) of the follicles and CLs were calculated as the mean lengths across the major and minor axes [32].

Blood sampling

For analysis of progesterone (P_4), estradiol-17β (E_2), LH and follicle-stimulating hormone (FSH) concentrations, 10 ml blood samples were taken by coccygeal venipuncture into heparinized vacutainers (Venoject II, Terumo, Tokyo, Japan). Plasma was separated by centrifugation at 3,000 rpm for 30 min immediately after the blood collection and frozen at –20 C until the hormone assay.

Scoring of the estrous signs

The 11 observed estrous signs were graded into three ranks (0, 1, 2) or two ranks (0, 1) by those obviousness or strength (Table 1).

Hormone assays

The plasma concentration of P_4 was measured by EIA [33]. The intra- and interassay coefficients of variation were 2.6% (3.4, 1.1 ng/ml) and 4.0% (3.5, 0.9 ng/ml), and the sensitivity was 0.2 ng/ml. The plasma concentrations of E_2 [34], LH and FSH [35] were measured by RIA. The intra- and interassay coefficients of variation and sensitivity were 10.3% (10.6, 2.6 pg/ml), 13.2% (10.2, 1.8 pg/ml) and 1.11 pg/ml for E_2, 4.2% (0.8, 8.8 ng/ml), 9.9% (0.6, 11.6 ng/ml) and 0.18 ng/ml for LH and 8.6% (3.1, 0.4 ng/ml), 15.0% (3.3, 1.7 ng/ml) and 0.12 ng/ml for FSH, respectively.

Statistical analysis

Measured or scored values are presented as means ± SD. The relationship between the scores for the estrous signs and plasma concentration of E_2 was determined by Spearman correlation coefficient analysis. Differences with $P<0.05$ were considered significant.

Results

Luteal and follicular dynamics and the profiles of plasma ovarian steroids, LH and FSH during the estrous cycles

The average length of the examined estrous cycles from ovulation (day 0) to subsequent ovulation of the 10 cows was 23.0 ± 2.1 days. The diameter of the CL increased and reached the maximum of around 25 mm on day 10 and then maintained this size until around day 18. The CL began to regress at 18.7 ± 1.9 days after ovulation and degenerated to 13.2 ± 1.4 mm in diameter on the day of the subsequent ovulation. The plasma concentration of P_4 increased in accordance with luteal development, reached 2.6 ± 0.7 ng/ml on day 6 and then maintained that level until around day 18, showing a peak of 5.3 ± 2.0 ng/ml on day 14. After the start of regression of the CL, the P_4 level decreased quickly and was low, being 0.6 ± 0.3 ng/ml on the day of the subsequent ovulation. Two of the 10 cows had three follicular waves, and the remaining 8 cows had two follicular waves. Following the start of CL degeneration, ovulatory follicular development and maturation and the profiles of the E_2 concentration were almost the same in cows with three follicular waves and two follicular waves. The diameters of the ovulatory follicles increased and reached a maximum of around 16 mm on the day before ovulation, and concomitant with follicular maturation, the E_2 levels increased gradually and reached a maximum 1 or 2 days before ovulation. The plasma LH concentration remained low, at around 1.0 ng/ml, from day 0 to day 21 and then showed high levels of around 7.0 ng/ml on day 22. The FSH concentration was under 1.0 ng/ml throughout the estrous cycle examined.

Periovulatory changes in the CL, follicle and plasma ovarian steroids, LH and FSH

Luteolysis started 18.7 ± 1.9 days after ovulation, and thereafter, the CL diameter decreased gradually and became 13.2 ± 1.4 mm on the day of the next ovulation, which occurred 4.3 ± 0.9 days after luteolysis (Fig. 1). The plasma concentration of P_4 began to decrease 0.5 days before luteolysis or around 112 h before the subsequent ovulation, became low (0.7 ± 0.4 ng/ml) around 48 h before the subsequent ovulation and remained at that low value until the subsequent ovulation. The diameter of the ovulatory follicle, which was around 14 mm on the day of initiation of CL degeneration, increased gradually and reached 16.5 ± 2.5 mm on the day before the subsequent ovulation, which occurred 3.5 ± 1.7 days after luteolysis. After that, the diameter of the follicle was maintained until ovulation. The plasma concentration of E_2 began to increase in

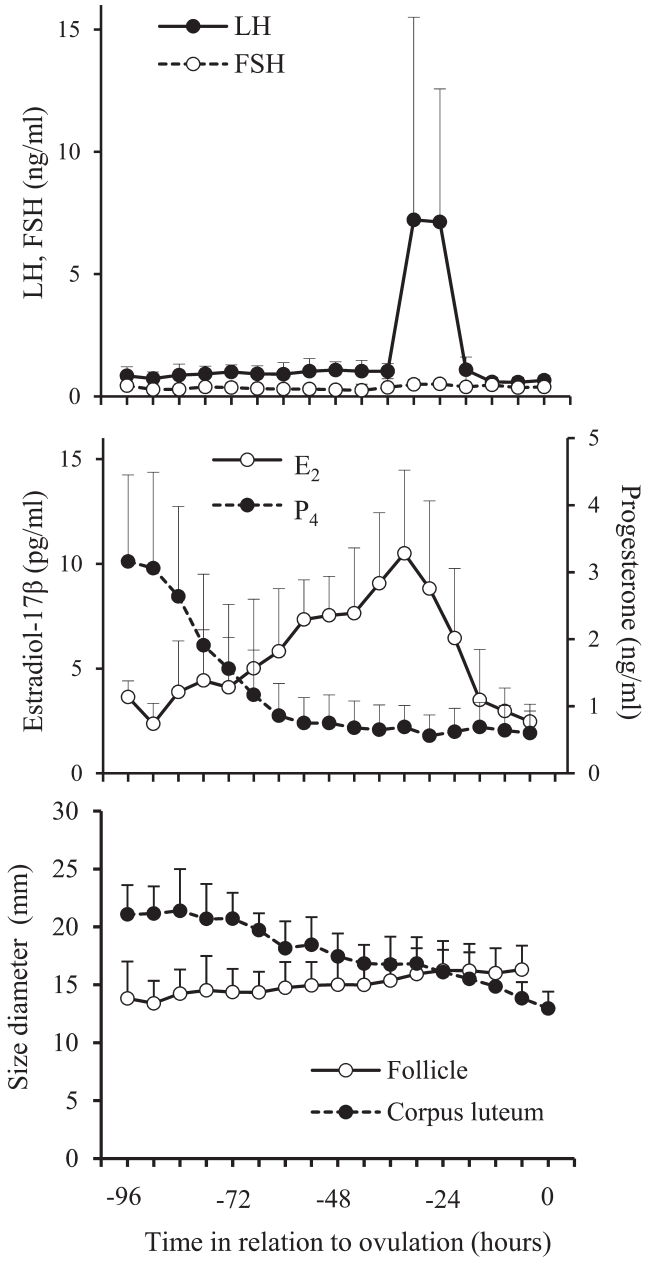

Fig. 1. Changes in diameter of the follicle and corpus luteum and plasma concentrations of progesterone, estradiol-17β, LH and FSH from 96 hours before to the time of ovulation. All data were normalized to the time of ovulation (0 h), and values are means ± SDs.

inverse proportion to the decrease of P_4 concentration and reached a peak of 10.5 ± 4.0 pg/ml 30 hours before the subsequent ovulation. Thereafter, the E_2 concentration decreased sharply and reached a low value of around 3.0 pg/ml 6–12 h before the subsequent ovulation. The LH concentration was low, around 1.0 ng/ml, for 48 hours after the P_4 level began to decrease and for 36 hours after the initiation of luteolysis. After that, the LH concentration abruptly increased in all cows and showed high levels of 7.2 ± 8.3 ng/ml and 7.1 ± 5.4 ng/ml at 30 hours and 24 h before the subsequent ovulation, respectively. Thereafter, the LH concentration decreased a low level, around 1.0 ng/ml, again at 18 h before the subsequent ovulation. The plasma FSH concentration was almost low and showed no obvious change except for a small increase (0.5 ng/ml) noticed when the LH concentration increased rapidly. The mean peak concentrations of E_2 and LH in individual cows were 12.4 ± 3.2 and 11.5 ± 7.2 ng/ml, respectively, and the intervals from the peak concentrations of E_2 and LH to the subsequent ovulation were 34.6 ± 8.7 and 26.4 ± 3.1 h, respectively.

Changes in the estrous signs during the periovulatory period

In general, the scores for most of the estrous signs increased in accordance with an elevation in the plasma concentration of E_2 and showed a tendency to decrease after the peak concentration towards ovulation (Fig. 2). However, the estrous signs did not change during the periovulatory period in the same manner. The scores for swelling of the vulva and the vaginal part of the cervix and opening of the external uterine orifice increased in accordance with the elevation of the E_2 concentration and then diminished with the decrease in the E_2 concentration after the E_2 peak (Fig. 2C, E and F). The scores for the diameters of the uterine horn and viscosity of the cervical mucus increased with the increase in the E_2 concentration to the peak of E_2, and after the peak concentration of E_2, the scores for the diameter of the uterine horn remained high points until ovulation; the scores for the viscosity of the mucus decreased at ovulation (Fig. 2H and I). The scores for hyperemia of the intravaginal part of the uterus and contraction of the uterus increased with elevation of the E_2 level and reached a plateau before the E_2 peak. The hyperemia of the intravaginal part of the uterus started to diminish after the E_2 peak. The contraction of the uterus remained at the plateau until ovulation (Fig. 2D and J). The scores for relaxation of the intravaginal part of the uterus exhibited extraordinary changes compared with other estrous signs. Namely, the scores started to increase from 12 h before the E_2 peak, reached the maximum 12 h after the E_2 peak, remained at the maximum until 6 h before the subsequent ovulation (for 12 h) and decreased at the subsequent ovulation (Fig. 2G). The scores for the degree of fluid secreted into the uterus started to increase halfway into the increase in E_2, stopped increasing 12 h before the E_2 peak and maintained a plateau just before ovulation (Fig. 2K). The changes in hyperemia of the vulva were obscure (Fig. 2B), and no useful observations were made regarding the scores for mucus discharge from the vulva (Fig. 2A).

Considering the changes in all estrous signs, relationships between the scores for estrous signs, which included swelling of the vulva, contraction of the uterus, diameter of the uterine horn when relaxed, hyperemia, swelling and relaxation of the intravaginal part of the uterus, opening of the external uterine orifice and viscosity of the cervical mucus, and the concentrations of E_2 peak and the LH surge were examined. Three estrous signs, hyperemia of the vulva, mucus discharge from the vulva and accumulation of fluid secreted into the uterus, were excluded because of indistinctness, inconstancy and necessity of ultrasound equipment, respectively. The average scores for the three of estrous signs in total regarding the change in the vulva and in the uterus by rectal examination (i.e., swelling of the vulva, contraction of the uterus and diameter of uterine horn when relaxed) and for the four estrous signs in total regarding the changes

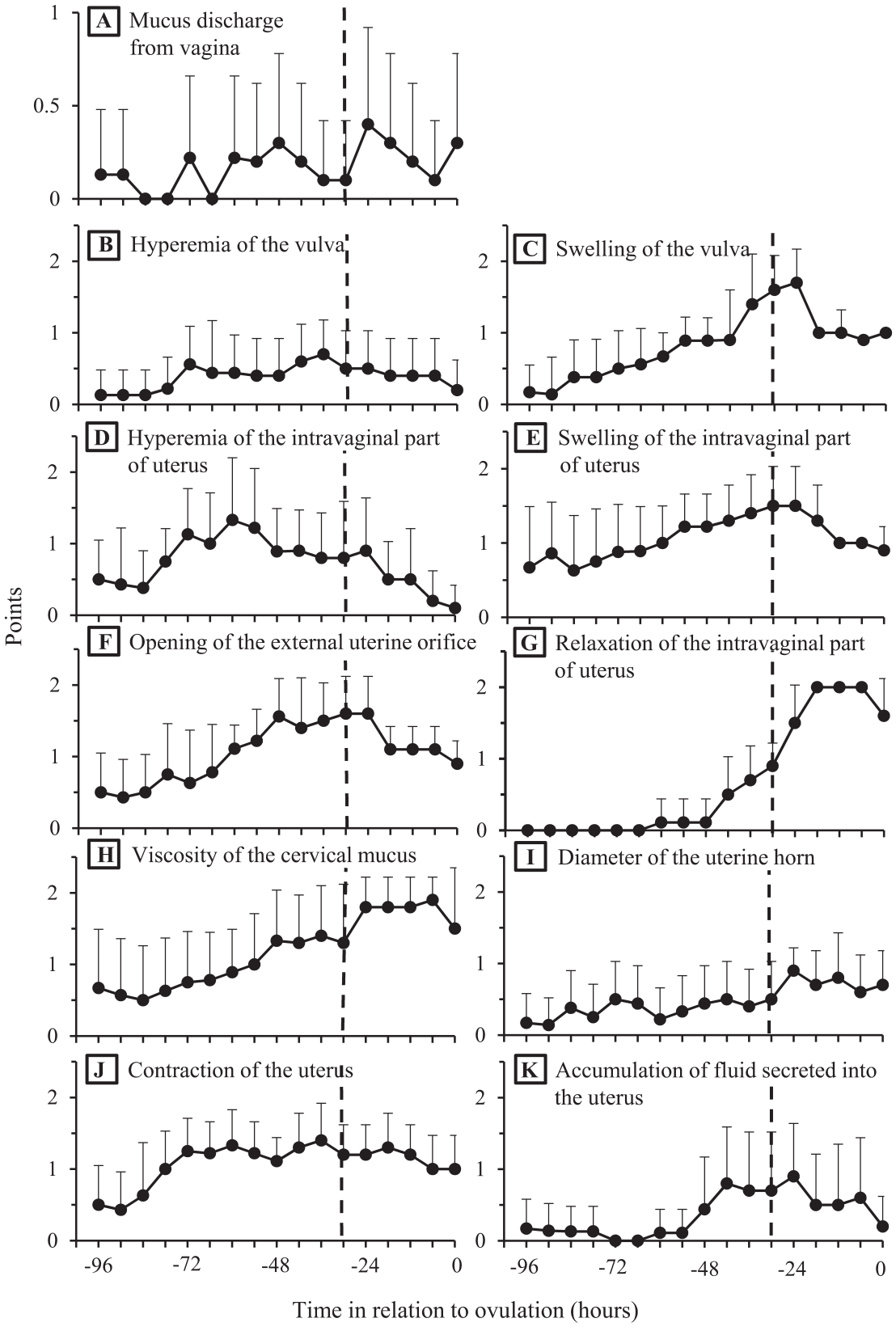

Fig. 2. Changes in scores for individual estrous signs from 96 hours before ovulation. The dashed lines indicate the E_2 peak. Values are means ± SDs.

Table 2. Total scores for estrous signs at maximum and at the E_2 peak, LH peak and time of ovulation

Estrous signs	Maximum	E_2 peak [b]	LH peak [c]	Ovulation	r s [d]
Vulva and uterus [e]	3.9 ± 0.3[a]	3.9 ± 0.6	3.7 ± 0.5	2.6 ± 0.8	0.31*
Vagina [f]	6.6 ± 1.0	5.9 ± 1.0	5.5 ± 1.6	3.4 ± 1.0	0.43*
All	10.4 ± 1.0	6.1 ± 1.3	9.3 ± 1.6	9.6 ± 1.2	0.43*

[a] Values are means ± SD. [b] Maximal estradiol-17β concentration before ovulation. [c] Maximal concentration of LH. [d] Correlation coefficients with E_2 concentration. [e] Swelling of the vulva, contraction of the uterus, diameter of the uterine horn (when relaxed). [f] Swelling of the intravaginal part of the uterus, hyperemia of the intravaginal part of the uterus, opening of the external uterine orifice, viscosity of the cervical mucus. *Statistically significant ($P<0.05$, Spearman's correlations).

in the intravaginal part of the uterus by vaginoscopic examination (i.e., hyperemia, swelling and opening of the external uterine orifice and viscosity of the cervical mucus) at the time of E_2 peak and LH surge were almost equivalent to the maximums and decreased toward ovulation. The chnges in the average scores for the three estrous signs in total and the four estrous signs in total were positively correlated with the E_2 concentration (rs =0.31 and 0.43, respectively) (Table 2). The scores of relaxation of the intravaginal part of the uterus showed a maximum at 6, 12 and 18 h before ovulation in all cows, and these changes were extremely obvious (Fig. 2G). Furthermore, the estrous signs of the intravaginal part of the uterus observed by vaginoscopic examination were obvious compared with the vulval and uterine estrous signs. As the E_2 concentration increased, the intravaginal part of the uterus became hyperemic and swollen and the external uterine orifice opened; after the E_2 peak and LH surge, the hyperemia and swelling disappeared, and the relaxation of the intravaginal part of the uterus became remarkable. The intravaginal part of the uterus, as a whole, dropped (Fig. 3).

Regarding the two kinds of pedometers that were used, no remarkable changes were encountered for the MANPO MK-365 pedometer, which was sensitive in all periods examined. For the HJ-151 pedometer, which had low sensitivity, remarkable increases were recognized during the 6 hours from 36 to 42 h before ovulation in cow No. 1 and No. 10. The number of steps increased by as much as 26.4 and 11.7 times the mean numbers for days 3, 4, 5, 7 and 14 days after ovulation, respectively. The pH and electrical resistance of the cervical mucus were not changed significantly throughout the periovulatory period examined (data not shown).

Discussion

In the present study, each estrous sign was scored to evaluate it numerically, and these scores were added together to estimate the total values for all estrous signs. The results of the present study demonstrated that the appraisable estrous signs were opening of the external uterine orifice and hyperemia, swelling and relaxation of the intravaginal part of the uterus. The present study showed that the changes in most of the estrous signs were related to the E_2 peak, LH surge and ovulation. In particular, the scores for the estrous signs in the intravaginal part of the uterus clearly increased as the E_2 concentration increased and decreased as the E_2 concentration decreased. On the other hand, relaxation of the intravaginal part of the uterus generally commenced around the E_2 peak and the LH surge and became obvious between the E_2 peak / LH surge and ovulation. It was useful to evaluate the relaxation of the intravaginal part of the uterus by vaginoscopic examination as an index to judge the stage after the E_2 peak and the LH surge in dairy cows kept in a tie-stall barn. Estrus and accompanying estrous signs are caused by estrogens, which play a key role in regulation of the endocrine control and appearance of estrus and estrous signs during the periovulatory period. It is generally accepted that a rise in E_2 concentration leads to estrus and that after a certain threshold of the E_2 concentration is achieved, an LH surge will occur, which will bring about ovulation [2, 15]. The results of the present study are supported by these findings.

Regarding the relationship between estrous signs and the estrogen concentration, Lyimo *et al.* [15] reported that there were high correlations between the E_2 concentration and the visual appearance of estrous symptoms such as mounting and standing. It is widely accepted that estrogenic activity causes estrous signs such as the hyperemia, swelling and relaxation of the vulva and the intravaginal part of the uterus throughout estrus [5, 6, 23]. However, the changes in hyperemia, swelling and relaxation during the periestrual period are assessed identically, and there are no reports examining the relationships among the appearance of estrous signs in the vulva, uterus and intravaginal part of the uterus and the estrogen concentration. In the present study, which evaluated 7 estrous signs, i.e., swelling of the vulva, contraction of the uterus, diameter of the uterine horn when relaxed, hyperemia and swelling of the intravaginal part of the uterus, opening of the external uterine orifice and viscosity of the cervical mucus, the scores increased with the increase in plasma E_2 concentration toward the E_2 peak and then decreased after the E_2 peak toward ovulation. Positive correlation was detected between the scores for these estrous signs and the E_2 concentration. In addition, in comparison of the appearances of each estrous sign, swelling was recognized, but hyperemia and relaxation were obscure in the vulva. However, hyperemia, swelling and relaxation of the intravaginal part of the uterus were recognized clearly by vaginoscopic examination. Furthermore, expressions of estrous signs in the intravaginal part of the uterus and the external uterine orifice were observed more obviously compared with those in the vulva and uterus.

In the present study, the scores for relaxation of the intravaginal part of the uterus were different from those of other estrous signs, revealing a peak plateau between the E_2 peak or LH surge and ovulation. In other words, the relaxation of the intravaginal part of the uterus has value as an index indicating the stage after the LH surge. In this regard, the intravaginal part of the uterus was constricted and the external

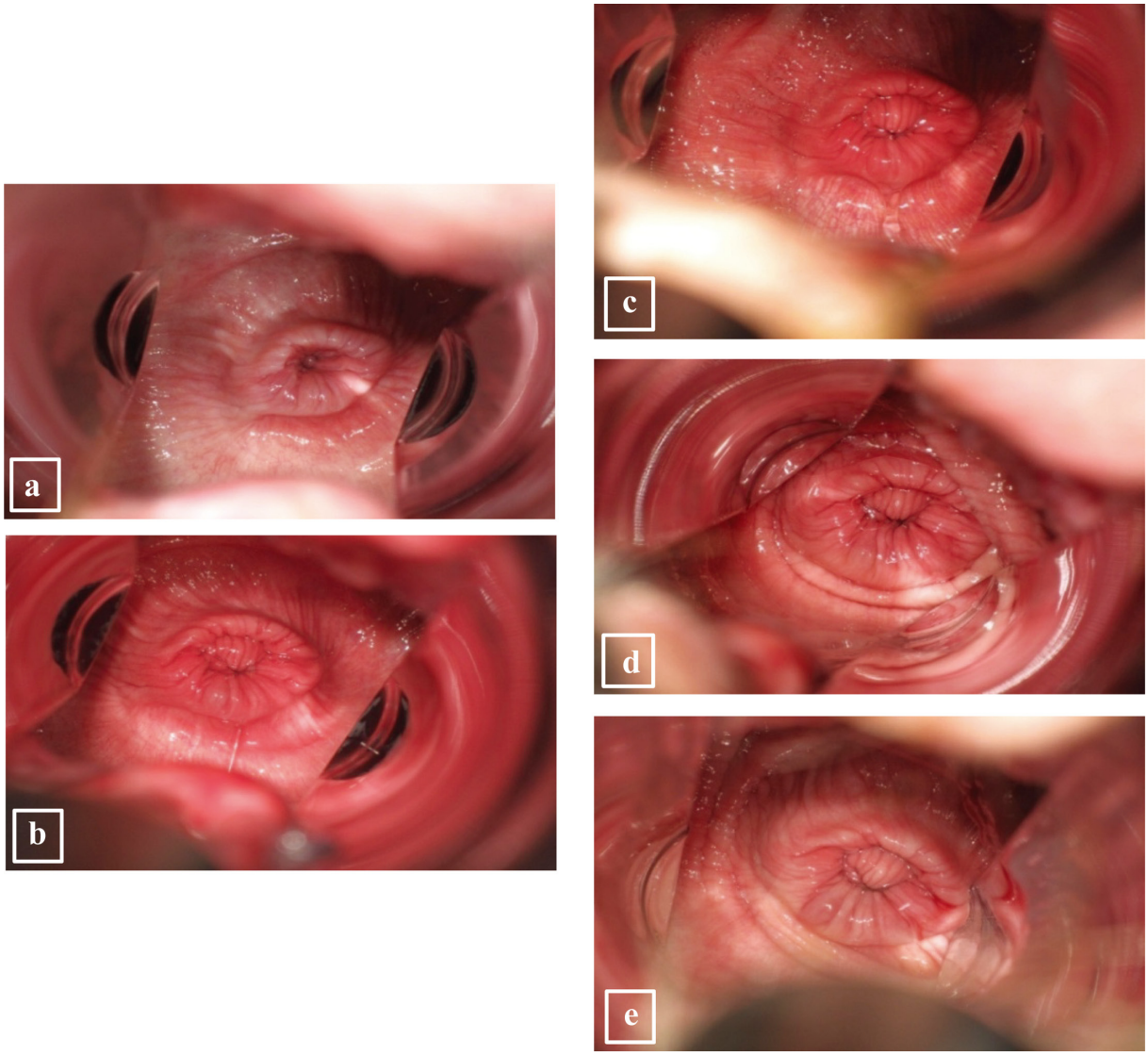

Fig. 3. Serial changes of estrous signs in the intravaginal part of the uterus in a representative cow (No. 8). a, Luteal phase (day 14). b, 24 h after initiation of luteolysis (24 h before E_2 peak). c, At the time of E_2 peak (42 h after initiation of luteolysis or 30 h before ovulation). d, 12 h before ovulation (60 h after initiation of luteolysis or 18 h after E_2 peak). e, At the time of ovulation (72 h after initiation of luteolysis or 30 h after E_2 peak).

uterine orifice was closed before estrus. However, the intravaginal part of the uterus was obviously swollen, and the external uterine orifice opened in accordance with the increase in E_2 concentration. Then the hyperemia and swelling decreased, and the intravaginal part of the uterus became obviously relaxed and, as a whole, dropped coincident with a decrease in the plasma E_2 concentration. The relaxation of the intravaginal part of the uterus became obvious, and the maximum scores were observed in all cows at 6, 12 and 18 h before ovulation. Thinking about the mechanisms of occurrence of hyperemia, swelling and relaxation, transient hyperemia first occurred in the vulva and intravaginal part of the uterus due to estrogenic activity [36]. The hyperemia is caused by expansion of arterioles and an increase in the blood volume flowing in, and the hyperemic part turns scarlet locally, becomes tense, increases in volume and swells due to the increase in inflow of arterial blood [37]. In addition, capillaries are expanded, and edema occurs due to an increase in the tissue blood volume and intravascular pressure. In particular, the vulva and the intravaginal part of the uterus have a low tissue osmotic pressure. Therefore, it is easy for edema to occur in these parts [37]. When the LH surge occurs, estrogen production from the ovulatory follicle stops and the estrogen concentration decreases successively; as a result, the tissue blood volume and intravascular pressure decrease, and transient hyperemia of the vulva and intravaginal part of the uterus disappear immediately. However, edema does not disappear immediately; therefore, the swelling, softness and loss of elasticity, that is, the conditions of relaxation, remain locally [38]. Based on

such an occurrence mechanism, it is thought that relaxation occurs after the decrease in the hyperemia and swelling.

In the present study, two estrous signs, i.e., hyperemia of the vulva and mucus discharge from the vulva, were not obvious. These two estrous signs are generally accepted as the estrous signs observed at the time of estrous [6, 23]; on the other hand, some studies have reported that these signs were only obvious in a small proportion of cows in estrus and were unreliable as sole methods of estrus detection [3, 9, 39]. Regarding the hyperemia of the vulva, the brightness of the lighting for observation was not fixed in this study. It is thought that the color tone observed during hyperemia is affected by the degree of brightness and quality of lighting. Therefore, the lighting conditions in this study might have affected the results, which showed no obvious change in hyperemia of the vulva. Regarding mucus discharge from the vulva, it is difficult to judge the existence of cervical mucus in the vagina depending on the occurrence of mucus discharge from the vulva. This is because even if mucus discharge from the vulva is not observed, cervical mucus is often present in the vagina; on the hand, even if mucus discharge is observed from the vulva, cervical mucus is not retained in the vagina in many cases. Therefore, a vaginoscopic examination is necessary to examine the presence and character of cervical mucus in the vagina.

Regarding the change in pedometric activity, no increase in steps in accordance with estrus was observed with the two kinds of pedometers, which had different sensitivities. Some previous studies indicated that the peak number of pedometric steps occurred 25 to 27 h before ovulation [16] and that the rates of conception were achieved when AI was carried out between 5 and 17 h [19], 11 and 16 h [18] and 6 and 17 h [17] after the increase in pedometric activity, respectively. Few reports have applied a pedometer for humans to cow. The number of pedometric steps increased in two cows from 36 to 42 h before ovulation according to the pedometer with low sensitivity in this study. The timing of these pedometric increases corresponded to 6 to 12 h before the E_2 peak and with the elevating stage of the E_2 concentration. However, this was considerably earlier than in previous reports. Many studies have examined estrus detection and the optimal timing for artificial insemination utilizing pedometers, particularly for cows housed in a free-stall barn, and pedometers are being used and put into practice [13–19]. A previous study in which the numbers of steps taken by cows were counted twice daily using 4 types of pedometer, attached to a lower rear leg, manufactured for human use reported that cows kept in comfort stalls were about 2.75 times as active during estrus as when not in estrus. It has also been reported that the results were satisfactory with only two of the four types of pedometers and that cows kept in tie-stall housing showed less distinct pedometric activity at the time of estrus than cows kept in the free-stall housing. In addition, estrus was only detected in 6% of cases by pedometric counts alone [14]. So it has been suggested that restriction of the movement of cows kept in tie-stalls reduces pedometric activity considerably compared with cows kept in free-stalls. The present results are supported by those of previous reports.

In the present study, no characteristic change in the pH and electrical resistance of the cervical mucus was observed during the periovulatory period (data not shown). Many studies have indicated that the pH and electrical resistance of the cervical mucus decrease with estrus and are lowest at the time of the LH surge [7, 23–30]. In these previous studies, the measurement probe was maintained or surgically implanted in the mucosa or submucosa of the vagina, and continuous monitoring was carried out in most of the studies. However, in the present study, cervical mucus was collected each time measurements were made, so the conditions of measurement were not constant. Accordingly, it was suspected that the measured value was not stable. Furthermore, the ease with which the properties of cervical mucus change and whether or not cervical mucus is present in the vagina during measurement might affect the values measured.

For optimal timing of AI, five properties, i.e., the timing of ovulation, the transport time of viable sperm from the site of AI to fertilization in the ampulla of the uterine tube, the time required for sperm to obtain fertilization ability in the female reproductive tract, the lifespan of the functionally viable sperm and the lifespan of the functionally viable ovulated ovum, should be concerned [13]. There are several reports about the optimal timing of AI in which the optimal timing was reported to be 12 to 18 h before ovulation [40], from 6 to 24 h before ovulation [41] or from 12 to 24 h before ovulation [8,19]. Thinking about the five above-mentioned properties and the previous reports, the theoretical optimal time for AI is considered to generally be 6 to 24 h before ovulation. The results of the present study indicated that precise observation, especially observation of the estrous signs of the intravaginal part of the uterus, i.e., hyperemia, swelling and relaxation of the intravaginal part of the uterus, opening of the external uterine orifice and viscosity of the cervical mucus, was necessary to recognize the estrous signs exactly.

In conclusion, in dairy cows housed in a tie-stall barn, the maximum scores for most of the estrous signs occurred around the E_2 peak and the LH surge and then decreased, but the scores for relaxation of the intravaginal part of the uterus became remarkable after the E_2 peak and LH surge, i.e., around 6 to 18 h before ovulation. It is recognized that the remarkable relaxation of the intravaginal part of the uterus was the most critical estrous sign corresponding to the theoretical optimal timing for AI.

References

1. The National Council of Milk Recording. The summary of milk recording report. *Livestock Improvement Association of Japan* 2011; **23**: 11–22 (In Japanese).
2. **López-Gatius F.** Is fertility declining in dairy cattle? A retrospective study in northeastern Spain. *Theriogenology* 2003; **60**: 89–99. [Medline] [CrossRef]
3. **Van Eerdenburg FJCM, Loeffler HSH, van Vliet JH.** Detection of oestrus in dairy cows: a new approach to an old problem. *Vet Q* 1996; **18**: 52–54. [Medline] [CrossRef]
4. **Van Vliet JH, Van Eerdenburg FJCM**. Sexual activities and oestrus detection in lactating Holstein cows. *Appl Anim Behav Sci* 1996; **50**: 57–69. [CrossRef]
5. **Yoshida C, Nakao T.** Some characteristics of primary and secondary oestrous signs in high-producing dairy cows. *Reprod Domest Anim* 2005; **40**: 150–155. [Medline] [CrossRef]
6. **Diskin MG, Sreenan JM.** Expression and detection of oestrus in cattle. *Reprod Nutr Dev* 2000; **40**: 481–491. [Medline] [CrossRef]
7. **Foote RH.** Estrus detection and estrus detection aids. *J Dairy Sci* 1975; **58**: 248–256. [Medline] [CrossRef]
8. **Roelofs J, López-Gatius F, Hunter RHF, van Eerdenburg FJCM, Hanzen C.** When is a cow in estrus? Clinical and practical aspects. *Theriogenology* 2010; **74**: 327–344. [Medline] [CrossRef]
9. **Williamson NB, Morris RS, Blood DC, Cannon CM.** A study of oestrous behaviour and oestrus detection methods in a large commercial dairy herd. I. The relative efficiency of methods of oestrus detection. *Vet Rec* 1972; **91**: 50–58. [Medline] [CrossRef]
10. **Hopkins SM, Evans LE.** Artificial insemination. *In:* Pineda MH, Dooley MH (eds.), Veterinary Endocrinology and Reproduction. 5th ed. Iowa University Press, Blackwell

Publishing Ltd, 2003: 341–375.
11. **Walker WL, Nebel RL, McGilliard ML.** Time of ovulation relative to mounting activity in dairy cattle. *J Dairy Sci* 1996; **79**: 1555–1561. [Medline] [CrossRef]
12. **At-Taras EE, Spahr SL.** Detection and characterization of estrus in dairy cattle with an electronic heatmount detector and an electronic activity tag. *J Dairy Sci* 2001; **84**: 792–798. [Medline] [CrossRef]
13. **Nebel RL, Dransfield MG, Jobst SM, Bame JH.** Automated electronic systems for the detection of oestrus and timing of AI in cattle. *Anim Reprod Sci* 2000; **60–61**: 713–723. [Medline] [CrossRef]
14. **Kiddy CA.** Variation in physical activity as an indication of estrus in dairy cows. *J Dairy Sci* 1977; **60**: 235–243. [Medline] [CrossRef]
15. **Lyimo ZC, Nielen M, Ouweltjes W, Kruip TAM, van Eerdenburg FJCM.** Relationship among estradiol, cortisol and intensity of estrous behavior in dairy cattle. *Theriogenology* 2000; **53**: 1783–1795. [Medline] [CrossRef]
16. **Sakaguchi M, Fujiki R, Yabuuchi K, Takahashi Y, Aoki M.** Reliability of estrous detection in Holstein heifers using a radiotelemetric pedometer located on the neck or legs under different rearing conditions. *J Reprod Dev* 2007; **53**: 819–828. [Medline] [CrossRef]
17. **Maatje K, Loeffler SH, Engel B.** Predicting optimal time of insemination in cows that show visual signs of estrus by estimating onset of estrus with pedometers. *J Dairy Sci* 1997; **80**: 1098–1105. [Medline] [CrossRef]
18. **Roelofs JB, van Eerdenburg FJCM, Soede NM, Kemp B.** Pedometer readings for estrous detection and as predictor for time of ovulation in dairy cattle. *Theriogenology* 2005; **64**: 1690–1703. [Medline] [CrossRef]
19. **Roelofs JB, Graat EAM, Mullaart E, Soede NM, Voskamp-Harkema W, Kemp B.** Effects of insemination-ovulation interval on fertilization rates and embryo characteristics in dairy cattle. *Theriogenology* 2006; **66**: 2173–2181. [Medline] [CrossRef]
20. **Redden KD, Kennedy AD, Ingalls JR, Gilson TL.** Detection of estrus by radiotelemetric monitoring of vaginal and ear skin temperature and pedometer measurements of activity. *J Dairy Sci* 1993; **76**: 713–721. [Medline] [CrossRef]
21. **Fisher AD, Morton R, Dempsey JMA, Henshall JM, Hill JR.** Evaluation of a new approach for the estimation of the time of the LH surge in dairy cows using vaginal temperature and electrodeless conductivity measurements. *Theriogenology* 2008; **70**: 1065–1074. [Medline] [CrossRef]
22. **Rajamahendran R, Robinson J, Desbottes S, Walton JS.** Temporal relationships among estrus, body temperature, milk yield, progesterone and luteinizing hormone levels, and ovulation in dairy cows. *Theriogenology* 1989; **31**: 1173–1182. [Medline] [CrossRef]
23. **Noakes DE.** Endogenous and exogenous control of ovarian cyclicity. *In:* Noakes DE, Parkinson TJ, England GCW (eds.), Veterinary Reproduction and Obstetrics, 9th ed. London: Saunders Ltd, 2001; 22.
24. **Senger PL.** The estrus detection problem: new concepts, technologies, and possibilities. *J Dairy Sci* 1994; **77**: 2745–2753. [Medline] [CrossRef]
25. **Foote RH, Oltenacu EAB, Mellinger J, Scott NR, Marshall RA.** Pregnancy rate in dairy cows inseminated on the basis of electronic probe measurements. *J Dairy Sci* 1979; **62**: 69–73. [Medline] [CrossRef]
26. **Ezov NE, Maltz R, Yarom GS, Lewis D, Schindler M, Ron E, Aizinbud E, Lehrer AR.** Cell density, fluid volume and electrolyte content of bovine vulvar tissue during oestrus and diestrus. *Anim Reprod Sci* 1990; **22**: 281–288. [CrossRef]
27. **Gartland P, Schiavo J, Hall CE, Foote RH, Scott NR.** Detection of estrus in dairy cows by electrical measurements of vaginal mucus and by milk progesterone. *J Dairy Sci* 1976; **59**: 982–985. [Medline] [CrossRef]
28. **Rorie RW, Bilby TR, Lester TD.** Application of electronic estrus detection technologies to reproductive management of cattle. *Theriogenology* 2002; **57**: 137–148. [Medline] [CrossRef]
29. **Heckman GS, Katz LS, Foote RH, Oltenacu EAB, Scott NR, Marshall RA.** Estrous cycle patterns in cattle monitored by electrical resistance and milk progesterone. *J Dairy Sci* 1979; **62**: 64–68. [Medline] [CrossRef]
30. **Smith JW, Spahr SL, Puckett HB.** Electrical conductivity of reproductive tissue for detection of estrus in dairy cows. *J Dairy Sci* 1989; **72**: 693–701. [Medline] [CrossRef]
31. **Ferguson JD, Galligan DT, Thomsen N.** Principal descriptors of body condition score in Holstein cows. *J Dairy Sci* 1994; **77**: 2695–2703. [Medline] [CrossRef]
32. **Sartori R, Haughian JM, Shaver RD, Rosa GJ, Wiltbank MC.** Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. *J Dairy Sci* 2004; **87**: 905–920. [Medline] [CrossRef]
33. **Prakash BS, Meyer HH, Schallenberger E, van de Wiel DF.** Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. *J Steroid Biochem* 1987; **28**: 623–627. [Medline] [CrossRef]
34. **Taya K, Watanabe G, Sasamoto S.** Radioimmunoassay for progesterone, testosterone and estradiol-17β using ^{125}I-iodohistamine redioligands. *Jpn J Anim Reprod* 1985; **31**: 186–197 (In Japanese). [CrossRef]
35. **Kengaku K, Tanaka T, Kamomae H.** Changes in the peripheral concentrations of inhibin, follicle-stimulating hormone, luteinizing hormone, progesterone and estradiol-17β during turnover of cystic follicles in dairy cows with spontaneous follicular cysts. *J Reprod Dev* 2007; **53**: 987–993. [Medline] [CrossRef]
36. **Kato H, Hoshi S, Nishikawa Y.** Estrus and estrous signs. *In:* Series of Livestock Reproduction Basic Volume. Tokyo: Asakura Publishing, 1970; 171–174 (In Japanese).
37. **Nagasawa H, Suzuki H.** A circulatory disorder. *In:* Color Atlas of Physiological Pathology, A Rev 2nd ed. Tokyo: Maruzen Publishing, 2011; 141–166 (In Japanese).
38. **Kikuchi K, Yoshiki T, Sato N, Ishikura H.** A circulatory disorder. *In:* Physiological Pathology, A Rev 17th ed. Tokyo: Nanzando, 2004; 126–134 (In Japanese).
39. **Heres L, Dieleman SJ, van Eerdenburg FJCM.** Validation of a new method of visual oestrus detection on the farm. *Vet Q* 2000; **22**: 50–55. [Medline] [CrossRef]
40. **Hunter RHF, Greve T.** Could artificial insemination be more fruitful? Penalties associated with aging eggs. *Reprod Domest Anim* 1997; **32**: 137–141. [CrossRef]
41. **Trimberger GW.** Breeding efficiency in dairy cattle from artificial insemination at various intervals before and after ovulation. *Nebraska Agric Exp Stn Res Bull* 1948; **153**: 3–25.