Studies on the Relationship between Lactation and Reproductive Physiology in Dairy Cows

（乳牛における泌乳と繁殖生理の関係に関する研究）

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Contents

Abbreviations

AI	Artificial insemination
ANOVA	Analysis of variance
ARGG	Anti-rabbit gamma globulin goat serum
ASGG	Anti-sheep gamma globulin donkey serum
AST	Aspartate aminotransferase
AUC	Area under the curve
BCS	Body condition score (s)
BSA	Bovine serum albumin
BUN	blood urea nitrogen
BW	Body weight (s)
CL	Corpus luteum
EDTA	Ethylene-diamine-tetra-acetic acid disodium salt
FSH	Follicle-stimulating hormone
γ-GTP	Gamma glutamyl transpeptidase
GnRH	Gonadotropin-releasing hormone
IGF-1	Insulin-like growth factor-1
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
LH	Luteinizing hormone
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
SF	Subordinate cohort of follicle (s)
TMR	Total mixed ration

Chapter 1. General Introduction

1.1. Background

Milk productivity of dairy cow has been increased rapidly during the past half century. In Japan, milk yield per year per cow has been increasing by 100 kg per year during the past 20 years from 1995 to 2005 and reached a level of 9,000 kg per year. Surprisingly, high yielding dairy cows have almost doubled their milk yield (> 10,000 kg per year) compared with those in the 1950s. The increase in milk yield per cow is mainly due to the combination of genetic improvement for high milk production and betterment of nutritional management. On the other hand, reproductive efficiency of dairy cows has been decreasing worldwide as documented by a number of recent publications (14, 22, 54). According to the survey performed during the period from 1976 to 1999 in Southeastern states in the United State, average of days open increased from 122 days in 1978 to 168 days in 1999, while average of milk yield per cow increased linearly (120). First-service conception rates in New York dairy herds decreased from approximately 65% in 1951 to 40% in 1996 (13). Similar trends are also occurring in Japan. According to the report in Dairy Herd Improvement Association in Japan, average of days open were 164 days in 2010, which was 28 days longer than that recorded 25 years ago. First-service conception rate in multiparous dairy cows is often lower than 50 %. There are multiple factors that can affect reproductive efficiency in dairy cows, and situations may vary widely according to the nations, countries and individual farms. Nevertheless, poor reproductive efficiency is recognized as a major challenge in current dairy industry.

The onset of normal ovarian cycles is one of the most important events for the dairy cows to regain their maximum breeding potential following parturition (66). However, recent studies report the retard resumption of ovarian cyclicity (18, 55) and high incidence of

ovarian disturbance during the early postpartum period in high-yielding cows (66). Some reproductive traits such as calving interval, days open and days to first service were reported to be negatively correlated with milk yield (76, 77). During the early lactation period, the mobilization of body reserves for milk production induces a negative energy balance (NEB). NEB are known to negatively affect the normal development of follicles and delays first ovulation through the inhibition of gonadotropin-releasing hormone (GnRH) release from the hypothalamus (15) and the reduction of metabolic hormones such as insulin like growth factor-1 (IGF-1) in blood (98, 102). Even when lactating cows resume ovarian cycles, the conception rate following AI is markedly lower than that in heifers (3, 77, 78). For example, Pryce et al. (77) reviewed that conception rates to first service was 64% and 71% in maiden heifers of high and average genetic merit, whereas conception rates were 39% and 45% in lactating cows of high and average genetic merit in the same herd. This suggests that any alterations in metabolic and nutritional status associated with high milk production may influence ovarian function and consequential fertility of dairy cows.

The ovarian cycle is central to reproductive function in mammals. It is characterized by the follicular development and ovulation as well as the formation, function and regression of the corpus luteum (CL). The main function of the CL is to synthesize and secrete progesterone, which regulates the estrous cycle length and maintains pregnancy in many species. Pituitary derived gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (60), are the primary regulation of follicular development, final follicular maturation and CL function (101). The preovulatory LH surge causes differentiation of follicular cells into luteal cells, and pulsatile secretion of LH in post-ovulatory period is required for the functional development of the CL in cattle (71, 79) and other ruminants (4, 45). Luteal secretion of progesterone is essential to successful pregnancy, including ovulation of a healthy oocyte, maintenance of uterine quiescence, and survival of the embryo/fetus.

Previous studies investigating the relationship between progesterone concentration following artificial insemination (AI) and conception rate found that abnormal progesterone profiles such as delayed progesterone rise after ovulation and low progesterone concentration during the luteal phase can reduce fertility of lactating dairy cows (37, 49). Furthermore, the influence of circulating progesterone concentrations on preovulatory follicular development and subsequent fertility has been gradually revealed using ovarian ultrasonography. In general, two or three follicular waves occur during the normal estrus cycle in cattle (42). Recent studies found that fertility tended to be greater in lactating cows inseminated after ovulation of the third wave dominant follicle compared with those inseminated after ovulation of the second wave dominant follicle (1, 8). These results suggest that increased duration of ovulatory follicle development from the time of emergence to ovulation can reduce the pregnancy rates following AI in dairy cows, probably by compromising oocyte developmental competence.

Estrus detection is another key component for successful reproductive management in dairy herds. In general, estrus detection is primarily based on the observation of standing behavior of cow (21, 28). Cow in estrus may also show other secondary signs of estrus. For example, they express more agonistic interactions, and spend more time walking and less time eating and resting (38). Together with the behavioral signs of estrus, some genital changes may be observed, such as mucus discharges from the vulva, hyperemia and swelling of the vulva and vagina (28). According to technical literature (72), the target detection rate of estrus is suggested to be 80%, but frequently it is less than 60% in recent reports (47, 57, 113). The low detection rate of estrus is considered to be mainly due to changes in behavioral aspects of modern dairy cows such as shorter duration of estrus and poor expression of standing estrus and other estrous signs (23, 113, 114, 129). The causes of poor expressions of estrus and estrous signs in modern dairy cows have been investigated with relevance to

endocrine aspects, herd management or estrus detection methods. Studies on endocrine events around estrus and ovulation have revealed that alterations in the concentrations of ovarian steroids and gonadotropins may influence expression of estrus and estrous signs (52, 57, 130). Lopez et al. (52) reported that estradiol-17β concentrations at estrus and duration and intensity of estrus were inversely affected by the level of milk production. To overcome the problem of estrus detection in lactating cows, an integrated approach is essential to understand comprehensively how lactation status can influence endocrine profiles around estrus, timing of ovulation and appearance of behavioral and genital estrous signs.

In general, onset of lactation after calving involves greater nutrient requirement for milk production. Increased milk production and resultant greater feed intake enhance the gastrointestinal and liver function, and it accompanies the increase in blood flow through gastrointestinal organs and liver (51, 88). A recent study has demonstrated that lactating cows had higher liver blood flow and greater metabolic rates of steroid hormones than non-lactating cows (93), in which the baseline liver blood flow was about two times greater in lactating cows (1,600 l/h) as compared to non-lactating cows (800 l/h), and there was a high correlation between the liver blood flow and the metabolic clearance rate of progesterone. Increased metabolic rates of estradiol-17β and progesterone are hypothesized to lower circulating concentrations of estradiol-17β and progesterone during the estrous cycles in lactating dairy cows (93, 125). In particular, the effect of progesterone metabolism on circulating progesterone was clearly observed in association with feeding (60, 80, 115), in which there was an acute decrease in circulating concentrations of progesterone after feeding. Therefore, it is suggested that alterations in circulating hormone concentrations due to increased steroid metabolism can influence various reproductive parameters such as follicular and luteal development, expression of estrus and estrous signs, as well as conception rates following AI.

Taken these findings together, the great increase in milk yield of dairy cows, which were mainly due to both genetic and management improvement, might have induced some undesirable changes in the reproductive physiology of dairy cows. In particular, metabolic and nutritional changes associated with lactation can directly influence ovarian function of steroidogenesis in dairy cows, or indirectly influence circulating hormone concentrations by the increased steroid metabolism. However, the interactions have not been clearly defined. To deal with reproductive problems in modern dairy cows, the influence of lactation on follicular and luteal development and their function, estrous signs and related hormonal profiles in dairy cows is needed to be comprehensively studied.

1.2. Objectives

In this present dissertation, ovarian follicular and luteal dynamics, endocrine patterns of ovarian steroids and LH during the estrous cycles and expressions of estrous signs were compared between lactating and non-lactating cows to examine the influence of lactation on those observations. In particular, circulating dynamics of progesterone including secreted status and its metabolic status were studied during the mid-luteal phase, and the role of pulsatile LH secretion on luteal function was also examined. Finally, the obtained results from these studies will provide some insights about the causes of low fertility and contribute to develop strategies by which both high productivity and good fertility can be maintained satisfactorily.

The specific aims of the studies in the present thesis are as follows:

1) The study in Chapter 3 was designed to compare the follicular and luteal dynamics and ovarian steroid concentrations during the estrous cycles and LH secretion pattern during the early to mid-luteal phase between lactating and non-lactating dairy cows.
2) The study in Chapter 4 was designed to compare the followings from luteolysis to ovulation between lactating and non-lactating cows; profiles of ovarian steroids and LH and the appearance of behavioral and genital estrous signs.
3) The study in Chapter 5 was designed to compare the progesterone profiles at the secreted level and the circulating level in lactating and non-lactating dairy cows with reference to feeding and to examine the association between LH and progesterone secretion patterns.
4) The study in Chapter 6 was designed to determine the association of LH pulses with luteal progesterone secretion in lactating dairy cows by examining whether increased frequency of LH pulses would stimulate progesterone secretion.

Chapter 2. General Materials and Methods

2.1. Animals

Lactating and non-lactating cycling Holstein dairy cows maintained at the dairy farm of the Tokyo University of Agriculture and Technology were used in the study. The lactating cows were housed in a free-stall with open-air and sheltered areas, milked twice at 0900 and 1700 h. The milk yield per lactation per cow in the herd was around 9,000 kg. The daily milk yields were recorded with an automatic monitoring system at the milking parlor, and are presented in each Chapter. The non-lactating cows were reared in a paddock or tie stalls and spent at least one month after dry off before being subjected to the study. They were maintained for experimental purposes and confirmed clinically to have no general reproductive problems before being assigned to the study. Diets for the lactating and non-lactating cows were formulated according to the Japanese feeding standards for dairy cattle (2006). The lactating cows were fed a total mixed ration (TMR) based on Sudan hay, alfalfa hay cubes, corn silage, and concentrate supplements after the milking. The diet contained 73.1% total digestible nutrients and 16.2% crude protein on a dry matter basis. The non-lactating cows were fed 7.0 kg of Sudan hay and 3.0 kg of concentrate supplements (73.0% total digestible nutrients and 18.0% crude protein). At the beginning of each experiment, body weights (BW) were measured with an electronic scale (TRU-Test EC2000, Fujihira Industry, Co. Ltd., Tokyo, Japan), and body condition scores (BCS) were assessed based on a five-point scale (1 = emaciated and 5 = obese) as described by Edmonson et al. (26).

The cows were confirmed to be clinically healthy and have normal estrous cycles before the study. All procedures were approved by the University Committee for the Use and Care of Animals of Tokyo University of Agriculture and Technology (Nos. 23-40 and 23-88).

2.2. Ovarian ultrasonography

Prior to the experiments in Chapters 3 - 6, ovaries were monitored by rectal palpation and transrectal ultrasonography every other day or daily to check the normality of estrous cycle and to determine the time of spontaneous ovulation (Day 0). Transrectal ultrasonography was carried out using a B-mode scanner (Ultrasonic Scanner HS-101V, Honda Electronics, Co., Ltd.; Aichi, Japan) equipped with a 5.0-MHz linear-array probe. All follicles and CL that grew to larger than 6 mm in diameter were recorded by at least three cross-sectional images with maximal areas.

The dominant follicle was defined as the largest growing follicle in a wave, and subordinate cohort of follicles (SF) was defined as a cohort of follicles that emerged simultaneously with the dominant follicle. The day of emergence of a wave was assigned as the day on which at least one follicle of the wave was detected. Duration of the first wave was defined as the interval from the day of emergence of the first wave to the emergence of the second wave. Growth rate of the dominant follicle of the first follicular wave was calculated by dividing the total change in diameter of the dominant follicle during the monitoring period by the growth period. The growth period of the dominant follicle was defined as the period between its emergence and the day on which it reached its maximal diameter.

Measurements of follicles and CL were used to calculate the diameter (mean of length [L] and width [W]) and the volume (V). Volume was calculated with the formula: $V = 4/3\times\pi\times R^3$, using a radius ($R$) calculated by the formula: $R = (L/2+W/2)/2$. For CL with a fluid-filled cavity, the volume of the cavity was subtracted from the total volume of the CL, and the estimated diameter of CL was calculated back from the volume of the CL. For cows having two CL after double ovulations occurred, the mean diameter of the two CL was calculated.

The initiation of luteolysis was determined as described by Sartori et al. (96) with some modifications as follows: it was considered to have initiated on the day before the day on which the diameter of the CL decreased for three consecutive days as determined by ultrasonography and the plasma progesterone concentration decreased to less than 50% of the average for the four maximum concentrations measured in the cycle and continued to decrease.

2.3. Blood sampling from the jugular vein

Blood samples (10 ml) obtained daily or every other day were collected by jugular venipuncture into heparinized vacutainers (Venoject II, Terumo, Tokyo, Japan). Blood samples (6 or 10 ml) obtained at 15-min intervals in Chapters 3 and 5, 12-min intervals in Chapter 6, and 3-h intervals in Chapter 4 were collected from a catheter (14 gauge, 30-cm length; Medicut Catheter Kit, Nippon Sherwood Medical Industries, Tokyo, Japan) inserted into the jugular vein. Catheterization was performed on the day of blood sampling in Chapters 3 and 4, or on the day before the blood sampling in Chapters 5 and 6. During the blood sampling, the catheter was filled with 10 IU/ml of heparinized saline to prevent the coagulation. Blood samples (6 ml) were collected from the catheter into test tubes that contained 10 IU heparin. Plasma was separated by centrifugation at 3,000 rpm (1,750 × g) for 20 min at 4 °C immediately after the blood collection and frozen at -20 °C until assay.

2.4. Blood sampling from the caudal vena cava

Catheterization into the caudal vena cava was conducted according to the method of Norman and Fields (65) with some modifications. All procedures were implemented after milking in the morning. Practically, cows were restrained in a treatment stall and sedated with 20 mg of xylazine (Celactal; Bayer, Tokyo, Japan), and epidural anesthesia was achieved by

injection of 4.5 ml of 2% lidocaine hydrochloride (Xylocaine; AstraZeneca, Osaka, Japan). The coccygeal vein was punctured using a 20-gauge needle equipped with a catheter introducer kit (Radifocus® introducer II H, Terumo, Tokyo, Japan; **Fig. 2-1**) and a mini-wire (0.64 mm in diameter; 45 cm in length) was passed through the needle and advanced into the vein. The needle was then removed and a sheath was passed over the mini-wire and placed into the vein. After the mini-wire was removed, a hydrophilic-coated guide wire (Radifocus® guide wire M; 0.46 mm in diameter; 150 cm in length; Terumo, Tokyo, Japan; **Fig. 2-2**, A) was threaded into the lumen of the coccygeal vein through the sheath toward the caudal vena cava. Then, a catheter (Optiflash® XL; inside diameter, 1.12 mm; outside diameter, 1.35 mm; 110 cm in length; Terumo, Tokyo, Japan; **Fig. 2-2**, B) was passed through the coccygeal vein up to the 100 cm length with the wire guide in place. Once the guide wire had been pulled out, nine blood samples (6 ml) were taken every 5 cm from 100 to 60 cm length of the catheter inserted into the vein. After the last blood sample had been taken, the catheter was re-inserted up to 100 cm in length and wrapped using adhesive bandage with the tail. The catheter was filled with 100 IU/ml of heparinized saline to prevent the coagulation. After all procedures were accomplished, cows were released and fed their morning diet at 1100 h. Obtained blood samples were centrifuged at 3,000 rpm (1,750 × g) for 20 min at 4 °C immediately after sampling and plasma was harvested. Plasma concentrations of progesterone for the obtained samples were analyzed by double-antibody enzyme immunoassay, where the incubation time was shortened for rapid measurement. The catheter was adjusted to the length where the progesterone concentration in the obtained blood sample showed the highest value. Some examples are shown in **Fig. 2-3**. During the blood sampling, the catheter was filled with 10 IU/ml of heparinized saline to prevent the coagulation. Blood samples (6 ml) were collected from the catheter into test tubes that contained 10 IU heparin. Plasma was separated by

centrifugation at 3,000 rpm (1,750 × g) for 20 min at 4 °C immediately after the blood collection and frozen at -20 °C until assay.

2.5. Hormone assays

2.5.1. Estradiol-17β

Estradiol-17β was measured in plasma by double-antibody radio-immunoassay using ^{125}I-labeled radio-ligand as described previously (110). Anti-estradiol-17β-sheep serum (GDN No. 244) was supplied by Dr. G. D. Niswender, Animal Reproduction and Biotechnology Laboratory, Colorado State University, U.S.A. The antiserum was diluted to ×500,000 with phosphate buffered saline (PBS) containing 0.05M ethylene-diamine-tetra-acetic acid, disodium salt (EDTA) and 0.25% normal sheep serum. Anti-sheep gamma globulin donkey serum (ASGG) was diluted to ×40 with 0.05M EDTA-PBS and polyethylene glycol (PEG) was added to a final concentration of 5%.

The assay procedure for estradiol-17β is summarized in **Fig. 2-4**. Standard estradiol-17β (Sigma, MO, USA) was diluted to a total volume of 1,000 μl with 1% bovine serum albumin (BSA: Sigma-Aldrich, Co., MO, USA) in PBS. The 1,000 μl of standard and plasma samples were placed in 13 × 100 mm disposable culture tubes and extracted once with 2 ml of diethyl ether (Wako, pure chemical industries, Ltd., Osaka, Japan). The tubes were mixed by direct-mixture (Thermal Kagaku Sangyo Co., Ltd., Tokyo, Japan) intermittently for 5 minutes and allowed to remain for more than 10 min to separate diethyl ether and aqueous layers. Then, the tubes were place in a cooled ethanol bath (approximately -40 °C) to froze the aqueous layer. The ether layer was decanted into 10 × 75 mm culture tubes and dried by a centrifugalized evaporator (Sakuma corporation, Tokyo, Japan) at 70 °C for 15 min. Then, 0.25 ml of 50% methanol and 1.0 ml of n-hexane (Wako, pure chemical industries, Ltd.,

Osaka, Japan) were added to the tube and centrifuged at 3,000 rpm (1,750 × g) for 5 min at 4 °C. The hexane layer was aspirated and the extraction was dried at 70 °C for 40 min.

The dried samples were re-dissolved in 250 μl of 1% BSA in PBS and mixed for 5 min. A hundred μl of the re-dissolved sample and 100 μl of the diluted solution of the primary antibody were added to a 1.2 ml micro-titer tube (Molecular BioProducts, Inc., California, USA) and mixed, and incubated at 4 °C for 24 h. Then, 100 μl of diluted ^{125}I-labelled estradiol-17β (Estradiol-6-(O-carboxymethyl)-oximino-2-(2-[^{125}I]-iodo-histamine), MP Biomedicals, Inc., CA, USA) was added to each tube, and the tube was mixed and incubated at 4 °C for 24 h. A hundred of the diluted solution of ASGG was added to each tube, and the tube was mixed and incubated at 4 °C for 24 h. After that, the hormone bound antibody was separated from the free hormone by centrifugation at 3,000 rpm (1,750 × g) for 30 min at 4 °C. The supernatant was aspirated and the precipitate was counted in an automatic gamma-spectrometer (Cobra, Packard, MN, USA).

2.5.2. Luteinizing hormone

Bovine LH was measured in plasma by double-antibody radio-immunoassay using ^{125}I-labeled NIADDK-ovineLH-I-3 as tracer (46). USDA-bLH-1 was served as a standard. Anti-ovine LH rabbit serum (YM No. 18) was used as the first antibody and diluted to ×60,000 with 0.05M EDTA in PBS containing 0.25% normal rabbit serum. Anti-rabbit gamma globulin goat serum (ARGG) was used as the second antibody and diluted to ×200 with 0.05M EDTA in PBS and PEG was added to a final concentration of 3.5%.

The assay procedure for LH is summarized in **Fig. 2-5**. A hundred μl of plasma and standard were placed in a 1.2 ml micro-titer tube (Molecular BioProducts, Inc., California, USA), and 100 μl of 1% BSA in PBS and 50 μl of the diluted solution of the primary antibody were added to each tube and mixed. The tubes were incubated at 4 °C for 24 h. Then,

50 μl of diluted ^{125}I-labelled ovine LH was added and mixed, and the tubes were incubated at 4 °C for 24 h. A hundred of the diluted solution of ARGG was added to each tube, and the tube was mixed and incubated at 4 °C for 24 h. After that, the hormone bound antibody was separated from the free hormone by centrifugation at 3,000 rpm (1,750 × g) for 30 min at 4 °C. The supernatant was aspirated, and the precipitate was counted in an automatic gamma-spectrometer (Cobra, Packard, MN, USA).

2.5.3. Progesterone

Progesterone was measured in plasma by a double-antibody enzyme immunoassay according to the previously described method (73). The assay was performed using rabbit anti-progesterone antibody (UCB Bioproducts, Brussels, Belgium) and horseradish peroxidase-conjugated progesterone. Micro-titer 96-well immunoplates (Nunc-Immuno Plate Maxisorp, Roskilde, Denmark) were used as the solid phase. Wells of the immunoplates were coated with 100 μl of 5 μg/ml affinity-purified anti-rabbit goat IgG antibody (Jackson Immuno Research, West Grove, PA, USA) in carbonate buffer. The plates were incubated over night at room temperature. Then, the solution was replaced to 200 μl of 0.1% BSA in PBS and kept in a refrigerator for later use.

The assay procedure for progesterone is summarized in **Fig. 2-6**. Standard progesterone (Sigma, MO, USA) was diluted to a total volume of 50 μl with 0.1% BSA-PBS. The 50 μl of standard and plasma samples were placed in 13 × 100 mm disposable culture tubes and extracted once with 1 ml of diethyl ether (Wako, pure chemical industries, Ltd., Osaka, Japan). The tubes were mixed by direct-mixture (Thermal Kagaku Sangyo Co., Ltd., Tokyo, Japan) intermittently for 5 minutes and allowed to remain for more than 10 min to separate diethyl ether and aqueous layers. Then, the tubes were place in a cooled ethanol bath (approximately -40 °C) to froze the aqueous layer. The ether layer was decanted into 10 × 75

mm culture tubes and dried by a centrifugalized evaporator (Sakuma corporation, Tokyo, Japan) at 70 °C for 15 min. Then, the tubes were rinsed with 0.5 ml of diethyl ether and dried again for 5 min.

The dried samples were re-dissolved in 100 µl of 1% BSA-PBS. The solution in the wells of the immunoplate was discarded and washed two times with 0.05 % Tween 80 (Becton, Dickinson and Company, MD, USA) solution, and 10 µl of samples were added to the wells. To these wells, 100 µl of diluted horseradish peroxidase-conjugated progesterone prepared in our laboratory was added, and then 100 µl of diluted anti-progesterone antibody was added. The plate was sealed with adhesive film and incubated for 24 h at 4 °C in refrigerator. After incubation, the wells were washed four times with Tween 80 solution. Then, 100 µl of diluted tetramethylbenzidine (TMBZ, Wako, pure chemical industries, Ltd., Osaka, Japan) solution was added as peroxidase substrate, and the plate was incubated for 40 min in dark. The reaction was stopped by addition of 100 µl of 4N H_2SO_4. The optical density was measured at 450 nm in a micro-plate reader (Model 680; BIO-RAD, Tokyo, Japan).

2.6. Statistical analyses

All experimental data are presented as mean and SD. The data were analyzed using the statistical software program SPSS for Windows version 20.0. Analysis methods used in the studies were described in each Chapter.

For the identification of LH pulses in frequently collected samples in Chapters 3, 5 and 6, a cluster analysis program was used (116). The cluster algorithm searched for significant increases and decreases among data points in a series via pooled *t*-tests. Patterns of LH pulses differed between the stages of the early and the mid-luteal phase as previously reported (82), and the following criteria were used for effective detection of pulses. For the analysis of LH pulses on Days 2 - 6, 2 points were used for the determination of a peak and 1 point to

establish a nadir. For the analysis of LH pulses on Days 8 and 14, 2 points were used for the determination of a peak and 2 points to establish a nadir. For the identification of progesterone pulses in blood collected from the caudal vena cava and the jugular vein in Chapters 5 and 6, the same procedure was used as the identification of LH pulses on Days 8 and 14. Characteristics of pulsatile patterns of LH and progesterone were analyzed in terms of mean concentration, pulse frequency (number of pulses per sampling period), pulse amplitude and basal level. Pulse amplitude was calculated by subtracting the maximal concentration of a pulse from the concentration preceding the pulse and expressed as ng/ml.

In Chapters 5 and 6, temporal relationships between pulses of LH in the jugular vein and progesterone in the caudal vena cava were determined according to the method described by Rhodes et al. (89), where the percentage of LH pulses that were followed by a progesterone pulse within 60 min of the peak of the LH pulse was calculated. In addition, the percentage of progesterone pulses that followed a LH pulse within 60 min of the peak of the LH pulse was calculated. Differences between groups or treatments were tested using chi-squared analysis or Fisher's exact test.

Fig.2-1 Catheter introducer kit (Radifocus® introducer Ⅱ H, Terumo, Tokyo, Japan) used for catheterization into the caudal vena cava.

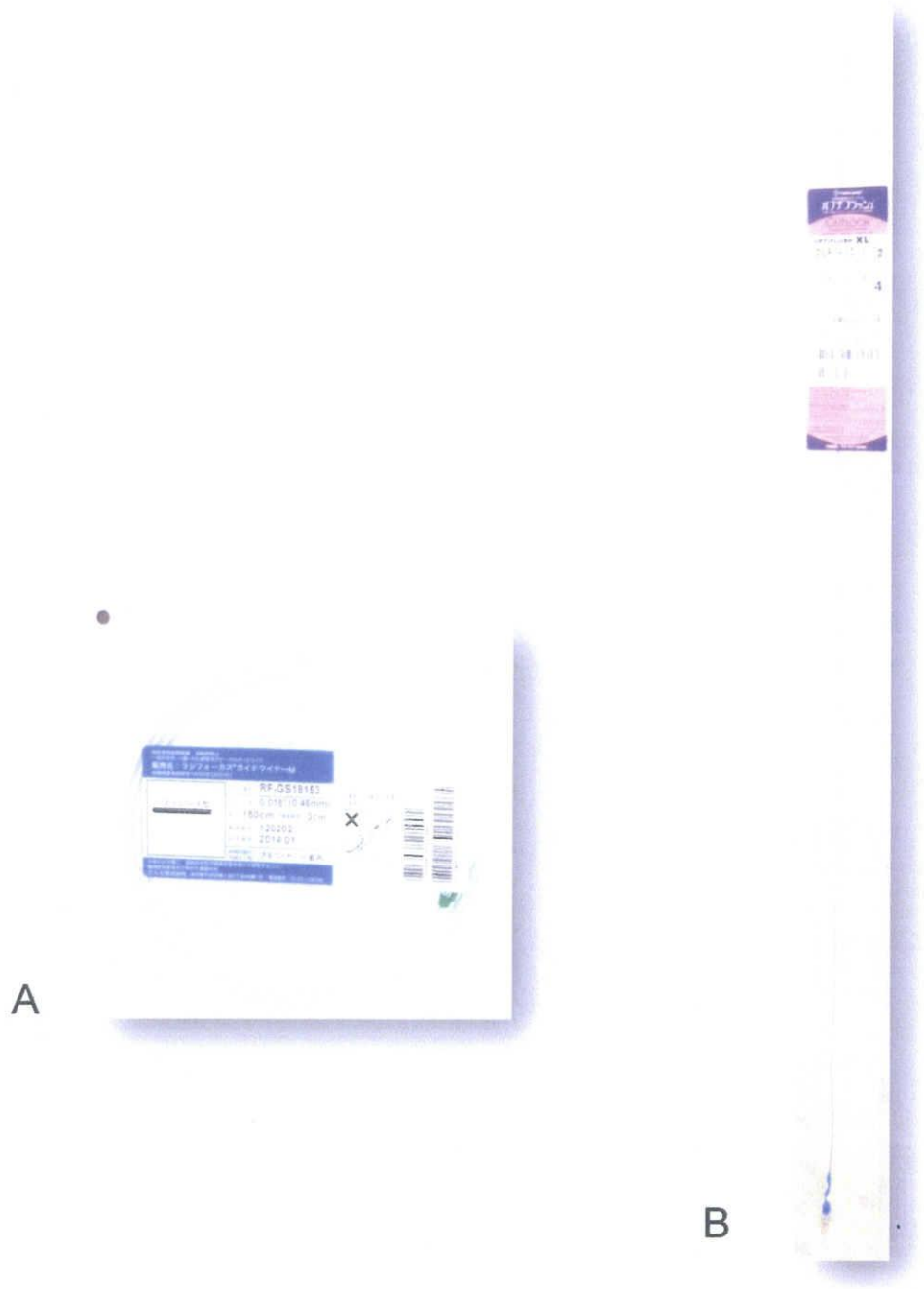

Fig.2-2 Hydrophilic-coated guide wire (Radifocus® guide wire M; 0.46 mm in diameter; 150 cm in length; Terumo, Tokyo, Japan; A) and catheter (Optiflash® XL; inside diameter, 1.12 mm; outside diameter, 1.35 mm; 110 cm in length; Terumo; B) used for catheterization into the caudal vena cava.

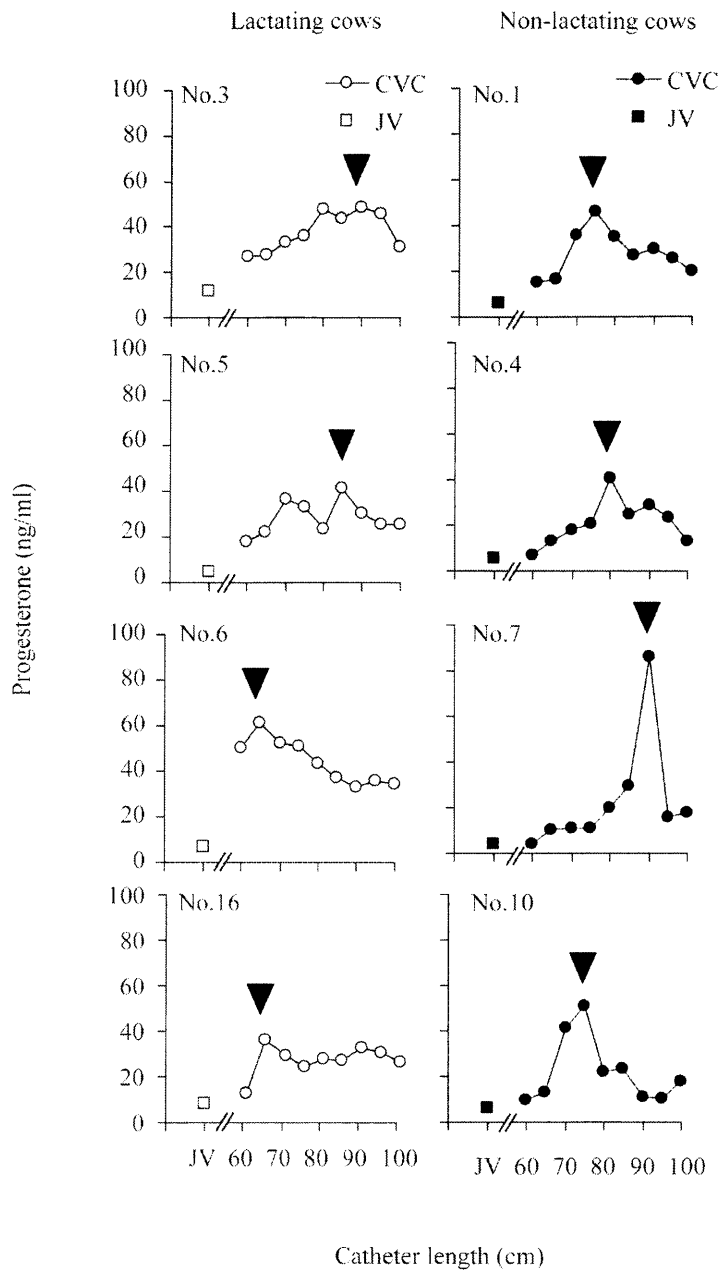

Fig.2-3 Progesterone concentrations in blood collected from the caudal vena cava (CVC) at different catheter insertion length in lactating (open circles, left panels) and non-lactating (filled circles, right panels) cows examined in Chapter 5. The progesterone concentration in the jugular blood (JV) collected at the time of catheter insertion were indicated by square in the graph, with open square (lactating cows) and filled square (non-lactating cows). The catheter for CVC was finally fixed at the length showing the highest value of progesterone in individual cows (arrow head).

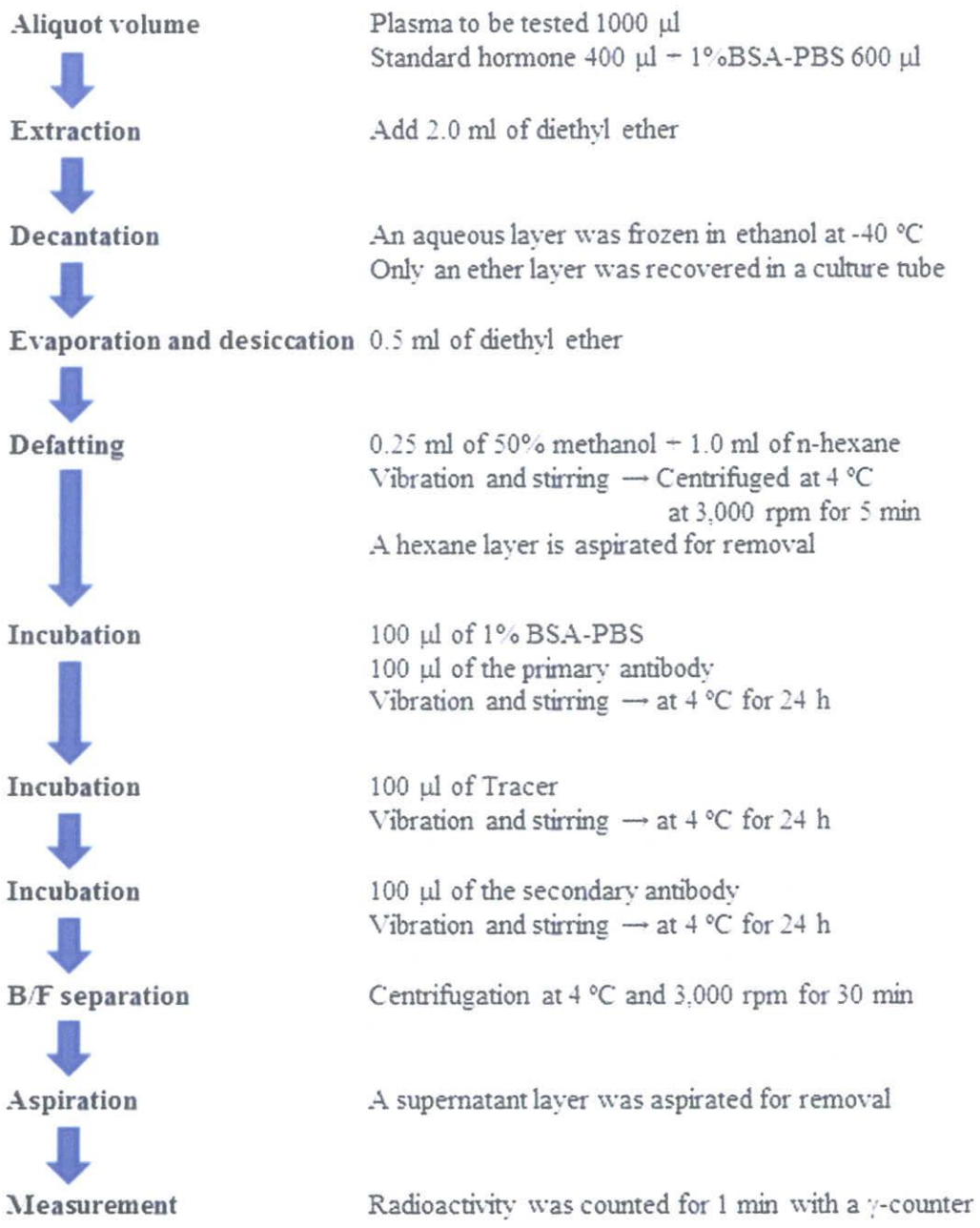

Fig.2-4 Assay procedure for estradiol-17β.

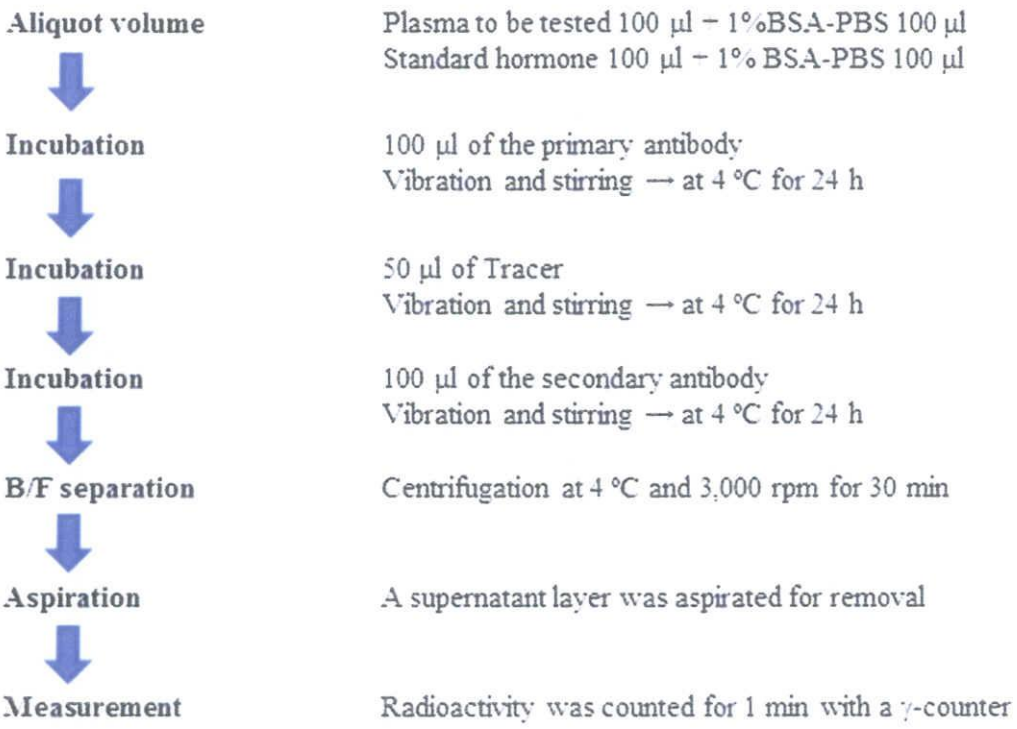

Fig.2-5 Assay procedure for LH.

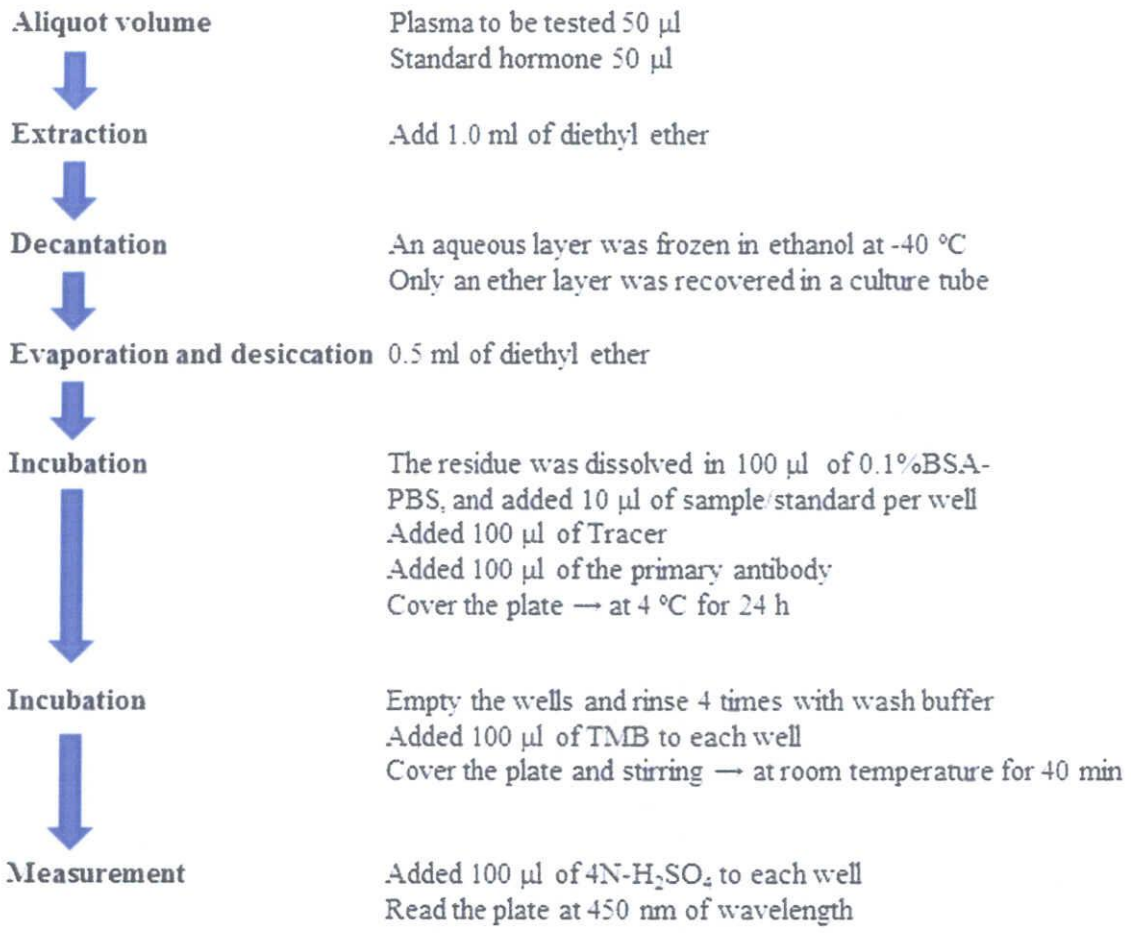

Fig.2-6 Assay procedure for progesterone.

Chapter 3. Comparison between lactating and non-lactating cows on follicular growth and corpus luteum development, and endocrine patterns of ovarian steroids and luteinizing hormone in the estrous cycles

3.1. Introduction

The ovarian cycle is central to reproductive function. It is characterized by the follicular development and ovulation as well as the formation, function and regression of the CL. The onset of normal ovarian cyclic activity is one of the essential events for reproduction in lactating dairy cows. However, ovarian disturbances such as anovulation, formation of cystic ovaries, and short or prolonged luteal phase are observed at a high rate in high-yielding cows during the early postpartum period (66). Even when lactating cows show normal estrous cycle, the conception rate following AI is markedly lower than that in heifers. While there are many risk factors for low fertility in lactating cows, it is suggested that difference in the endocrine and metabolic characteristics between lactating and non-lactating cows may directly influence the ovarian activities of steroidogenesis (17). Alternatively, the increased metabolic rates of steroid hormones associated with lactation (93, 125) could also have a great influence on circulating concentrations of these hormones and subsequent fertility in lactating dairy cows.

In cattle, LH has an essential role in the development and function of the CL (71, 79). During the luteal phase, progesterone secreted from CL regulates follicular development via negative-feedback control on pulse frequency of LH secretion (48). Studies using exogenous progesterone treatment have shown that LH pulsatile secretion increases under low progesterone concentrations, which result in prolonged growth of dominant follicle and reduced fertility after ovulation (11, 87). This suggests that low circulating concentrations of progesterone resulting from subnormal luteal function (30) or increased progesterone

metabolism (93, 125) could be associated with inadequate follicular development during spontaneous estrous cycles in cows.

Therefore, the objective of the present study was to examine the influence of lactation on ovarian function and related hormone profiles, by comparing lactating dairy Holstein cows with non-lactating ones in terms of follicular and luteal dynamics, ovarian steroid concentrations, and LH secretion pattern.

3.2. Materials and methods

3.2.1. Animals

Five lactating (one primiparous and four multiparous; 4.4 ± 2.2 years of age; 98.0 ± 11.1 days postpartum; 28.4 ± 3.2 kg of daily milk yield) and five non-lactating (one primiparous and four multiparous; 5.8 ± 2.2 years of age; 381.0 ± 112.1 days postpartum) were used in this study. Two of five non-lactating cows were reared in a paddock, and the remaining three non-lactating cows were reared in tie-stalls. At the beginning of the study, BW were 650.0 ± 41.1 and 671.6 ± 52.9 kg for lactating and non-lactating cows, respectively ($P > 0.1$), and their BCS were 3.3 ± 0.2 and 3.3 ± 0.3, respectively ($P > 0.1$). Body weights and BCS did not change significantly between the beginning and the end of the study in both lactating and non-lactating cows.

3.2.2. Experimental procedure

The study was conducted during two consecutive estrous cycles (Day 0: day of ovulation). The first cycle was defined as the period from the day of initial ovulation until the day of the following ovulation (second ovulation), and the second cycle was defined as the period from the day of second ovulation to the day of third ovulation. For the analyses of follicular and luteal development, ovarian ultrasonography was performed daily through the

experiment as described in Chapter 2. For the analyses of progesterone and estradiol-17β concentrations in plasma during the estrous cycles, blood samples (10 ml) were collected by jugular venipuncture daily throughout the study. Further, for the analyses of pulsatile patterns of LH and progesterone concentrations, blood samples were collected via a jugular catheter at 15-min intervals for 8 h (1000 to 1800 h) on days 2, 4, 6, 8 and 14 of the second cycle. The obtained blood samples were processed for hormone assays as described in Chapter 2.

3.2.3. Assays

Estradiol-17β, progesterone and LH were measured in plasma as described in Chapter 2. For the estradiol-17β assay, the intra- and interassay coefficients of variation were 10.1% and 13.0%, and the sensitivity was 0.92 pg/ml. For the progesterone assay, the intra- and interassay coefficients of variation were 3.4% and 7.4%, and the sensitivity was 0.03 ng/ml. For the LH assay, the intra- and interassay coefficients of variation were 6.1% and 11.9%, and the sensitivity was 0.09 ng/ml.

3.2.4. Statisitcal analysis

In the present study, two estrous cycles in each cow were considered as independent observations and comparison was made between 10 lactating cow records and 10 non-lactating cow records. To assess sequential data of luteal diameter and hormone concentrations, two-way ANOVA with repeated measures was used for determining the main effects of group and day and their interaction. When significant differences were detected, Tukey's post hoc follow-up test or Student's *t*-test was used to detect significant differences among groups within days and among days within groups. To assess data for the development of dominant follicles such as follicular diameter and duration of a follicular wave, estrous

cycles were subdivided according to the number of follicular waves (two or three follicular waves, see **Fig. 3-1**). Then, the statistical difference was analyzed by one-way ANOVA followed by Student's *t*-test. Percentage of cycles in which at least one SF grew to more than 10 mm in diameter was compared between groups by Fisher's exact test. For the identification of LH pulses in the samples collected frequently on Days 2, 4, 6, 8, and 14, a cluster analysis program (116) was used as described in Chapter 2.

3.3. Results

3.3.1. Luteal development and progesterone concentration during the estrous cycles

The length of estrous cycle did not differ significantly between lactating and non-lactating cows (22.6 ± 2.5 vs. 21.3 ± 0.8 days; mean ± SD). Changes in CL diameter and plasma progesterone concentrations from Day 0 to 14 are shown in **Fig. 3-2**. Comparison of progesterone concentrations was made on samples collected once a day. Lactating cows developed larger CL in diameter compared with non-lactating cows on Days 8 - 14 (25.4 ± 1.8 vs. 23.5 ± 1.5 mm, $P < 0.01$). Moreover, differences between lactating and non-lactating cows were detected in both the maximal CL diameter (26.3 ± 2.5 vs. 23.9 ± 1.5 mm, $P < 0.05$) and the maximal CL volume (9239.0 ± 2084.0 vs. 7182.7 ± 1319.2 mm^3, $P < 0.05$). Mean progesterone concentration in the samples collected daily from Day 0 to 7 did not differ significantly between lactating and non-lactating cows (1.6 ± 1.2 vs. 1.5 ± 1.1 ng/ml; **Fig. 3-2**). However, mean progesterone concentration in plasma samples collected daily from Day 8 to 14 was higher in lactating cows than in non-lactating cows (4.6 ± 1.0 vs. 3.9 ± 0.9 ng/ml, $P < 0.01$).

3.3.2. Follicular dynamics and estradiol-17β concentrations

Four of 10 cycles in lactating cows (40.0%) and three of 10 cycles in non-lactating cows (30.0%) had three follicular waves, and the remaining cycles had two follicular waves. Characteristics of the dominant follicle of the first wave and plasma estradiol-17β concentrations are shown in **Table 3-1**. In the estrous cycles with two follicular waves, the maximal diameter of the dominant follicle of the first wave was larger in lactating cows than that in non-lactating cows (17.2 ± 1.8 vs. 15.5 ± 0.8 mm, $P < 0.05$), but the duration of the first wave did not differ significantly between groups. In the estrous cycles with three follicular waves, the maximal diameter of the dominant follicle (15.9 ± 2.1 vs. 13.5 ± 1.9 mm) and the duration of the first wave did not differ significantly between lactating and non-lactating cows. Estradiol-17β concentration showed an increase during the development of the dominant follicle of the first wave. In this period, peak concentration of estradiol-17β and day of estradiol-17β peak were not significantly different between lactating and non-lactating cows both in the estrous cycles with two and three follicular waves (**Table 3-1**).

In all cows, one or two follicles ovulated at the end of the cycle. Double ovulations occurred in two of 10 cycles in lactating cows (20.0%) and one of 10 cycles in non-lactating cows (10.0%). Characteristics of the ovulatory follicle in the estrous cycles with two or three follicular waves are shown in **Table 3-2**. In the estrous cycles with two follicular waves, maximal diameter of ovulatory follicle in lactating cows was larger than that in non-lactating cows (17.9 ± 1.2 vs. 15.2 ± 0.8 mm, $P < 0.05$), but days from emergence to ovulation of the ovulatory follicle did not differ significantly between groups. In the estrous cycles with three follicular waves, there were no significant differences between lactating and non-lactating cows in terms of the maximal diameter of the ovulatory follicle (13.6 ± 1.7 vs. 12.3 ± 2.8 mm) and in terms of the days from emergence to ovulation. Peak concentration of estradiol-17β around ovulation did not differ significantly between lactating and non-lactating cows (10.9 ± 3.7 vs. 12.4 ± 4.1 pg/ml).

The number of SF in each wave did not differ significantly between lactating and non-lactating cows (3.0 ± 1.2 vs. 2.4 ± 1.4 follicles/wave). However, the diameter of largest SF was larger in lactating cows than in non-lactating cows (8.8 ± 2.2 vs. 7.5 ± 0.8 mm, $P < 0.01$). Moreover, large SF that grew to more than 10 mm in diameter was detected in five of 10 cycles (50.0%) in lactating cows, but none of 10 cycles (0%) in non-lactating cows ($P < 0.05$).

3.3.3. Luteinizing hormone and progesterone concentrations in frequently collected samples

Representative profiles of pulsatile LH secretion and progesterone concentrations during frequent blood sampling periods of Days 2, 4, 6, 8, and 14 in one lactating (No. 10) and one non-lactating cow (No. 4) are shown in **Fig. 3-3**. In the lactating cow (No. 10), the number of LH pulses detected during the sampling period for 8 h was decreased from 7 pulses on Day 2 to 5 pulses on Day 6, but thereafter remained unchanged until Day 14, although the plasma concentration of progesterone increased gradually from 0.5 ng/ml on Day 2 to 6.3 ng/ml on Day 14. On the other hand, in the case of non-lactating cow (No. 4), the number of LH pulses decreased gradually from 6 pulses on Day 2 to 2 pulses on Day 14, while the plasma concentration of progesterone increased from 0.7 ng/ml on Day 2 to 5.9 ng/ml on Day 14.

Comparisons of LH secretion and plasma progesterone concentration between lactating and non-lactating cows were made on the samples collected at 15 min-intervals for 8 h (**Table 3-3**). The frequency of LH pulses decreased on Days 6, 8, and 14 compared with that on Day 2 in both lactating and non-lactating cows ($P < 0.05$). The frequency of LH pulses in each sampling period of Days 2, 4, 6, 8, and 14 did not differ significantly between lactating and non-lactating cows, but overall mean of LH pulse frequency was higher in lactating cows than in non-lactating cows (5.5 ± 1.6 vs. 4.4 ± 1.8 pulses/8 h, $P < 0.01$). Luteinizing hormone

concentration was different between lactating and non-lactating cows on Day 2 ($P < 0.05$), but no further difference was detected between groups thereafter. Progesterone concentration increased on Days 6, 8, and 14 compared with that on Day 2 in both lactating and non-lactating cows ($P < 0.05$). On Days 4 and 6, progesterone concentration was lower in lactating cows than in non-lactating cows ($P < 0.01$), but no significant difference was detected between the groups on Day 8. On Day 14, lactating cows showed higher progesterone concentration than non-lactating cows ($P < 0.01$).

3.4. Discussion

Contrary to our expectation, lactating cows developed larger CL and had higher progesterone concentrations during mid-luteal phase than non-lactating cows. Concentrations of progesterone in blood are dependent on the amount of steroidogenic tissue and capacity of the steroidogenic tissue to secrete (synthesize and release) progesterone. Previous studies showed that there was a positive association between plasma progesterone concentration and CL size in the mid-luteal phase of dairy cows (56, 90). It is widely accepted that LH plays an important role in the development of CL in most mammals. A previous study reported that CL development was dependent on pulsatile release of LH from Day 2 to 12 of the estrous cycle in cattle (71). Inhibition of the release of LH pulses by treatment of GnRH antagonist during luteal development decreased the maximal diameter of CL and progesterone concentration during the luteal phase compared with the values for control cows (79). During mid-luteal phase, LH pulses stimulate the secretion of progesterone from the functional CL (75) together with other luteotropic hormones such as growth hormone and IGF-1 (98, 102). In the present study, LH pulse frequency was higher in lactating cows than in non-lactating cows throughout the frequent sampling period on Days 2 - 14. Therefore, the increased frequency of LH pulses might contribute to the development of larger CL and higher concentrations of

plasma progesterone during mid-luteal phase of lactating cows compared with those of non-lactating cows. On the other hand, frequent blood sampling at 15-min intervals detected a significant decrease in the concentrations of progesterone on Days 4 and 6. It is also possible that lactating condition brings about transient decrease in the blood levels of progesterone during the early luteal phase in dairy cows.

In the estrous cycles with two follicular waves, the dominant follicle of the first wave and the ovulatory follicle of the lactating cows grew larger than those of non-lactating cows. In the estrous cycles with three follicular waves, although the sample number was too small to detect significant differences, the maximal diameters of the dominant follicle of the first wave and the ovulatory follicle in lactating cows were larger than those in non-lactating cows. Increased size of the ovulatory follicle in lactating cows was previously reported in comparisons of lactating and non-lactating cows (17, 96) or of lactating cows and nulliparous heifers (128). Previous studies demonstrated that the larger preovulatory follicles in lactating cows could have resulted from the increased frequency of LH pulses and the longer duration of dominance (100). Furthermore, increased duration of development of ovulatory follicle from emergence to estrus was associated with prolonged increases in estradiol-17β concentration (1). In the present study, however, days from emergence of follicle to ovulation were not different between lactating and non-lactating cows. In addition, despite the larger follicles in lactating cows, no difference was detected in estradiol-17β peak around ovulation between the groups. Similarly, in lactating cows, the dominant follicle of the first wave grew larger than that of non-lactating cows, but the duration of the first wave and the peak estradiol-17β concentration during the first wave did not differ from those of non-lactating cows. Taking these findings together, it is conceivable that an increase in LH pulse frequency detected in the lactating cows in this study is not so intensive as to prolong the duration of dominant follicles or to increase circulating estradiol-17β concentration. However, it remains

unclear whether the development of larger follicles in lactating cows has a negative impact on oocyte quality. Functional aspects of larger follicles in lactating cows should be examined in the future study.

Interestingly, lactating cows had larger SF in terms of diameter than non-lactating cows, although the number of SF in each wave was not different between the groups. It is noticeable that large SF that grew to more than 10 mm in diameter was detected in 50% of the cycles in lactating cows, whereas none of the SF grew larger than 10 mm in non-lactating cows. In cattle, follicles acquired ovulatory capacity at about 10 mm in diameter (95). It is likely that high-producing cows might select more than one dominant follicle, which results in high incidence of double ovulation (124). The incidence of double ovulation was not assessed in this study because of the small number of animals, but it is speculated that development of larger SF may participate in the process that produces double ovulation in lactating cows.

Low fertility in lactating cows is often explained by luteal deficiency and lower progesterone concentration insufficient for establishment and maintenance of pregnancy. Recent studies suggest that elevated steroid metabolism associated with high milk production might reduce circulating concentrations of ovarian steroids, which can be a potential cause for low fertility in lactating dairy cows (93, 125). In the present study, data from frequently collected samples from early to mid-luteal phase showed that lactating cows had lower progesterone levels in early luteal phase on Days 4 and 6 and higher levels in mid-luteal phase on Day 14. Progesterone concentration in the circulating blood reflects the balance between production and metabolic rate of progesterone. It is assumed that the lower progesterone concentrations in early luteal phase in the lactating cows may be due to either insufficient capacity of progesterone synthesis by the developing CL or increased metabolic clearance of progesterone from the systemic blood. Observations based on ovarian ultrasonography partially allow the assessment of functional aspects of the CL (44, 56, 107),

and no apparent difference was found in the CL size in early luteal phase between lactating and non-lactating cows. Therefore, it is possible that the lower concentrations of progesterone in the lactating cows during frequent blood sampling periods of Days 4 and 6 might be associated with the increased metabolic rate of progesterone in lactating cows (93). In addition, lactating animals lose a certain amount of progesterone into milk, although the total amount of progesterone excreted in milk is very small compared with the production rate from the CL (35). In particular, during early luteal phase, developing CL cannot produce as much progesterone as functional CL does. Then, the lactating cows might compensate for the greater loss of progesterone to some extent by developing larger CL and elevating progesterone secretion. The lactating cows used in the present study were in mid-lactation period (> 80 days postpartum), and maintained their BCS and BW within a normal range. Although the exact mechanism participating in the maintenance of circulating progesterone level in the lactating cows is still unclear, it is likely that lactating cows, under a moderate level of milk production and in good nutritional condition, have the ability to produce sufficient progesterone to maintain normal circulating progesterone level.

In summary, the lactating cows had larger CL and higher progesterone concentrations during mid-luteal phase, and showed higher frequency of LH pulses during early to mid-luteal phase than the non-lactating cows. Further, the follicles grew larger in the lactating cows than in the non-lactating cows, although the estradiol-17β concentrations were similar throughout the estrous cycle. These results indicate that the increased LH pulses detected in the lactating cows are probably involved in larger development of CL and follicles and higher concentrations of plasma progesterone during mid-luteal phase, which is likely to be a physiological modulation in the endocrine pattern of lactating cows.

Table 3-1 Characteristics of dominant follicle of the first wave in lactating and non-lactating cows.

	Two follicular waves		Three follicular waves	
	Lactating	Non-lactating	Lactating	Non-lactating
Item	(n = 6)	(n = 7)	(n = 4)	(n = 3)
Maximal diameter (mm)	17.2 ± 1.8 [c]	15.5 ± 0.8 [d]	15.9 ± 2.1	13.5 ± 1.9
Duration of first wave [a)] (d)	9.7 ± 1.0	10.1 ± 0.9	9.8 ± 1.0	7.3 ± 2.1
Estradiol-17β peak (pg/ml)	3.5 ± 1.3	4.8 ± 2.2	4.9 ± 0.6	4.9 ± 3.2
Day of estradiol-17β peak [b)]	4.3 ± 0.8	4.6 ± 2.1	4.8 ± 0.5	3.7 ± 0.6

Values are mean and SD.

[a)] Interval from the emergence of the first wave to the emergence of the second wave.

[b)] Days after ovulation.

[c, d] Different ($P < 0.05$) within each row.

Table 3-2 Characteristics of the ovulatory follicle in the estrous cycles with two and three follicular waves in lactating and non-lactating cows.

	Two follicular waves		Three follicular waves	
	Lactating	Non-lactating	Lactating	Non-lactating
Item	(n = 6)	(n = 7)	(n = 4)	(n = 3)
Maximal diameter (mm)	17.9 ± 1.2 [a]	15.2 ± 0.8 [b]	13.6 ± 1.7	12.3 ± 2.8
Days from emergence to ovulation	12.0 ± 1.3	10.9 ± 1.2	7.0 ± 1.4	7.3 ± 2.1

Values are mean and SD.

[a, b] Different ($P < 0.01$) within each row.

Table 3-3 LH pulse frequency, mean LH and progesterone concentration in frequently collected samples on Days 2, 4, 6, 8, and 14 of the second estrous cycle in lactating and non-lactating cows.

Group	Days of the estrous cycle					Overall
	2	4	6	8	14	
	LH pulse frequency (pulses/8h)					
Lactating (n=5)	7.6 ± 0.9^{x}	6.2 ± 0.4	5.6 ± 1.1^{y}	4.2 ± 0.4^{y}	3.8 ± 1.3^{y}	5.5 ± 1.6^{c}
Non-lactating (n=5)	6.4 ± 0.9^{x}	6.0 ± 0.7	4.2 ± 0.8^{y}	3.2 ± 0.8^{y}	2.4 ± 1.1^{y}	4.4 ± 1.8^{d}
	LH concentration (ng/ml)					
Lactating (n=5)	0.9 ± 0.3^{a}	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.4	0.8 ± 0.3	0.8 ± 0.2
Non-lactating (n=5)	1.0 ± 0.4^{b}	0.9 ± 0.4	0.8 ± 0.3	0.7 ± 0.3	0.8 ± 0.2	0.8 ± 0.3
	Progesterone concentration (ng/ml)					
Lactating (n=5)	0.8 ± 0.3^{x}	1.6 ± 0.7^{a}	2.5 ± 0.7^{cy}	3.5 ± 0.8^{y}	5.2 ± 1.0^{cy}	2.7 ± 1.7
Non-lactating (n=5)	0.8 ± 0.4^{x}	1.8 ± 0.6^{b}	2.9 ± 1.0^{dy}	3.7 ± 1.3^{y}	4.8 ± 1.2^{dy}	2.8 ± 1.7

Values are mean and SD.

$^{a-d}$ Different ($^{a, b}$ $P < 0.05$, $^{c, d}$ $P < 0.01$) within each column.

$^{x, y}$ Different ($P<0.05$) within each row.

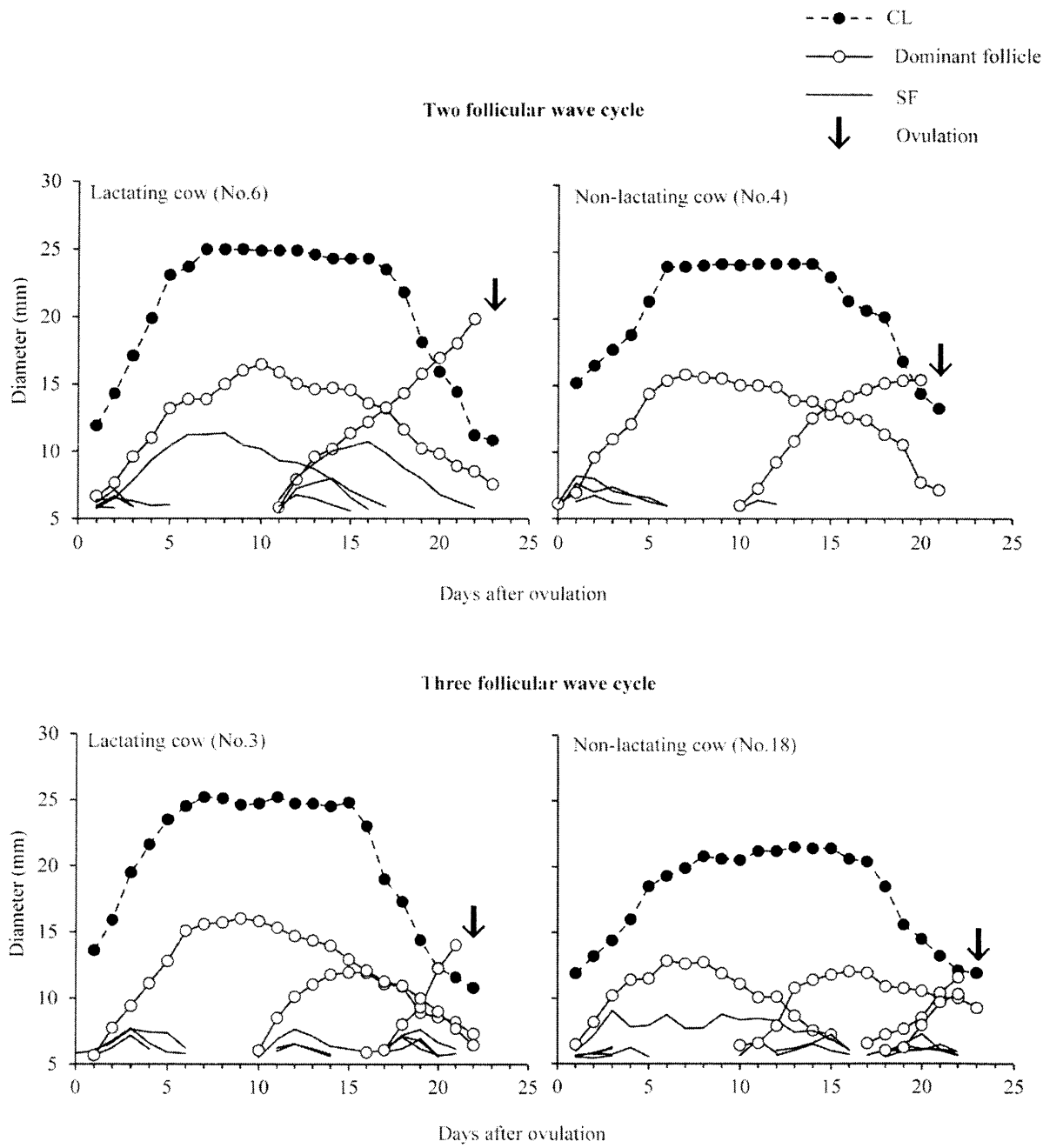

Fig. 3-1 Patterns of follicular dynamics and CL development in lactating and non-lactating cows with two or three follicular waves per one estrous cycle.

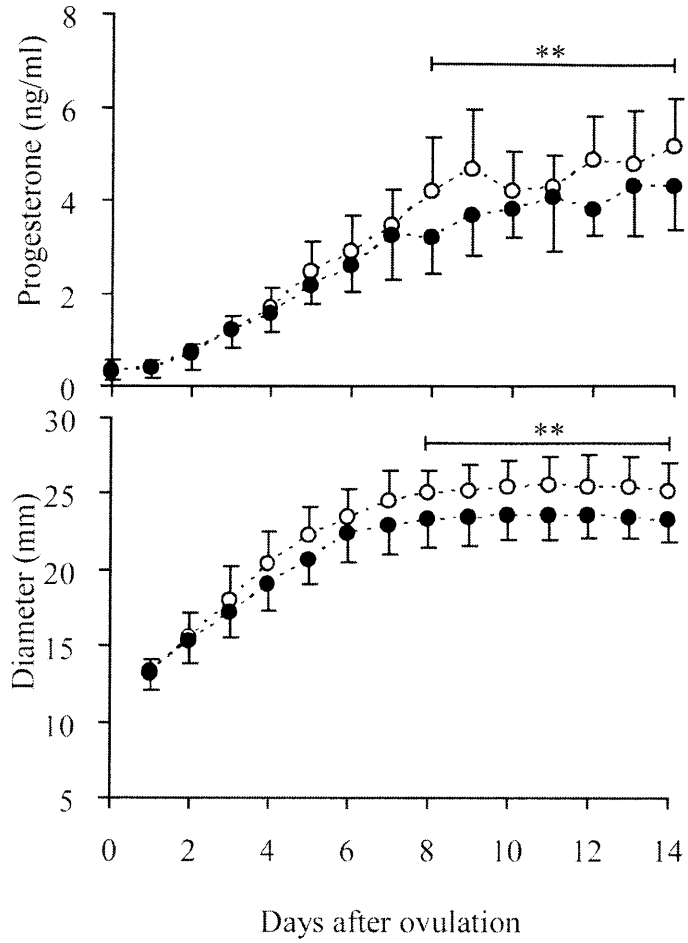

Fig. 3-2 Changes in CL diameter (bottom panel) and plasma progesterone concentrations (upper panel) in lactating (n = 10, open circles) and non-lactating (n = 10, filled circles) cows from Day 0 to 14 of the estrous cycle. Comparison of progesterone concentration was made on samples collected once a day. Mean CL diameter and plasma progesterone concentrations from Day 8 to 14 is higher (** $P < 0.01$) in lactating cows than in non-lactating cows. Day 0 is the day of ovulation and values are mean and SD.

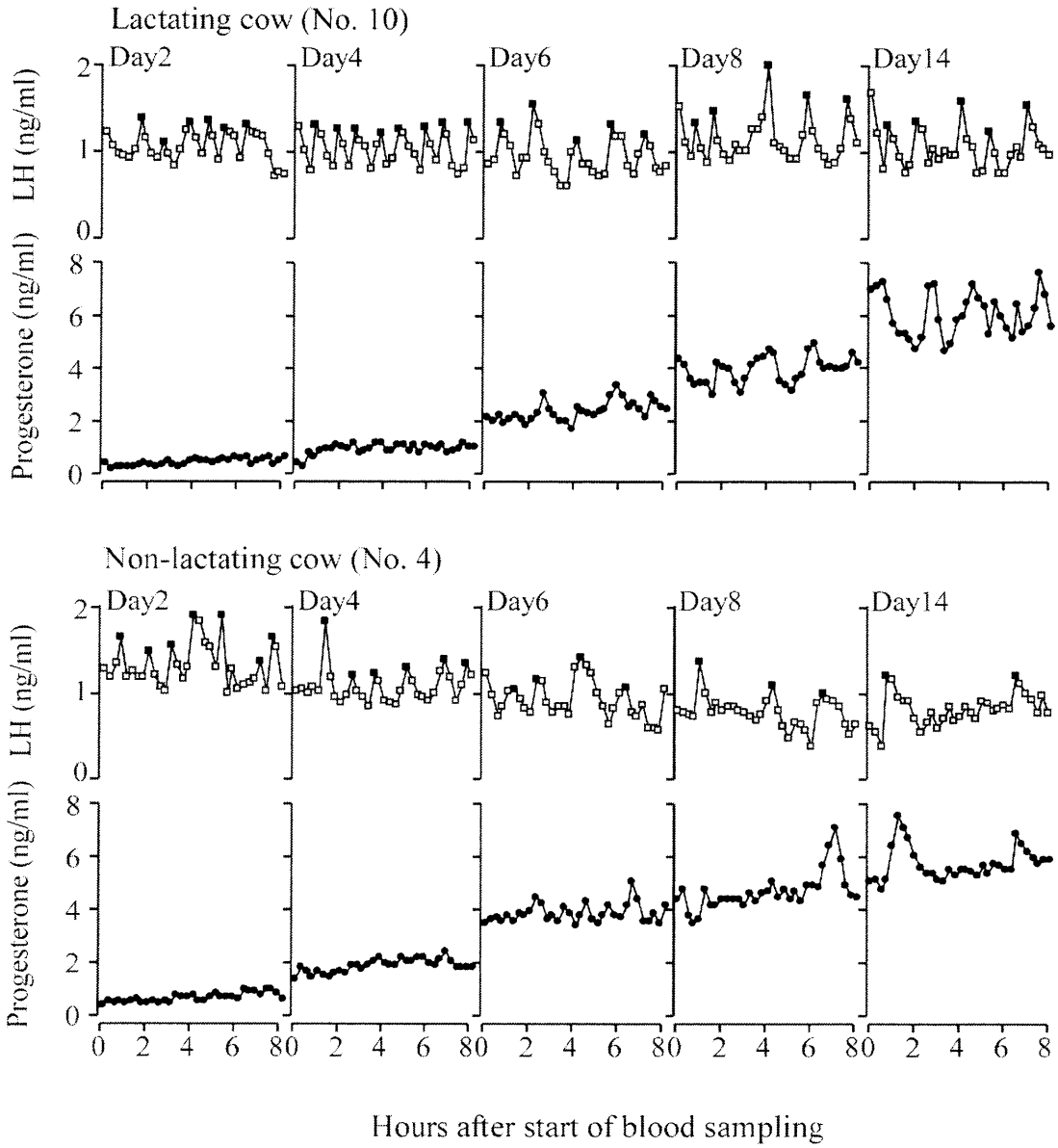

Fig. 3-3 Representative LH (squares) and progesterone (circles) secretion pattern obtained from the frequently collected samples on Days 2, 4, 6, 8, and 14 of the estrous cycle in one lactating cow (No. 10, upper panel) and one non-lactating cow (No. 4, bottom panel). Filled squares represent peaks of statistically identified LH pulses.

Chapter 4. Comparison between lactating and non-lactating cows on endocrine patterns of ovarian steroids and luteinizing hormone and estrous signs from luteolysis to ovulation

4.1. Introduction

Estrus detection, in general, is primarily based on the observation of standing behavior of cows (21, 28). According to an earlier study reported in 1948 (111), the duration of estrus was 17.8 h for dairy cows and 15.3 h for dairy heifers. However, recent studies reported that the expression of estrous behavior has been decreasing in modern dairy cows (23, 47, 114, 129, 131). In these studies, the duration of standing estrus was less than 10 h (129, 131), and more than one-third of the cows did not show standing estrus (114).

The causes of poor expressions of estrus and estrous signs in modern dairy cows have been investigated with relevance to endocrine aspects, herd management or estrus detection methods. Previous studies on endocrine events around estrus and ovulation have revealed that alterations in the concentrations of ovarian steroids and gonadotropins may influence expression of estrus and estrous signs. For example, Lyimo et al. (57) found high correlations between the estradiol-17β concentration and visual estrous symptoms. Another study suggests that high milk production decreases the duration of estrus, probably due to decreased circulating concentrations of estradiol-17β (52). Suprabasal progesterone concentrations during the periestrous period, which may be caused by incomplete luteolysis and acute stress, could inhibit the expression of estrous behavior (130) and lead to delayed ovulation (24). Some of these alterations have been reported in association with high milk production of modern dairy cows. In Chapter 3, the lactating cows developed larger ovulatory follicles than non-lactating cows, but no difference was detected between lactating and non-lactating cows in the estradiol-17β concentration from daily collected samples throughout the estrous cycle.

More intensive monitoring during the period from luteolysis to ovulation would be helpful in examining the effect of lactation on hormone dynamics such as peak concentrations of estradiol-17β around estrus and occurrence of LH surge.

Facing the problem of low detection of standing estrus, alternative methods to predict estrus by using pedometers to measure physical activity (57, 92) or monitoring internal changes such as body temperature (83) are being used and developed in practice. Vaginoscopic examination is generally utilized as an additional aid for visual observations of estrous signs, especially in cows housed in tie-stall barns. This is because behavioral observations are often inadequate compared with those in cows housed in free-stall barns. The appearances of the vulval mucosa and cervical mucus are altered under the influence of estrogens (43), which could be accurate indicators of the stage of the estrous cycle. There are only general data available on characteristics of the estrous signs in the vagina as well as those in the vulva. Temporal changes in vaginal estrous signs from luteolysis to ovulation have not been well examined in association with preovulatory hormonal profiles and other estrous signs.

Therefore, the objectives of the present study were to investigate comprehensively the profiles of ovarian steroids and LH and the appearance of behavioral and genital estrous signs in relation to luteolysis and ovulation in lactating and non-lactating dairy cows and to examine the influence of lactation on those observations.

4.2. Materials and methods

4.2.1. Animals and experimental procedures

The present study was conducted during the period from luteolysis to ovulation in the first estrous cycle in Chapter 3. Thus, the animals were the same in Chapter 3 (five lactating and five non-lactating cows). For analysis of estradiol-17β, progesterone and LH

concentrations, blood samples (10 ml) were collected via a jugular catheter at 3-h intervals, beginning on the day after a decrease in diameter of the CL was confirmed by ultrasonography until ovulation. The obtained blood samples were processed for hormone assays as described in Chapter 2. Estrous signs were observed in terms of behavior, the vulva and the vagina at 8-h intervals (at 0700, 1500 and 2300 h) from the day of confirmation of luteolysis by daily ultrasonographic examination until ovulation. Time of ovulation was determined by ultrasound examinations, beginning on the time when a declining intensity of estrous signs was detected. Thereafter, scanning of the ovary was performed at 3-h intervals from immediately after blood sampling until ovulation was confirmed. The time of ovulation was defined as the time when the preovulatory follicle disappeared.

4.2.2. Observation of estrous signs

First, cows were observed for behavioral changes for 30 min including restlessness and bellowing. Restlessness was checked for with the following behavioral characteristics (84): not showing any interest in feed while other cows were eating, sometimes standing while other cows were lying, teasing nearby cows, paying close attention to the people working nearby and sometimes trying to mount them. After observation, the cows were restrained with a rope and checked for external genital changes including hyperemia and swelling of the vulva and mucous discharge from the vulva. Then, vaginoscopic examination was carried out according to the method described by Lu et al. (53) to monitor intravaginal changes including hyperemia, swelling and relaxation of the vaginal portion of the cervix and mucous discharge from the external uterine orifice. Typical appearances of the anterior portion of the vagina and the external uterine orifice observed by using vaginoscopy at several stages of estrous cycle are shown in **Fig. 4-1**. Intensity of estrous signs was assessed comprehensively from the above changes using a three-point scale (1 = no signs, 2 = moderate signs, 3 = conspicuous

signs). In particular, the following criteria were used for assessing behavioral intensity: a score of 1 indicates that no behavioral change was observed; a score of 2 indicates that some of behavioral changes were observed; and a score of 3 indicates that almost all of the behavioral changes were frequently observed with high activity during a 30-min observation period. Appearance of estrous signs was determined as the time when an estrous sign was first seen. Disappearance of estrous signs was determined as the time when the last estrous sign disappeared. Duration of estrous signs was estimated as the interval from the appearance to the disappearance of estrous signs.

4.2.3. Hormone assays

Estradiol-17β, progesterone and LH were measured in plasma as described in Chapter 2. For the estradiol-17β assay, the intra- and interassay coefficients of variation were 10.1% and 13.0%, and the sensitivity was 0.92 pg/ml. For the progesterone assay, the intra- and interassay coefficients of variation were 3.4% and 7.4%, and the sensitivity was 0.03 ng/ml. Luteinizing hormone was measured in a single assay. The intra-assay coefficient of variation of LH was 11.6%, and the sensitivity was 0.11 ng/ml.

The LH surge was defined as a sustained rise (for at least two consecutive blood samples) in the plasma LH concentrations exceeding twice the average baseline level. The baseline level was calculated by averaging the plasma LH concentrations for a 6 h period (two samples) preceding a surge.

4.2.4. Statistical analyses

Patterns of hormone concentrations in the two groups were compared by repeated measures ANOVA. Other characteristics such as maximal hormone concentration were compared by the Student's *t*-test. Data related to appearance or disappearance of each estrous

sign and its duration were compared among the different estrous signs (behavior, vulva and vagina) by one-way ANOVA. Post hoc multiple comparisons were made by using Tukey's test. The relationship between intensity of estrous signs and estradiol-17β level was determined by Pearson's correlation coefficient analysis.

4.3. Results

4.3.1. Initiation of luteolysis to ovulation and plasma concentration of progesterone

Luteolysis started on Day 16.8 ± 2.2 (mean ± SD) and Day 17.0 ± 1.0 in the lactating and non-lactating cows, respectively, with no significant difference between the groups. The progesterone concentrations decreased sharply from the day after initiation of luteolysis and were maintained at a basal level (less than 1 ng/ml) from two days after initiation of luteolysis until ovulation in both groups (**Fig. 4-2**). All lactating cows and four of five non-lactating cows ovulated four or five days after initiation of luteolysis, and the remaining one non-lactating cow ovulated three days after initiation of luteolysis. The mean intervals from initiation of luteolysis to ovulation were not significantly different between lactating and non-lactating cows (4.6 ± 0.5 and 4.2 ± 0.8 days), which resulted in similar interovulatory intervals (21.4 ± 1.8 and 21.2 ± 1.0 days) between the groups.

4.3.2. Plasma concentrations of estradiol-17β and LH

Changes of estradiol-17β and LH concentrations from four days before ovulation to the time of ovulation did not differ significantly between lactating and non-lactating cows, as shown in **Fig. 4-3**. The maximal estradiol-17β concentration (estradiol-17β peak) and area under the curve (19) of the estradiol-17β concentration from the initiation of luteolysis to ovulation were not significantly different between lactating and non-lactating cows (**Table 4-1**). The estradiol-17β peaks were detected 3.2 ± 0.5 and 2.9 ± 0.8 days after the initiation of

luteolysis in lactating and non-lactating cows, respectively. The mean interval from the estradiol-17β peak to ovulation did not differ significantly between lactating and non-lactating cows (34.2 ± 4.5 and 30.6 ± 3.9 h; **Table 4-2**), although there were some variations among animals (ranges: 27 to 39 and 27 to 36 h in lactating and non-lactating cows, respectively). After the estradiol-17β concentration reached its peak, an acute increase in LH concentration (LH surge) was observed in all examined cows. The mean intervals from the estradiol-17β peak to the peak of the LH surge were 6.0 ± 3.7 and 2.4 ± 2.5 h in lactating and non-lactating cows, respectively. These values were not significantly different between the groups but varied among animals (ranges: 0 to 12 and 0 to 9 h in lactating and non-lactating cows, respectively). The interval from the peak of the LH surge to the time of ovulation was 27 h, with no variation in the animals of both groups. When a comparison was made in terms of the magnitude of the LH surge between groups, the peak concentration (11.7 ± 3.7 and 14.2 ± 2.8 ng/ml; **Table 4-1**), AUC (44.5 ± 21.7 and 58.8 ± 13.0) and duration (5.4 ± 2.5 and 6.0 ± 0 h) were not significantly different between lactating and non-lactating cows.

4.3.3. Estrous signs

Representative profiles of estrous signs in behavior, the vulva and vagina in one lactating (No.10) and one non-lactating (No.4) cows that showed behavioral sign and in one lactating (No.11) and one non-lactating cows (No.7) that did not show any behavioral sign are shown in **Fig. 4-4**. The proportions of cows ranked as having "conspicuous signs," "moderate signs" and "no signs" in terms of behavior were 2/5 (40.0 %), 2/5 (40.0%) and 1/5 (20.0%) in lactating cows, and these values were the same as those in non-lactating cows. On the other hand, vulval and vaginal estrous signs were clearly detected, and their intensities were ranked as "conspicuous signs" in all lactating and non-lactating cows. From these findings, no difference was detected in the intensity of estrous signs between lactating and non-lactating

cows. To determine the relationship between the intensity of behavioral estrous signs and the estradiol-17β level, data of all lactating and non-lactating cows were combined and correlations were calculated. The intensity of behavioral estrous signs was not correlated with the peak concentration of estradiol-17β ($r = 0.29$, $P > 0.1$) or the estradiol-17β AUC ($r = 0.20$, $P > 0.1$) from the initiation of luteolysis to ovulation.

There was no significant difference in the appearance of each estrous sign and its duration between the groups (**Table 4-3**). The data of both groups were combined for comparisons among estrous signs. Behavioral, vulval and vaginal estrous signs appeared 2.5 ± 1.3, 2.2 ± 1.2 and 1.6 ± 1.2 days after the initiation of luteolysis, respectively. Vaginal estrous signs appeared 0.8 ± 0.6 days earlier than behavioral signs ($P < 0.05$), and the interval from appearance of estrous signs to ovulation was significantly longer for the vaginal signs than for the behavioral signs (68.9 ± 25.4 vs. 44.8 ± 21.6 h, $P < 0.01$). The interval from disappearance of estrous signs to ovulation was 6.0 ± 5.1 h for the behavioral signs, which was greater ($P < 0.01$) than the intervals for the vulval and vaginal signs (0.4 ± 1.1 and 0.6 ± 1.8 h, respectively) because the vulval and vaginal estrous signs lasted to ovulation in four (80.0%) of the five lactating cows and four (80.0%) of the five non-lactating cows. The duration of vaginal estrous signs (68.9 ± 25.4 h) was significantly longer ($P < 0.01$) than that of behavioral signs (41.3 ± 23.6 h), but not significantly different from that of vulval signs (54.6 ± 22.9 h).

4.4. Discussion

Some previous studies have suggested the negative influence of lactation on reproductive function by comparing circulating concentrations of ovarian steroids and gonadotropins in lactating cows with those in non-lactating cows (17, 97) or nulliparous heifers (96, 97, 128). Wolfenson et al. (128) reported that concentrations of estradiol-17β

around estrus and LH surge were lower in lactating dairy cows than in heifers. In the present study, however, profiles of ovarian steroids and LH from luteolysis to ovulation in lactating dairy cows were comparable with those in non-lactating ones, suggesting that lactation does not influence the preovulatory hormonal dynamics in dairy cows. When comparison was made between lactating cows and heifers, it is difficult to distinguish among the effects of lactation, age, nutrition, energy balance and other factors on the reproductive traits (128). Moreover, the lactating cows in the above studies (96, 97, 128) were in early to mid-lactation (mean, 46 to 77 days postpartum), whereas the present study was conducted when the cows were in mid-lactation (mean, 98.0 ± 11.1 days postpartum). The daily milk yields of the cows in the above studies (mean, 46 to 49 kg/day) were much higher than those of the present study (mean, 28.4 ± 3.2 kg/day). The stage of lactation and level of milk production may also be important factors in determining the influence of lactation on reproductive function.

In the present study, the interovulatory intervals were within the normal range (18-24 days (64)) in all lactating and non-lactating cows. A recent study reported that lactating cows showed longer intervals from spontaneous luteolysis to ovulation (5 or 6 days) than heifers (4 or 5 days) (96). The longer interval from luteolysis to ovulation could extend the growth period of dominant follicles (97), which might be related to reduced oocyte quality and subsequent low fertility in dairy cows (8, 87). However, in the present study, the mean intervals from luteolysis to ovulation were not different between lactating and non-lactating cows (4.6 and 4.2 days, respectively), and these values are comparable to that of heifers in the above study (96) or that of lactating cows in an earlier study in which luteolysis was induced by prostaglandin $F_{2\alpha}$ (86).

It is suggested that the increase in steroid metabolism in lactating dairy cows can affect not only circulating progesterone concentrations during the luteal phase but also circulating concentrations of estradiol-17β around estrus (93, 125). Lopez et al. (52) reported that cows

with higher milk production (> 39.1 kg/day) had lower concentrations of estradiol-17β around estrus than cows with lower milk production (< 39.1 kg/day). In the present study, however, the sequence of endocrine changes occurring after luteolysis in lactating cows was very similar to that in non-lactating cows, and the concentrations of estradiol-17β and LH around estrus were not different between lactating and non-lactating cows. Moreover, our results found fewer variations in the time from the estradiol-17β peak to ovulation (range 12 h) and the time from the LH surge to ovulation (range 0 h) compared with those reported in recent studies (9, 108). It is generally accepted that estradiol-17β induces the preovulatory LH surge as an "all or nothing" event. After a certain threshold of estradiol-17β concentration is reached, there will be an LH surge, which results in ovulation (57, 127). It is speculated that the similar peak levels of estradiol-17β detected in the lactating and non-lactating cows would be sufficient to induce an LH surge and ovulation without any alterations.

Behavioral signs of estrus such as restlessness and bellowing were not observed in a low proportion of cows (1/5 of lactating and 1/5 of non-lactating cows), whereas vulval and vaginal estrous signs were clearly observed in both groups of cows. It is well established that estradiol-17β, in the relative absence of progesterone, acts on the hypothalamus to induce estrous behavior (2). Studies on modern dairy cows have found that the plasma estradiol-17β concentration around estrus was correlated with intensity of estrous behavior (57) and duration of estrus (52). However, in the present study, lactating cows showed similar appearances of estrous signs to non-lactating cows, and this finding likely reflects similar endocrine profiles in the two groups. Our observations of estrous signs were not sufficient in quantity to evaluate the relationship between the intensity of estrous signs and the estradiol-17β level, but our results may agree with the hypothesis that once a sufficient concentration of plasma estradiol-17β is achieved to induce estrus, additional amounts of estradiol-17β have no further stimulatory effect on expression of the behavior (2).

It is generally accepted that the intensity and duration of estrous behavior vary among individuals and herd levels (23, 113), because environmental and management factors such as type of housing, herd size and the number of animals in estrus at the same time can affect estrous behavior (12, 38). Indeed, the cows examined in the present study were kept in individual pens during the frequent sampling period, and their opportunities to interact with other herd mates were limited. On the other hand, the present study showed that the estrous signs in the vulva and vagina were observed more clearly in all lactating and non-lactating cows compared with those related to behavior. Although there are no reports examining the appearance of vaginal estrous signs and their duration in cows by using vaginoscopy, vaginal temperature (83) and electrical conductivity of the vaginal mucus (104) have been reported to be closely associated with the onset of estrus and time of ovulation. At estrus, high levels of estrogens increase blood flow in the reproductive tract, causing increased vaginal hydration and mucous discharge (21). Besides, estrogens act to dilate the cervix and external uterine orifice during estrus (43). The present study showed that after the initiation of luteolysis, these characteristic estrous signs in the vagina appeared earlier and continued for a longer period than those related to behavior, although significant differences were not detected between the changes in the vagina and vulva. Several studies suggest that estrus detection based on secondary estrous signs such as mucous discharge from the vagina is less accurate than estrus detection based on standing estrus (84, 113), resulting in a high incidence of estrus detection error and a low conception rate (84). It might be difficult to predict the stage of estrus or timing of ovulation by observation of secondary estrous signs alone. However, the fact that estrous signs in the vagina were clearly observed in all cows examined is of practical importance to use vaginoscopic examination as an aid to detect estrus effectively.

In summary, the profiles of ovarian steroids and LH from luteolysis to ovulation in lactating dairy cows were comparable with those in non-lactating ones. Behavioral estrous

signs were observed in a shorter period and showed more variability among animals than vulval and vaginal ones regardless of lactating status. Therefore, it seems that, when lactating cows are managed under a moderate level of milk production and in good nutritional condition, lactation might not interfere with the preovulatory hormonal profiles, timing of ovulation and appearance of estrous signs. This finding suggests that the problem of low rates of estrus detection in modern dairy cows can be partly overcome by developing management techniques to enhance the accuracy and efficiency of estrus detection.

Table 4-1 Profiles of estradiol-17β concentrations and LH surge in lactating and non-lactating cows.

	Estradiol-17β (pg/ml)			LH surge (ng/ml)	
Group	At luteolysis [a)]	Peak [b)]	AUC [c)]	Peak [b)]	AUC
Lactating (n = 5)	2.7 ± 3.0	13.3 ± 3.1	27.9 ± 8.7	11.7 ± 3.7	44.5 ± 21.7
Non-lactating (n = 5)	2.3 ± 1.4	12.5 ± 3.1	21.6 ± 2.3	14.2 ± 2.8	58.8 ± 13.0

Values are mean and SD.

[a)] Estradiol-17β concentration at the initiation of luteolysis.

[b)] Maximal hormone concentration before ovulation.

[c)] AUC of estradiol-17β concentration from the initiation of luteolysis to ovulation.

Table 4-2 Time intervals between the peak of estradiol-17β, LH surge and ovulation in lactating and non-lactating cows.

Item	Lactating (n = 5)	Non-lactating (n =5)
Days from initiation of luteolysis to E_2 peak	3.2 ± 0.5	2.9 ± 0.8
Days from initiation of luteolysis to LH peak	3.5 ± 0.5	3.1 ± 0.7
Hours from E_2 peak to ovulation	34.2 ± 4.5	30.6 ± 3.9
Hours from E_2 peak to LH peak	6.0 ± 3.7	2.4 ± 2.5
Hours from LH peak to ovulation	27	27

Values are mean and SD.

E_2 peak: maximal estradiol-17β concentration before ovulation.

LH peak: maximal concentration of LH surge.

Table 4-3 Appearance of each estrous sign in relation to luteolysis and ovulation and its duration in lactating (n = 5) and non-lactating (n = 5) cows.

		Estrous signs		
Item	Group	Behavior	Vulva	Vagina
Appearance after initiation of luteolysis (d) [a)]	Lactating	2.8 ± 1.3	2.3 ± 1.2	1.7 ± 1.5
	Non-lactating	2.3 ± 1.5	2.0 ± 1.2	1.4 ± 1.0
	All	2.5 ± 1.3 [e]	2.2 ± 1.2 [ef]	1.6 ± 1.2 [f]
From appearance to ovulation (h) [b)]	Lactating	42.0 ± 23.5	55.2 ± 22.2	69.6 ± 31.3
	Non-lactating	47.5 ± 22.6	54.6 ± 25.5	68.2 ± 21.8
	All	44.8 ± 21.6 [e]	54.9 ± 22.6 [ef]	68.9 ± 25.4 [f]
From disappearance to ovulation (h) [c)]	Lactating	8.0 ± 4.9	0	1.0 ± 2.2
	Non-lactating	5.5 ± 5.0	0.6 ± 1.3	0
	All	6.0 ± 5.1 [g]	0.4 ± 1.1 [h]	0.6 ± 1.8 [h]
Duration (h) [d)]	Lactating	40.5 ± 25.3	55.2 ± 22.2	69.6 ± 31.3
	Non-lactating	42.0 ± 25.6	54.0 ± 26.2	68.2 ± 21.8
	All	41.3 ± 23.6 [g]	54.6 ± 22.9 [gh]	68.9 ± 25.4 [h]

Values are mean and SD.

[a)] The interval from the initiation of luteolysis to the appearance of estrous signs.

[b)] The interval from the appearance of estrous signs to ovulation.

[c)] The interval from the disappearance of estrous signs to ovulation.

[d)] The interval from the appearance to the disappearance of estrous signs.

[e - h] Values with different superscripts within each row differ significantly ([ef] $P < 0.05$, [gh] $P < 0.01$).

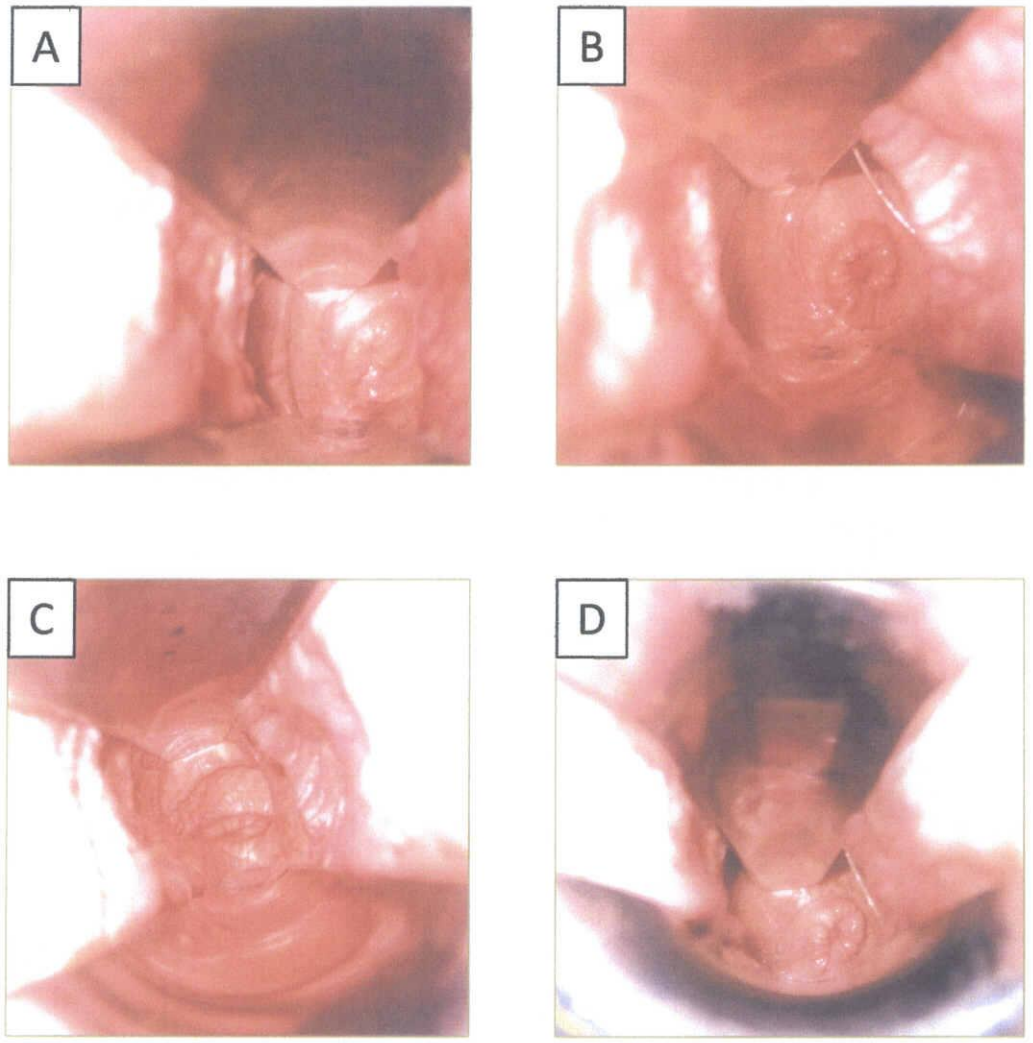

Fig. 4-1 Appearance of the anterior portion of the vagina and the external uterine orifice observed by using vaginoscopy in a representative cow during the mid-luteal phase (A), on one day before estrus (B), on the day of estrus (C) and on the day of ovulation (D).

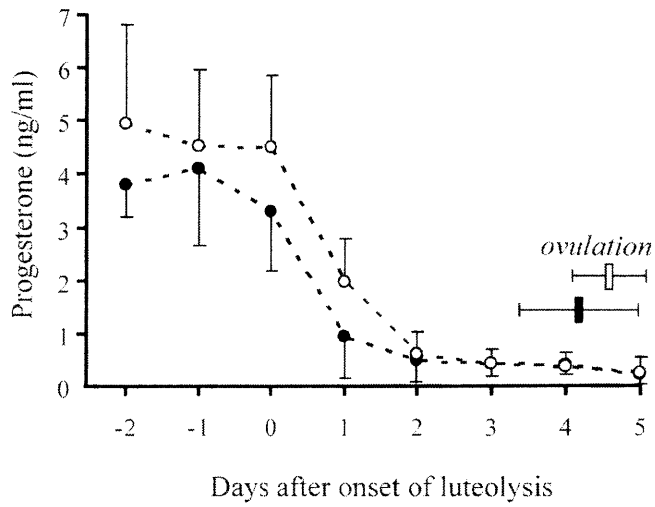

Fig. 4-2 Changes in plasma progesterone concentrations in lactating (n = 5, open circles) and non-lactating (n = 5, filled circles) cows from -2 to 5 days after initiation of luteolysis. Vertical bar indicates time of ovulation in lactating (open bar) and non-lactating (solid bar) cows with mean and SD.

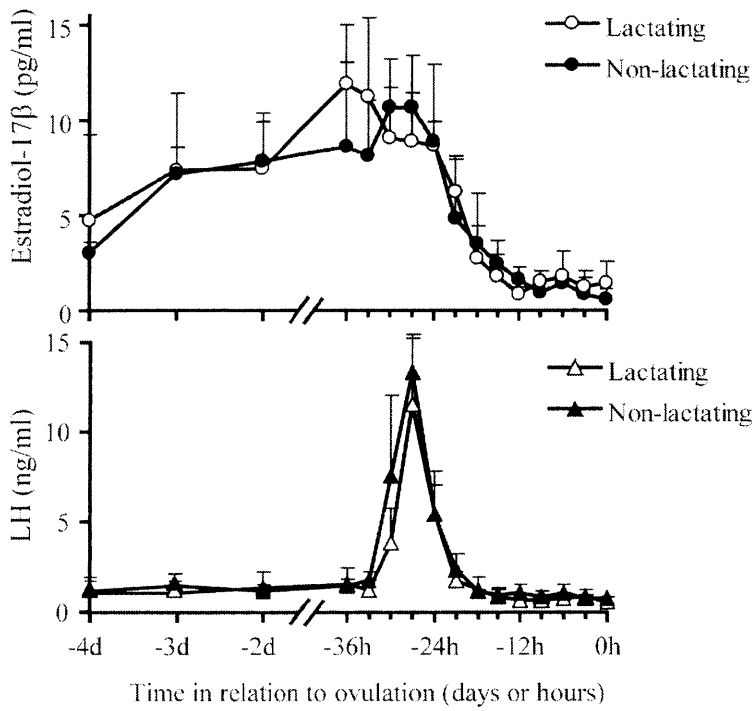

Fig. 4-3 Temporal relationships between plasma concentrations of estradiol-17β and LH from four days before to the time of ovulation in lactating (n = 5) and non-lactating (n = 5) cows. All data were normalized to the time of ovulation (0 h), and values are mean and SD.

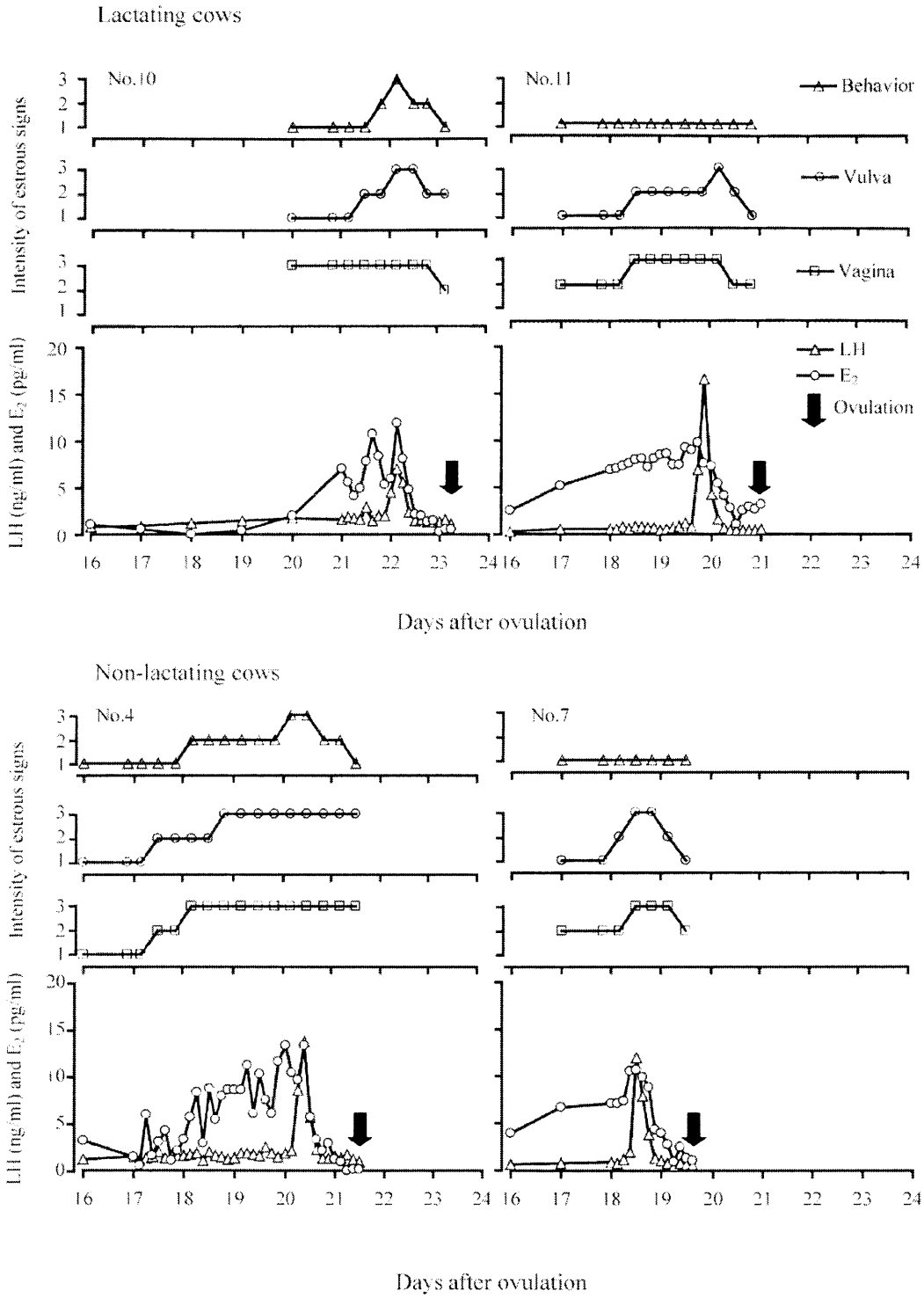

Fig. 4-4 Representative profiles in the intensity of estrous signs in behavior, the vulva and vagina and estradiol-17β (E_2) and LH in one lactating (No.10) and one non-lactating (No.4) cows that showed behavioral sign, and in one lactating (No.11) and one non-lactating cows (No.7) that did not show any behavioral sign.

Chapter 5. Comparison between lactating and non-lactating cows on plasma progesterone levels in the caudal vena cava and the jugular vein and luteinizing hormone profiles, and their association with feeding

5.1. Introduction

The main function of the CL is to synthesize and secrete progesterone, which regulates the estrous cycle length and maintains pregnancy in many species. Low circulating concentrations of progesterone during the luteal phase might reduce embryo survival rates (41, 49). In Chapter 3, the lactating cows had lower progesterone levels when the CL was developing, which may imply the influence of increased progesterone metabolism associated with lactation (93, 125). However, when the CL was fully developed, the lactating cows had higher progesterone concentrations than non-lactating cows. It seems that the higher progesterone concentration in lactating cows might result from increased secretion status of progesterone by the fully-developed CL.

The importance of luteotropic hormones secreted by the pituitary gland for normal luteal development and function has been demonstrated in many species. Luteinizing hormone is the primary luteotropic agent, and a temporal association between LH pulses and secretion of progesterone was observed during the early and mid-luteal phase in cattle (75, 119). The results in Chapter 3 showed that lactating cows had higher frequency of LH pulses during early to mid-luteal phase than non-lactating cows. Although the exact mechanism for the increase in LH pulses in lactating dairy cows remains unclear, it is likely that an alteration in LH secretion patterns may be associated with luteal activity of steroidogenesis.

Catheterization at a point naturally close to the ovary, for example, the caudal vena cava, has been utilized to facilitate collection of blood containing high concentrations of ovarian steroid hormones before they are metabolized by the liver (6, 65, 89, 119). Blood samples

frequently collected from the caudal vena cava would provide detailed information regarding the secretion status of progesterone from the CL. Furthermore, progesterone concentration in the circulating blood reflects the balance between secretion and metabolic status of progesterone. This prompted the idea that metabolic status of progesterone in lactating and non-lactating cows could be inferred from the difference in progesterone concentrations at the secreted level (i.e. in the caudal vena cava) and the circulating level (i.e. in the jugular vein). In addition, previous studies in cows (60, 115) and sheep (67, 68) have demonstrated that feeding increased blood flow to the liver and thereby elevated metabolic rates of steroid hormones. Lactating cows require greater feed intake for milk production compared with non-lactating cows. Therefore, I hypothesized that greater changes in progesterone concentration in the jugular vein would be observed after feeding in lactating cows than in non-lactating cows, owing to the greater changes in metabolic rates of progesterone.

The first objective of the present study was to assess the progesterone profiles at the secreted level (in the caudal vena cava) and the circulating level (in the jugular vein) in lactating and non-lactating dairy cows. The second objective was to examine the association between LH pulses in the jugular vein and progesterone secretion patterns in the caudal vena cava, with reference to feeding.

5.2. Materials and methods

5.2.1. Animals

Four lactating (two primiparous and two multiparous: 4.5 ± 2.4 years of age; 102.5 ± 15.6 days postpartum; milk yield of 25.5 ± 3.6 kg/day) and four non-lactating (one primiparous and three multiparous; 6.0 ± 2.2 years of age; 570.3 ± 238.6 days postpartum) were used in this study. At the beginning of the study, BW were 637.5 ± 80.7 and 731.5 ±

39.0 kg for lactating and non-lactating cows, respectively ($P > 0.1$), and their BCS were 3.2 ± 0.3 and 3.6 ± 0.3, respectively ($P > 0.1$).

5.2.2. Experimental procedure

The study was started from the beginning of the cycle (Day 0: day of ovulation) until the end of the cycle, for which subsequent ovulation was confirmed. For the analysis of follicular and luteal development and plasma progesterone, ovarian ultrasonography and blood sampling were conducted every other day from Day 0 to Day 14 and then daily through the cycle, as described in Chapter 2. For the analyses of progesterone in the caudal and jugular veins and LH in the jugular vein, catheterization into both veins was conducted on one day during the mid-luteal phase (Day 10, 11, 12 or 13), as described in Chapter 2. On the day after catheterization, frequent blood sampling was conducted at 15-min intervals for 12 h (0500 to 1700 h). All cows were kept unfed for 6 h before the start of the frequent blood sampling, and then fed 50% of the daily amount of diet at 6 h after the start of sampling (1100 h). The remaining 50% of the diet was fed after the end of sampling. Cows were kept in designated pens located next to the free-stall area from the day of catheterization until the end of frequent blood sampling for two days; otherwise, they were managed in the same manner as their herdmates.

5.2.3. Blood sampling and assays

Blood samples (10 ml) during the estrous cycle were collected by jugular venipuncture into heparinized vacutainers (Venoject II, Terumo, Tokyo, Japan). Blood samples (6 ml) collected from the catheterized caudal and jugular veins during the frequent sampling were collected into test tubes that contained 10 IU heparin. The obtained blood samples were processed for hormone assays as described in Chapter 2. In addition, for blood chemical

analyses, blood samples (10 ml) were collected at the beginning and the end of the frequent blood sampling (6 h before and 6 h after feeding) into vacutainers for serum separation (Venoject II, Terumo, Tokyo, Japan). Blood was allowed to coagulate at room temperature for 1 h, centrifuged at 3,000 rpm (1,750 × g) for 20 min at 4 °C, and serum was stored at −20 °C until assay.

Plasma concentrations of progesterone and LH were measured as described in Chapter 2. For the progesterone assay, the intra- and interassay coefficients of variation were 4.3% and 9.7%, and the sensitivity was 0.14 ng/ml. For the LH assay, the intra- and interassay coefficients of variation were 5.4% and 8.8%, and the sensitivity was 0.09 ng/ml.

Serum concentrations of total cholesterol, blood urea nitrogen (BUN), aspartate aminotransferase (AST) and gamma glutamyl transpeptidase (γ-GTP) were analyzed using an automatic analyzer (Dri-chem4000, Fuji Film, Tokyo, Japan). Serum concentrations of glucose and non-esterified fatty acids (NEFA) were analyzed using commercial assay kits according to the manufacturer's instructions (Wako Pure Chemical Co., Osaka, Japan).

5.2.4. Statistical analyses

To assess data for follicular and luteal diameter, progesterone and LH profiles, and blood chemical analysis, two-way ANOVA with repeated measures was used for determining the fixed effects of group and time and their interaction. When significant differences were detected, Turkey's post hoc follow-up test or Student's t-test were used to detect significant differences among groups within time and among time within groups. To assess hourly changes in AUC of progesterone relative to feeding for each group, Dunnett's multiple comparison method was used to determine the significance of values in comparison with the value of 1 h before feeding.

Pulsatile patterns of LH in the jugular vein and progesterone in the caudal vena cava were analyzed by cluster analysis program (116), and the temporal relationships between pulses of LH in the jugular vein and progesterone in the caudal vena cava were determined as described in Chapter 2. Differences between groups (lactating vs. non-lactating) were tested using chi-squared analysis.

5.3. Results

5.3.1. Luteal development and progesterone concentrations during the estrous cycles

The length of estrous cycle (interovulatory interval) did not differ between lactating and non-lactating cows (21.5 ± 1.2 and 20.8 ± 0.6 days; mean ± SD). Mean diameters of the CL during mid-luteal phase of Days 8 -14 were higher ($P < 0.01$) in lactating cows than in non-lactating cows (25.5 ± 2.4 and 23.0 ± 0.8 mm, respectively). Mean concentrations of progesterone during mid-luteal phase of Days 8 – 14 were higher ($P < 0.05$) in lactating than in non-lactating cows (7.1 ± 2.0 and 5.9 ± 0.9 ng/ml, respectively), although mean concentrations of progesterone during early luteal phase of Days 0 – 6 did not differ between lactating and non-lactating cows (2.7 ± 1.7 and 2.4 ± 1.4 ng/ml). There was no difference between lactating and non-lactating cows in terms of the growth rate of the first wave dominant follicles (1.2 ± 0.4 and. 0.9 ± 0.2 mm/d) and the maximal diameter of the first wave dominant follicles (14.8 ± 2.6 and 14.7 ± 2.0 mm).

5.3.2. Progesterone profiles in the caudal and jugular veins

Representative profiles of progesterone concentrations in the caudal vena cava and in the jugular vein and pulsatile LH secretion in the jugular vein in one lactating (No. 6) and one non-lactating cow (No. 1) are shown in **Fig. 5-1**. In addition to LH pulses, pulsatile pattern of progesterone (asterisks in **Fig. 5-1**) was also determined for the blood samples in the caudal

vena cava. Progesterone concentrations in the caudal vena cava were approximately ten times higher on average, than those in the jugular vein (**Table 5-1**). When comparisons were made between groups, mean progesterone concentrations in the caudal vena cava during the 12-h frequent sampling period did not differ between lactating and non-lactating cows (49.0 ± 29.2 and 53.3 ± 51.9 ng/ml). However, in the jugular vein, mean progesterone concentrations during the 12-h sampling period were higher in lactating cows than in non-lactating cows (6.4 ± 1.8 and. 5.6 ± 1.4 ng/ml, $P < 0.001$). For progesterone pulses in the caudal vena cava, a total of 26 pulses were detected in all sampling periods of lactating cows. This value was the same as that of non-lactating cows (26 pulses). In addition to frequency of progesterone pulses (6.5 ± 0.7 and 6.8 ± 1.4 pulses/12 h; **Table 5-1**), basal level and amplitude of progesterone pulses did not differ significantly between lactating and non-lactating cows.

5.3.3. Luteinizing hormone profile in the jugular veins and its association with progesterone pulses in the caudal vena cava

For LH pulses, totals of 28 and 17 pulses were detected in the whole sampling periods of lactating and non-lactating cows, respectively. The frequency of LH pulses was higher in lactating cows than in non-lactating cows (7.0 ± 1.4 and 4.3 ± 1.9 pulses/12 h, $P < 0.05$; **Table 5-2**). The other characteristics for LH secretion such as mean concentration, basal level and amplitude of LH pulses were not different between the groups. Most of the LH pulses were detected in association with progesterone pulses in the caudal vena cava. During the whole sampling period, the percentage of LH pulses that were followed by a pulse of progesterone was 71.4% (20/28) and 88.2% (15/17) in the lactating and non-lactating cows, respectively, without significant difference between the groups. Also, the percentages of progesterone pulses that followed a LH pulse were not different between lactating and non-lactating cows (73.1% (19/26) and 61.5% (16/26), respectively).

5.3.4. Influence of feeding on progesterone and LH profiles

To examine the influence of feeding on progesterone and LH profiles, mean concentration, pulse frequency, pulse amplitude, and basal level during pre-feeding 6 h were compared with those during post-feeding 6 h. In the caudal vena cava, mean concentration and pulse amplitude of progesterone were higher ($P < 0.05$) during post-feeding 6 h than during pre-feeding 6 h for the both lactating and non-lactating cows (**Table 5-1**), but basal level and pulse frequency did not change significantly. In the jugular vein, however, the influence of feeding on progesterone concentrations differed between lactating and non-lactating cows. In lactating cows, mean progesterone concentration in the jugular vein was lower ($P < 0.05$) during post-feeding 6 h than during pre-feeding 6 h. Conversely, in non-lactating cows, mean progesterone concentration in the jugular vein tended to be higher ($P = 0.054$) during post-feeding 6 h than during pre-feeding 6 h. Consequently, mean concentration during post-feeding 6 h did not differ between lactating and non-lactating cows, although mean concentration during pre-feeding 6 h was higher ($P < 0.05$) in lactating cows than in non-lactating cows.

Hourly changes in progesterone AUC relative to feeding in the caudal and jugular veins are shown in **Fig. 5-2**. In lactating cows, the progesterone AUC in the caudal vena cava were greater ($P < 0.05$) during the period from 0 to 2 h after feeding than the value of 1 h before feeding. Conversely, the progesterone levels in the jugular vein were lower ($P < 0.05$) during the period from 1 to 4 and 5 to 6 h after feeding than the value of 1 h before feeding. In non-lactating cows, no significant differences were detected on the progesterone AUC throughout the sampling period in the both caudal and jugular veins compared with the value of 1 h before feeding.

Profiles of LH secretion were not influenced by feeding; pulse frequency, mean concentration, and pulse amplitude and basal level of LH did not differ significantly between pre-feeding and post-feeding 6 h for the both groups (**Table 5-2**).

5.3.5. Blood chemical profiles

There was no significant difference on the concentrations for any parameters between the samples collected 6 h before and 6 h after feeding for the both groups. So, values of two samples were combined and the averages were presented (**Table 5-3**). A significant difference between lactating and non-lactating cows was detected for the concentrations of total cholesterol (224.5 ± 51.8 and 79.9 ± 10.4 mg/dl; $P < 0.01$). In addition, AST value was significantly higher in lactating cows than in non-lactating cows (74.6 ± 6.2 and 50.0 ± 7.0 U/l; $P < 0.01$), but all the individual values for both lactating and non-lactating cows (ranges: 65 - 85 and 40 - 61 U/l, respectively) were below the suggested reference value, <132 U/l (81). No significant difference was detected between the groups for the concentrations of glucose, NEFA, BUN, and γ-GTP.

5.4. Discussion

The present study demonstrated that lactating dairy cows had greater frequency of LH pulses, higher progesterone concentrations and larger CL size during mid-luteal phase compared with non-lactating cows, in agreement with the findings in Chapter 3. In the present study, frequency of LH pulses was higher in the lactating cows (mean; 7.0 pulses/12 h) than in the non-lactating cows (4.3 pulses/12 h), or the previously described observation in non-lactating cows (3.6 pulses/12 h (119)). Peripheral progesterone concentrations are considered as a net result of secretion and metabolism. As a possible explanation for the higher circulating concentrations of progesterone in the lactating cows, following is elicited.

It is likely that greater amounts of progesterone were secreted from the CL in the lactating cows than in the non-lactating cows, even though lactating cows have higher progesterone metabolic rate than non-lactating cows (93, 115). As discussed in Chapter 3, the larger CL in diameter of lactating cows seems to be one of main factors involved in the greater progesterone production, because luteal tissue volume has been found to be correlated with the circulating progesterone concentration in some studies (56, 90).

To assess the secreted level of progesterone in this experiment, catheters were adjusted to the respective length for each cow by measuring progesterone concentrations in vena cava blood collected by 5-cm gradation of catheter length inserted, as proposed by Benoit and Dailey (6). The progesterone concentrations in the caudal vena cava blood were approximately ten times higher than those in the jugular blood, and sufficiently fulfilled the criteria that blood samples from the caudal vena cava have at least three-times higher concentrations of progesterone than peripheral blood (6, 65, 119). However, no difference could be detected between the two groups for progesterone profiles from the caudal blood samples. This might be partially due to the wider fluctuations of progesterone concentrations through the frequent sampling time in the caudal vena cava than in the jugular vein, the short sampling period and the small number of animals examined in this study. Besides, although blood collected from the caudal vena cava contained greater amount of ovarian steroids before metabolized by the liver, the hormonal concentration might be influenced by blood flow rate at the sampling position.

It is well recognized that onset of lactation after calving is accompanied by increases in the blood volume, cardiac output, mammary blood flow (10), and blood flow through the gastrointestinal organs and liver (51, 88), aiming to provide the udder with nutrients and hormones for regulation of milk synthesis (109). In addition, feeding causes rapid changes in circulatory system, such as increase in heart rates and blood pressure (7) and increase in

blood flow to the portal and hepatic veins (51, 123). If changes in blood flow occur, concentrations of ovarian hormones at the sampling position within the caudal vena cava could vary apparently. In the present study, feeding 50% of the daily amount of diet caused a significant increase in the progesterone concentrations in the caudal vena cava for the both groups. Such a post-feeding increase in progesterone concentrations in the caudal vena cava has been reported in a study on early pregnant gilts (117), and the authors have speculated the presence of a direct effect of metabolic mediator (such as insulin) on ovaries. In the present study, changes in progesterone AUC were determined for the accurate estimation of the secreted amount of progesterone from the CL into the bloodstream. As a result, significant increase in the progesterone AUC was found from 0 to 2 h after feeding in the lactating cows. This increase seemed to occur in consistent with the period when the lactating cows were eating their diets. In contrast, such a significant increase in progesterone AUC was not detected in the non-lactating cows. It is possible that, in addition to differences in feed (amount, energy density, or nutrient composition), physiological responses to feeding such as intake and digestibility of the diets, blood flow changes to the splanchnic circulation, and metabolic and hormonal responses could differ between lactating and non-lactating cows.

Comparison of pulsatile patterns of progesterone in the caudal vena cava between pre- and post-feeding period showed that feeding influenced the pulse amplitude, but not the basal level and pulse frequency for the both groups. These results indicate that the increased pulse amplitude of progesterone could mainly contribute to the increase in mean progesterone concentrations during post-feeding period. During the frequent sampling periods, a high proportion of LH pulses were followed by pulses of progesterone in the caudal vena cava (71.4 and 88.2% in the lactating and non-lactating cows), suggesting a temporal association between LH pulses and secretion of progesterone (75, 119). Based on the results in Chapter 3, I supposed that the increase in frequency of LH pulses in lactating cows would stimulate

progesterone secretion by the CL, which could be detected from the progesterone profiles in the caudal vena cava. Indeed, the present study also confirmed that lactating cows had higher frequency of LH pulses and circulating concentrations of progesterone than non-lactating cows. However, further differences between the groups were not found in the association of LH pulses with the in the progesterone secretion patterns in the caudal vena cava through the pre- and post-feeding periods. Alternatively, I noticed that a low proportion of progesterone pulses occurred in the absence of LH pulse, and that feeding increased the progesterone concentrations in the caudal vena cava without apparent alterations in the LH pulse pattern. It has been demonstrated by in vitro study (34) that progesterone secretion by small luteal cells is stimulated by LH in a dose-dependent manner, but progesterone secretion by large luteal cells seems to be independent of LH stimulation (61). It is possible that other hormonal or non-hormonal factors could stimulate progesterone production by the CL, and thereby cause a significant change in progesterone profiles in the caudal vena cava in relation to time of feeding. Further studies are required to investigate the mechanisms on how feeding cause such a change in progesterone profiles in the caudal vena cava, including the measurement of blood flow at the sampling site.

On the other hand, feeding 50% of the daily amount of diet decreased circulating progesterone concentrations in the lactating cows. This finding concurs with the preceding observation that provision of 100 or 50% of the TMR to pregnant lactating cows decreased the circulating progesterone by 1 h after feeding (115). However, in the present study, such a decrease was not detected in the non-lactating cows. In fact, the mean progesterone concentrations in the jugular vein in the non-lactating cows tended to be higher during post-feeding 6 h than during pre-feeding 6 h, which might reflect the increase in the progesterone concentrations in the caudal vena cava after feeding. It is suggested that high feed intake results in increased liver blood flow and metabolic clearance of progesterone,

which decreases progesterone concentration in plasma of sheep (67, 68), pigs (74), and cows (125). Particularly in lactating dairy cows, a continuous high plane of nutrition appears to chronically elevate liver blood flow and metabolic clearance rate of progesterone (93). Therefore, differences in metabolic clearance rates of progesterone between lactating and non-lactating cows could bring about different profiles of circulating progesterone in response to feeding. In lactating cows, circulating progesterone concentration may be greatly influenced by the elevated progesterone metabolism, which could negate the post-feeding increase in the progesterone concentration in the caudal vena cava. In non-lactating cows, however, if metabolic rate of progesterone was increased after feeding, the effect would have little influence on circulating progesterone concentrations.

Metabolic and nutritional status has major impacts on ovarian function and fertility in dairy cows (20). In early postpartum cows, negative energy balance resulting from increased milk production can reduce follicular and luteal activity by decreasing IGF-1 concentrations in blood (91, 105). The lactating cows used in the present study were in mid-lactation period (98.1 ± 11.1 days postpartum) and maintained their BCS and BW within a normal range. As a result, no significant difference between lactating and non-lactating cows was detected in blood chemical profiles except for AST and total cholesterol. Higher concentrations of total cholesterol in lactating cows may reflect an alteration in lipid metabolism to support lactation (5). Although there was a significant difference for AST values among the groups, all of the individual values for both lactating and non-lactating cows were within normal range (81). The higher AST values in lactating cows compared with those in non-lactating cows was interpreted not as a pathological condition, but rather as an adjustment of liver function to the increased metabolic requirements for milk production (36). As for total cholesterol, a great difference was found between lactating and non-lactating cows (224.5 ± 51.8 and 79.9 ± 10.4 mg/dl). The higher concentrations of total cholesterol in the lactating cows compared with

those in the non-lactating cows may reflect greater feed intake and alterations in lipid metabolism to support lactation (5). From these points, the cows used in the study might not have nutritional problems that could be related to subnormal luteal function. This is supported by the findings that lactating cows had higher circulating progesterone and developed larger CL during mid-luteal phase than non-lactating cows. Therefore, the post-feeding decrease in the circulating progesterone detected only in the lactating cows is likely the result of elevated steroid metabolism associated with lactating status (93, 125). In addition, previous studies on cattle (25, 39) indicate that dietary energy positively influences LH pulse frequency and plasma concentrations of LH by affecting the hypothalamic mechanisms controlling GnRH release. This may account for the greater frequency of LH pulses in lactating cows observed in the present and previous studies (115).

In summary, lactating cows maintained well-developed CL, and higher progesterone concentrations in the jugular vein and greater frequency of LH pulses during the mid-luteal phase than non-lactating cows. However, progesterone concentrations in the caudal vena cava were not different between lactating and non-lactating cows. While profiles of LH secretion were not influenced by feeding, progesterone profiles in the caudal vena cava and the jugular vein changed in relation to feeding and these changes exhibited different patterns between lactating and non-lactating cows. Progesterone concentrations in the caudal vena cava increased after feeding for both groups of cows, whereas progesterone concentrations in the jugular vein were decreased in lactating cows but not in non-lactating cows. These results suggest that the increase in progesterone metabolism after feeding is so dramatic in lactating dairy cows that it can decrease progesterone levels in circulating blood.

Table 5-1 Progesterone (P_4) profiles in the caudal vena cava (CVC) and the jugular vein (JV) during whole 12 h, pre- and post-feeding 6 h sampling periods in lactating (n = 4) and non-lactating (n = 4) cows.

Vein	P_4 character	Group	Whole 12 h	Pre-feeding 6 h	Post-feeding 6 h
CVC	Mean concentration	Lactating	49.0 ± 29.2	41.8 ± 22.0 ^{x}	56.5 ± 33.7 ^{y}
	(ng/ml)	Non-lactating	53.3 ± 51.9	46.1 ± 41.5 ^{x}	60.8 ± 60.3 ^{y}
	Basal level	Lactating	32.8 ± 17.3	32.5 ± 15.9	33.1 ± 19.1
	(ng/ml)	Non-lactating	31.1 ± 16.6	26.2 ± 10.1	36.3 ± 20.7
	Pulse frequency	Lactating	6.5 ± 0.7	3.0 ± 0.8	3.5 ± 1.3
	(pulses/12 h or 6 h)	Non-lactating	6.8 ± 1.4	3.5 ± 0.6	3.3 ± 1.3
	Pulse amplitude [a)]	Lactating	57.3 ± 33.1	38.3 ± 20.6	67.0 ± 36.7 ^{y}
	(ng/ml)	Non-lactating	57.4 ± 73.1	38.1 ± 49.1 ^{x}	78.3 ± 80.9 ^{y}
JV	Mean concentration	Lactating	6.4 ± 1.8 ^{b}	6.7 ± 1.8 ^{bx}	6.1 ± 1.8 ^{y}
	(ng/ml)	Non-lactating	5.6 ± 1.4 ^{c}	5.4 ± 1.4 ^{c†}	5.8 ± 1.4 †

Values are mean and SD.

[a)] Pulse amplitude was calculated by subtracting the maximal concentration of a pulse from the concentration preceding the pulse and expressed as ng/ml.

[b, c] Different superscripts within a column indicate significant differences ($P < 0.05$) between lactating and non-lactating cows.

[x, y] Different superscripts within a row indicate significant differences ($P < 0.05$) between pre- and post-feeding 6 h.

[†] There was a tendency ($P = 0.054$) for a difference between 6 h pre- and 6 h post-feeding within a row.

Table 5-2 Luteinizing hormone profiles in the jugular vein during whole 12 h, pre- and post-feeding 6 h sampling periods in lactating (n = 4) and non-lactating (n = 4) cows.

LH character	Group	Whole 12 h	Pre-feeding 6 h	Post-feeding 6 h
Mean concentration	Lactating	0.34 ± 0.23	0.32 ± 0.22	0.36 ± 0.24
(ng/ml)	Non-lactating	0.41 ± 0.19	0.40 ± 0.21	0.42 ± 0.18
Basal level	Lactating	0.28 ± 0.19	0.26 ± 0.16	0.31 ± 0.24
(ng/ml)	Non-lactating	0.33 ± 0.13	0.32 ± 0.14	0.33 ± 0.14
Pulse frequency	Lactating	7.0 ± 1.4 [b]	3.8 ± 0.5	3.3 ± 1.0
(pulses/ 12 h or 6 h)	Non-lactating	4.3 ± 1.9 [c]	1.8 ± 1.3	2.5 ± 1.9
Amplitude [a)]	Lactating	0.24 ± 0.09	0.27 ± 0.12	0.21 ± 0.05
(ng/ml)	Non-lactating	0.37 ± 0.27	0.34 ± 0.29	0.40 ± 0.28

Values are mean and SD.

[a)] Pulse amplitude was calculated by subtracting the maximal concentration of a pulse from the concentration preceding the pulse and expressed as ng/ml.

[b, c] Different superscripts within a column indicate significant differences ($P < 0.05$) between lactating and non-lactating cows.

Table 5-3 Blood chemical profiles in lactating and non-lactating cows on the day of frequent blood sampling

Item	Lactating (n = 4)	Non-lactating (n = 4)
Glucose (mg/dl)	67.2 ± 4.2	71.4 ± 6.4
NEFA (μEq/l)	93.1 ± 63.6	83.6 ± 33.9
Total cholesterol (mg/dl)	224.5 ± 51.8[c]	79.9 ± 10.4[d]
BUN (mg/dl)	11.6 ± 3.1	10.4 ± 3.6
[a)] AST (U/l)	74.6 ± 6.2[c]	50.0 ± 7.0[d]
[b)] γ-GTP (U/l)	33.0 ± 7.0	36.4 ± 3.6

Values are mean and SD.

[a)] AST: aspartate aminotransferase.

[b)] γ-GTP: gamma glutamyl transpeptidase.

[c, d] Different superscripts within a row indicate significant differences ($P < 0.01$).

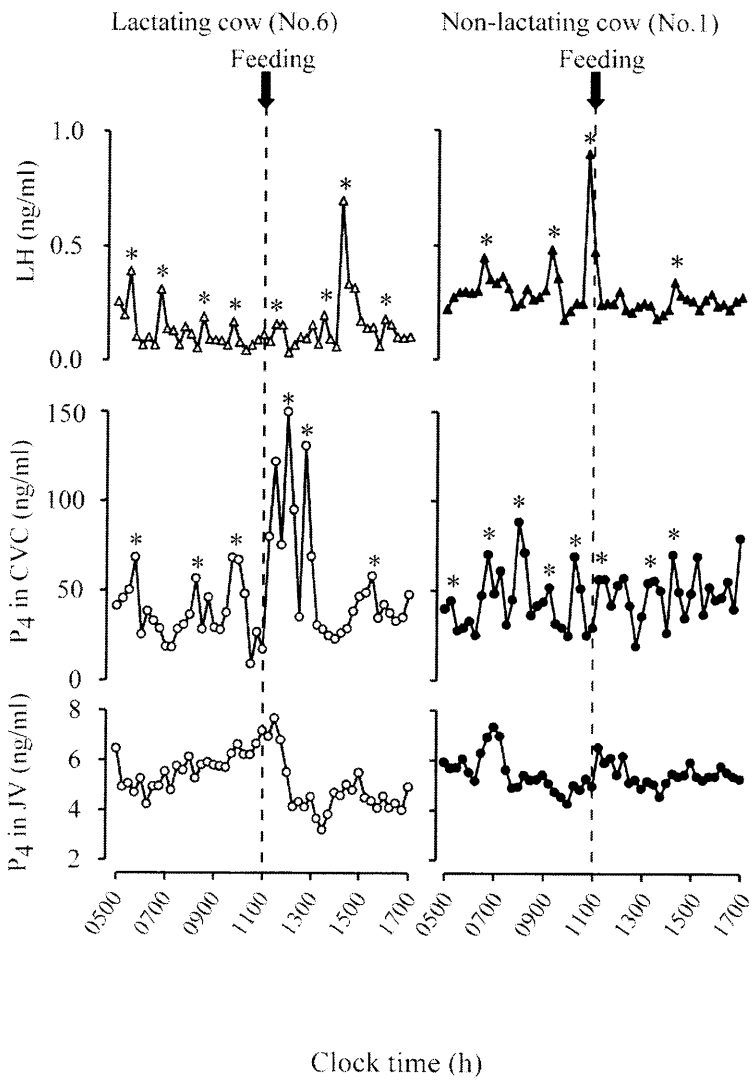

Figure 5-1 Representative profiles of progesterone (P_4) concentrations in the caudal vena cava (CVC) and in the jugular vein (JV) and pulsatile LH secretion in the JV in one lactating (No. 6) and one non-lactating cow (No. 1). Asterisk indicates a peak of LH pulse in the JV or P_4 pulse in the CVC. Arrow and broken line indicate the time when one half of the daily diet was provided.

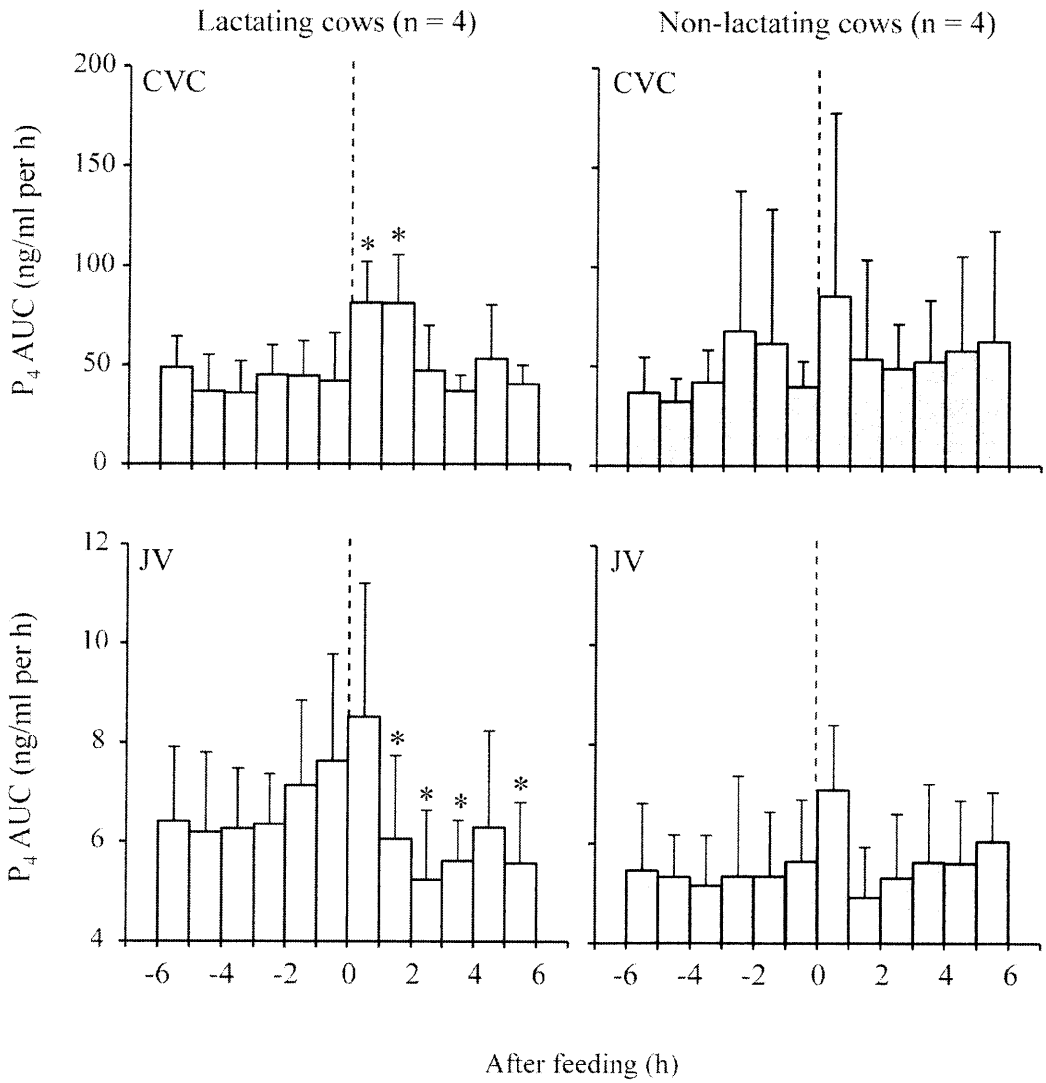

Figure 5-2 Changes in area under the curve of progesterone (P_4 AUC) in caudal vena cava (CVC) and the jugular vein (JV) after feeding. Asterisk indicates a difference ($P < 0.05$) as compared to the value of -1 to 0 h after feeding. Broken line indicates the time when one half of the daily diet was provided.

Chapter 6. Changes in progesterone concentrations in the caudal vena cava and the jugular vein in response to pulsatile LH stimulation induced by GnRH treatment during the mid-luteal phase in lactating dairy cows

6.1. Introduction

The results in Chapters 3 and 5 indicate that lactating dairy cows with normal estrous cycles have greater frequency of LH pulses, higher progesterone concentrations and larger CL size during mid-luteal phase compared with non-lactating cows. Furthermore, in Chapter 5, it is shown that a high proportion of LH pulses are followed by pulses of progesterone in the caudal vena cava. This finding is consistent with the previous observations in cattle (75, 119), and indicates that progesterone secretion by the CL is stimulated by the pulsatile secretion of LH. However, further studies are required to clarify the relationship between the LH pulses and progesterone secretion in lactating cows. In spite of the greater frequency of LH pulses in lactating cows compared with non-lactating cows, any difference in the progesterone concentrations and the detected number of progesterone pulses in the caudal vena cava was not detected between the groups. This may be attributed to statistical difficulties to detect the pulses because of the wide fluctuations of progesterone concentrations through the frequent sampling time in the caudal vena cava and also to the presence of progesterone pulses that were not associated with LH pulses. In addition to the observational study, another experimental approach is needed to determine how frequency of LH pulses directly influences the luteal function and subsequent progesterone concentrations in the caudal vena cava and the jugular vein in lactating cows.

The objectives of the present study were to examine the direct association between LH pulses and luteal progesterone secretion in lactating dairy cows and to determine whether an increase in the frequency of LH pulses would influence progesterone concentrations in the

caudal vena cava and the jugular vein. For these purpose, the present study employed low dose treatment of GnRH in order to induce endogenous LH pulses of physiological magnitude in cows [5,6]. To assess the secretion patterns of progesterone, blood samples were collected from the caudal vena cava and the jugular vein.

6.2. Materials and methods

6.2.1. Animals

Five lactating Holstein dairy cows (one primiparous and four multiparous ; 5.1 ± 2.1 years of age; 95.1 ± 34.0 days postpartum ; 25.5 ± 4.0 kg of daily milk yield) were used in this study. At the beginning of the study, their BW and BCS were 680 ± 59.1 kg and 3.4 ± 0.3, respectively.

6.2.2. Experimental procedure

The study was started from the beginning of the estrous cycle (Day 0: day of ovulation) until the end of the cycle, for which subsequent ovulation was confirmed. Cows were treated with GnRH- (GnRH group; n = 4) or saline- (saline group; n = 3) during the experiment. Two of three cows in the saline-treated group were assigned again for GnRH group after they spent at least one untreated estrous cycle before the GnRH-treatment cycle. For the analysis of follicular and luteal development and plasma progesterone, ovarian ultrasonography and blood sampling were conducted every other day from Day 0 to Day 14 and then daily through the cycle, as described in Chapter 2. For the analyses of progesterone in the caudal and jugular veins and LH in the jugular vein, catheterization into both veins was conducted on one day during the mid-luteal phase (Day 10, 11, 12 or 13), as described in Chapter 2. On the day after catheterization, cows were treated intravenously six times with GnRH (2.5 μg of gonadorelin acetate (LH-RH injection, Tanabe Seiyaku Co., Ltd., Osaka, Japan) in 2 ml of

sterile 0.9% w/v NaCl solution) for the GnRH group or 2 ml of sterile 0.9% w/v NaCl solution for the saline group at 1-h intervals via the jugular catheter, beginning at 1100 h. Blood samples (6 ml) for LH and progesterone determination were collected from the both veins at 12-min intervals for 12 h (0500 to 1700 h); pre-treatment 6 h and post-treatment 6 h. The sampling interval was shorten from 15-min (Chapters 3 and 5) to 12-min in the present study for more accurate analysis of pulsatile patterns. The obtained blood samples were processed as described in Chapter 2. To exclude the possible influence of feed intake on measured parameters, residual feed were removed before the beginning of blood sampling, and 25% of the daily amount of TMR was provided at 3 h and 9 h after the beginning of blood sampling (0800 and 1400 h).

6.2.3. Hormone assays

Plasma concentrations of progesterone and LH were measured as described in Chapter 2. For the progesterone assay, the intra- and interassay coefficients of variation were 3.4% and 7.4%, and the sensitivity was 0.03 ng/ml. For the LH assay, the intra- and interassay coefficients of variation were 6.1% and 11.9%, and the sensitivity was 0.09 ng/ml

6.2.4. Statistical analyses

In one cow in GnRH group, blood samples could not be collected from the caudal vena cava because of difficulty of catheterization, therefore, only the samples collected from the jugular vein of this cow were included for data analysis. Data for LH and progesterone profiles were evaluated by repeated measures ANOVA using GLM procedure of SPSS software version 20.0 for Windows. The ANOVA model included the fixed effects treatment group (GnRH, saline) and period (pre-treatment, treatment) and their interaction. When a

significant difference was detected, differences among groups within each period or among periods within each group were analyzed by pairwise *t*-test or Mann-Whitney *U* test.

Pulsatile patterns of LH in the jugular vein and progesterone in the caudal vena cava were analyzed by cluster analysis program (116), and the temporal relationships between pulses of LH in the jugular vein and progesterone in the caudal vena cava were determined, as described in Chapter 2. Differences between groups were tested using Fisher's exact test.

6.3. Results

6.3.1. Luteal development during the estrous cycle

Based on ovarian ultrasonography, there was no significant difference between the GnRH and saline groups in the estrous cycle length subjected to the experiment (22.5 ± 2.5 and 22.3 ± 1.2 days; mean ± SD), and mean CL diameter during the mid-luteal phase of Days 8 - 14 was 24.2 ± 1.9 and 24.8 ± 0.3 mm, respectively. The day of luteolysis, as determined by the definition described in Chapter 2, was not significantly different between GnRH and saline groups (17.3 ± 1.5 and 17.5 ± 2.4 days).

6.3.2. Secretion patterns of LH in the jugular vein and progesterone in the caudal vena cava and the jugular vein

Representative profiles of LH in the jugular vein and progesterone in the caudal vena cava and the jugular vein for cows treated with GnRH or saline are shown in **Fig. 6-1**.

Hourly injections of GnRH induced 5.3 ± 0.5 LH pulses during the post-treatment 6 h, which was greater ($P < 0.05$) than those in saline-treated cows (3.0 ± 0.0 pulses / 6 h; **Table 6-1**). The peak of LH pulse was detected within 12 or 24 min (mean, 18.6 ± 6.1 min) after each GnRH injection. Mean LH concentrations were increased by GnRH treatment, and the mean LH concentrations during the post-treatment 6 h was higher ($P < 0.05$) in GnRH group

than in saline group (0.50 ± 0.12 and 0.38 ± 0.09 ng/ml). Basal LH concentrations in GnRH group were increased from 0.23 ± 0.04 ng/ml during the pre-treatment to 0.38 ± 0.06 ng/ml during the post-treatment 6 h ($P < 0.05$), but were not different from those in saline group. Amplitude of LH pulses was not changed by GnRH or saline treatments, and the amplitude of LH pulses during the post-treatment 6 h in GnRH and saline groups was 0.30 ± 0.12 and 0.44 ± 0.12 ng/ml, respectively.

Changes in progesterone concentrations in the caudal vena cava and the jugular vein during the 12 h frequent blood sampling are shown in **Fig. 6-2**, and characteristics of progesterone pulses in the caudal vena cava and the jugular vein are summarized in **Table 6-2.** Progesterone concentrations in the caudal vena cava were increased by GnRH treatment, and mean progesterone concentrations during the post-treatment 6 h were higher ($P < 0.05$) in GnRH group than in saline group (74.8 ± 54.9 and 45.3 ± 20.2 ng/ml). Basal progesterone concentrations in the caudal vena cava in GnRH group were increased from 29.1 ± 11.4 ng/ml during the pre-treatment to 43.6 ± 21.8 ng/ml during the post-treatment ($P < 0.05$), but no significant difference was detected between GnRH and saline groups in basal progesterone concentrations in the caudal vena cava during the post-treatment 6 h. Frequency of progesterone pulses in the caudal vena cava was not changed by GnRH or saline treatments, and the frequency of progesterone pulses in the caudal vena cava during the post-treatment 6 h in GnRH and saline groups was 4.3 ± 0.6 and 3.3 ± 0.6 pulses/ 6 h, respectively. Amplitude of progesterone pulses in the caudal vena cava was increased by GnRH treatment, and the amplitude of progesterone pulses during 6-h treatment period were higher ($P < 0.05$) in GnRH group than in saline group (97.7 ± 16.9 and 51.8 ± 33.8 ng/ml).

Progesterone concentrations in the jugular vein were not different between GnRH and saline groups during the pre-treatment 6 h (mean, 6.0 ± 1.5 and 5.7 ± 1.7 ng/ml; **Table 6-2** and **Fig. 6-2**). However, progesterone concentrations in the jugular vein were increased by

GnRH treatment, and mean progesterone concentrations during the post-treatment 6 h were higher ($P < 0.05$) in GnRH group than in saline group (7.0 ± 2.0 and 5.4 ± 1.4 ng/ml). No significant difference was detected between GnRH and saline groups in characteristics of pulsatile pattern of progesterone (pulse frequency, basal level and pulse amplitude). Progesterone concentrations in the jugular vein at 24, 48, and 72 h after the treatment did not differ significantly between GnRH and saline groups (mean, 6.6 ± 1.6 and 6.2 ± 1.3 ng/ml).

6.3.3. Association of LH pulses in the jugular vein with progesterone pulses in the caudal vena cava and the jugular vein

The proportion of progesterone pulses that followed an LH pulse within 60 min of the peak of the LH pulse (i.e. LH-associated progesterone pulses) were calculated to examine the association of LH pulse with progesterone pulses in the caudal vena cava and the jugular vein (**Table 6-3**).

In the caudal vena cava, the proportion of LH-associated progesterone pulses during the post-treatment 6 h in GnRH group (12/13; 92.3%) was greater ($P < 0.05$) than that during the pre-treatment 6 h (8/13; 61.5%), but was not different from those in saline group (7/10; 70.0%). The interval from the peak of LH pulse to the peak of progesterone pulse during the post-treatment 6 h was 11.0 ± 13.0 (range, 0 to 36) and 16.0 ± 12.4 (range, 0 to 36) min in GnRH and saline groups, respectively ($P > 0.1$).

In the jugular vein, the proportion of LH-associated progesterone pulses was not changed significantly by GnRH or saline treatments. The interval from the peak of LH pulse to the peak of progesterone pulse during the post-treatment 6 h was 18.5 ± 13.5 (range, 0 to 36) and 30.0 ± 12.6 (range, 12 to 48) min in GnRH and saline groups, respectively ($P > 0.1$). Data of all cows in saline and GnRH groups were combined and comparison was made between the caudal vena cava and the jugular vein in terms of those observations. Of 52

pulses of progesterone detected in the caudal vena cava in all cows examined (three cows in saline group and three cows in GnRH group), 73.1% (38/52) of progesterone pulses were associated with LH pulses. As for progesterone pulses in the jugular vein, of 54 pulses in all cows examined (three cows in saline group and four cows in GnRH group), 68.5% (37/54) of progesterone pulses were associated with LH pulses, and no significant difference was detected compared with that in the caudal vena cava. However, the mean interval from the peak of LH pulse to the peak of progesterone pulse in the caudal vena cava in all cows examined was 11.4 ± 11.3 min, which was different from that in the jugular vein (19.5 ± 13.1 min, $P < 0.01$).

6.4. Discussion

The hourly pulsatile treatment of low dose GnRH to lactating dairy cows induced pulsatile LH secretion of greater frequency compared with that in the saline group, which resulted in the increases in progesterone concentrations both in the caudal vena cava and the jugular vein. The GnRH treatment would cause both LH and FSH secretion, but the increase in progesterone secretion following GnRH injection is probably due to the direct action of LH rather than FSH, as has been suggested in vivo and in vitro studies (29, 34). The frequency of LH pulses in GnRH group (around one pulse every 1 h) was about two times greater than that in the saline group (around one pulse every 2 h), and was almost comparable to those of the early luteal phase (82) or the follicular phase in cattle (40). The LH pulses induced by low dose of GnRH (2.5 μg of gonadorelin acetate) were similar amplitude to that of spontaneously released LH pulses in the saline-treated cows. It is widely accepted that pulsatile LH secretion is necessary for both structural and functional development of the CL in cattle (4, 71, 79), and this fact was supported by the study in Chapter 3. When the CL is fully developed, the subsequent role of LH pulses in the luteal function remains incompletely

understood. Peters et al. (71) reported that blocking pulsatile LH release by the treatment with a GnRH antagonist from Days 12 through 17 of the estrous cycle did not influence the circulating progesterone concentrations. In contrast to their study, a recent study reported that blocking pulsatile LH secretion by the treatment with a GnRH antagonist during mid-luteal phase in heifers decreased the circulating progesterone concentrations as well as pulsatile progesterone release by the CL (31). The present study examined the role of LH pulses in luteal progesterone secretion by increasing the frequency of LH pulses by the pulsatile treatment with low dose GnRH. Consequently, it was demonstrated that the only functional CL increased progesterone secretion in response to the increased frequency of LH pulses.

The luteal response to the LH pulses induced by the pulsatile GnRH treatment was well assessed by secretion patterns of progesterone in the caudal vena cava blood. The frequency of progesterone pulses was not changed by the pulsatile GnRH treatment, but the proportion of progesterone pulses that occurred in association with LH pulses was increased from 61.5% during the pre-treatment 6 h up to 92.3% during the post-treatment 6 h. This indicates that almost all progesterone pulses were released in association with LH pulses, and the mean value of amplitude of progesterone pulses during the pulsatile GnRH treatment was about two times higher than that of the saline group. Thus, it is suggested that stimulation of the CL by an LH pulse cause a pulsatile increase in progesterone concentrations in the caudal vena cava, and increased frequency of LH pulses results in a significant increase in mean progesterone concentration during the pulsatile GnRH treatment.

Progesterone concentrations in the jugular vein also increased in response to the increased LH pulses, but the increase was smaller compared with those in the caudal vena cava. It was calculated that two times increase in the frequency of LH pulses leaded to 1.5 and 1.3 times increases in the mean concentrations of progesterone in the caudal vena cava and the jugular vein, respectively. In this Chapter, pulsatile pattern of progesterone was also

determined in the jugular vein and compared to that in the caudal vena cava. The obtained results showed that the frequency of progesterone pulses in the jugular vein were similar to those in the caudal vena cava, and were not changed by GnRH or saline treatment. In addition, although most of progesterone pulses in the jugular vein as well as in the caudal vena cava were detected in association with LH pulses (68.5 and 73.1%, respectively), the interval from LH peak to progesterone peak was longer in the jugular vein than in the caudal vena cava (19.5 and 11.4 min, respectively). In regard to the pulse amplitude of progesterone in the jugular vein, any significant change was not detected during the GnRH treatment, in contrast to the marked increase in the caudal vena cava. Therefore, while pulsatile pattern of progesterone concentrations can be detected both in the caudal vena cava and the jugular vein, blood samples collected from the caudal vena cava may be more favorable for examining changes in the secretion pattern (i.e. pulse amplitude) and identifying the peak time of a progesterone pulse. The less clear pattern of progesterone pulses and the smaller increase in progesterone concentrations in the jugular vein in response to the increased LH pulses could be explained not only by the peripheral hemodilution of secreted progesterone (70) but also by the elevated metabolic rates of progesterone in lactating dairy cows (93, 125).

The progesterone profiles in the jugular vein through the estrous cycle indicated that the increased progesterone concentration was not sustained for the remaining luteal phase after GnRH treatment. Additionally, the increased frequency of LH pulses caused by serial GnRH treatment appeared not to influence the CL size and the day of luteolysis. The stimulation of LH pulses within a physiological level (frequency and amplitude) could enhance steroid production of the CL within the comparable period, and might not bring long-term effects such as stimulation of CL growth or retarded luteolysis. This assumption is supported by a previous study showing that treatment with GnRH agonist, azagly-nafarelin, from Day 3 to Day 21 of the estrous cycle increased both the luteal size and plasma progesterone

concentrations, whereas treatment with GnRH agonist from Day 12 to Day 21 of the estrous cycle increase the plasma concentrations of progesterone without any alteration in the luteal size compared with the controls (16).

In summary, the increased frequency of LH pulses led to increases in progesterone concentrations both in the caudal vena cava and the jugular vein in lactating dairy cows. Most of progesterone pulses in the caudal vena cava as well as the jugular vein occurred in association with LH pulses. Although it cannot be determined from the present study whether LH pulses are essential for the fully developed CL, these results imply that increase in the frequency of LH pulses could contribute to the increase in circulating progesterone concentrations during the estrous cycles in lactating cows.

Table 6-1 Pulsatile patterns of LH in the jugular vein for cows treated with saline or GnRH during the mid-luteal phase.

Characteristic	Group	Pre-treatment 6 h	Post-treatment 6 h
Mean concentration (ng/ml)	Saline (n = 3)	0.38 ± 0.09	0.38 ± 0.09[b]
	GnRH (n = 4)	0.32 ± 0.06[x]	0.50 ± 0.12[cy]
Basal level (ng/ml)	Saline (n = 3)	0.31 ± 0.02	0.33 ± 0.02
	GnRH (n = 4)	0.23 ± 0.04[x]	0.38 ± 0.04[y]
Pulse frequency (pulses/6 h)	Saline (n = 3)	4.0 ± 1.0	3.0 ± 0.0[b]
	GnRH (n = 4)	3.0 ± 0.8[x]	5.3 ± 0.5[cy]
Pulse amplitude [a)] (ng/ml)	Saline (n = 3)	0.31 ± 0.09	0.44 ± 0.12
	GnRH (n = 4)	0.29 ± 0.08	0.30 ± 0.12

Values are mean and SD.

[a)] Pulse amplitude was calculated by subtracting the maximal concentration of a pulse from the concentration preceding the pulse and expressed as ng/ml.

[b, c] Different superscripts within a column indicate significant differences ($P < 0.05$) between saline and GnRH group.

[x, y] Different superscripts within a row indicate significant differences ($P < 0.05$) between pre- and post-treatment 6 h.

Table 6-2 Pulsatile patterns of progesterone (P_4) in the caudal vena cava (CVC) and the jugular vein (JV) for cows treated with saline or GnRH during the mid-luteal phase.

Veins	Characteristic	Group	Pre-treatment 6 h	Post-treatment 6 h
CVC	Mean concentration (ng/ml)	Saline (n = 3)	46.6 ± 18.9	45.3 ± 20.2 [a]
		GnRH (n = 3)	48.3 ± 30.0 [x]	74.8 ± 54.9 [by]
	Basal level (ng/ml)	Saline (n = 3)	34.8 ± 10.3	31.3 ± 8.8
		GnRH (n = 3)	29.1 ± 11.4 [x]	43.6 ± 21.8 [y]
	Pulse frequency (pulses/6 h)	Saline (n = 3)	5.3 ± 0.6	3.3 ± 0.6
		GnRH (n = 3)	4.3 ± 1.5	4.3 ± 0.6
	Pulse amplitude (ng/ml)	Saline (n = 3)	30.7 ± 18.1	51.8 ± 33.8 [a]
		GnRH (n = 3)	47.3 ± 31.0 [x]	97.7 ± 16.9 [by]
JV	Mean concentration (ng/ml)	Saline (n = 3)	5.7 ± 1.7	5.4 ± 1.4 [a]
		GnRH (n = 4)	6.0 ± 1.5 [x]	7.0 ± 2.0 [by]
	Basal level (ng/ml)	Saline (n = 3)	4.3 ± 1.6	4.3 ± 0.8
		GnRH (n = 4)	4.7 ± 0.5	5.0 ± 0.8
	Pulse frequency (pulses/6 h)	Saline (n = 3)	4.7 ± 2.5	3.3 ± 0.6
		GnRH (n = 4)	3.5 ± 1.0	4.0 ± 0.8
	Pulse amplitude (ng/ml)	Saline (n = 3)	3.6 ± 0.5	3.1 ± 0.9
		GnRH (n = 4)	3.0 ± 0.7	3.6 ± 2.4

Values are mean and SD. [a)] Pulse amplitude was calculated by subtracting the maximal concentration of a pulse from the concentration preceding the pulse and expressed as ng/ml.

[a, b] Different superscripts within a column indicate significant differences ($P < 0.05$) between saline and GnRH group.

[x, y] Different superscripts within a row indicate significant differences ($P < 0.05$) between pre- and post-treatment 6 h.

Table 6-3 Association of LH pulses with progesterone (P_4) pulses in the caudal vena cava (CVC) and the jugular vein (JV) for cows treated with saline or GnRH during mid-luteal phase.

Veins	Characteristic	Group	Pre-treatment 6 h	Post-treatment 6 h
CVC	LH-associated P_4 pulses [a] (%)	Saline (n = 3)	11/16 (68.8)	7/10 (70.0)
		GnRH (n = 3)	8/13 (61.5^{x})	12/13 (92.3^{y})
	LH peak to P_4 peak (min)	Saline (n = 3)	12.0 ± 9.3	16.0 ± 12.4
		GnRH (n = 3)	7.5 ± 11.0	11.0 ± 13.0
JV	LH-associated P_4 pulses [a] (%)	Saline (n = 3)	9/14 (64.3)	6/10 (60.0)
		GnRH (n = 4)	9/14 (64.3)	13/16 (81.3)
	LH peak to P_4 peak (min)	Saline (n = 3)	18.7 ± 13.6	30.0 ± 12.6
		GnRH (n = 4)	14.7 ± 10.0	18.5 ± 13.5

Values are mean and SD.

[a] P_4 pulses that followed an LH pulse within 60 min of the peak of the LH pulse/ total number of P_4 pulses.

[b] Intervals from the peak of the LH pulse to the peak of the P_4 pulse (mean and SD).

[x, y] Different superscripts within a row indicate a significant difference ($P < 0.05$).

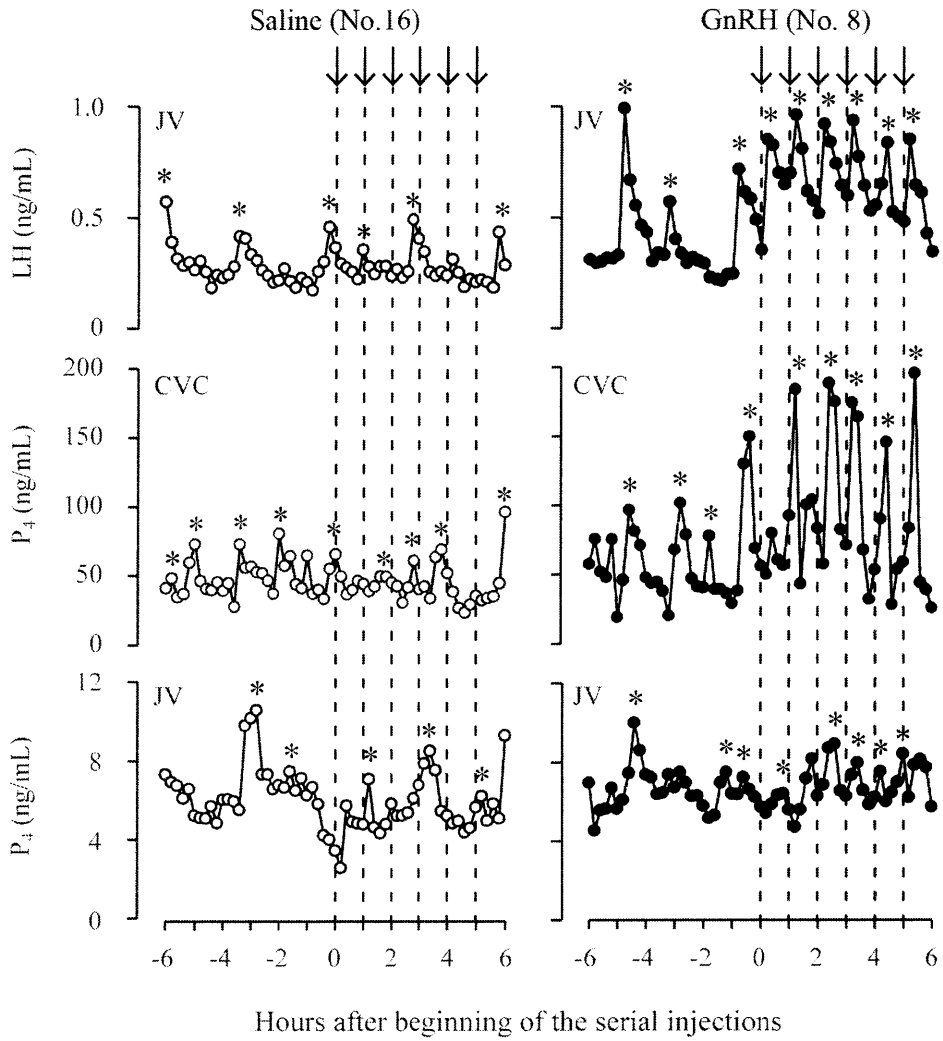

Fig. 6-1 Representative profiles of LH in the jugular vein (JV) and progesterone (P_4) in the caudal vena cava (CVC) and in the JV for cows treated with saline (open circles) or GnRH (filled circles) intravenously six times at 1-h intervals on one day during the mid-luteal phase (on 11 days after ovulation for the both cows). Asterisk indicates a peak of LH or P_4 pulse. Arrow and broken line indicate time of saline or GnRH injection.

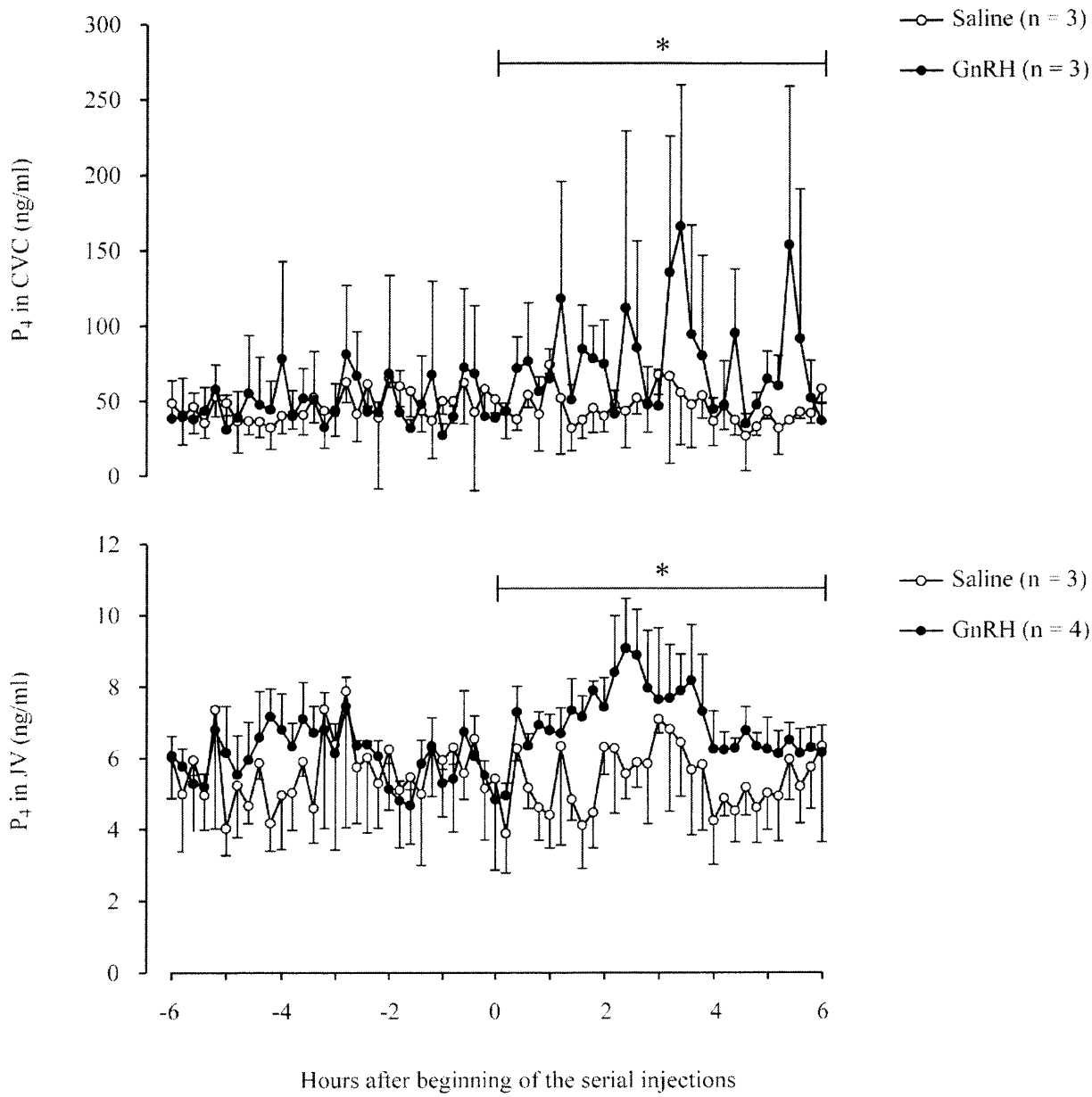

Fig. 6-2 Progesterone concentrations (P_4) in the caudal vena cava (CVC) and the jugular vein (JV) during the 12 h frequent blood sampling for cows treated with saline (open circles) or GnRH (filled circles). Values are mean and SD and arrow indicates time of saline or GnRH injection. Asterisk indicates a significant difference ($P < 0.05$) between saline and GnRH groups.

Chapter 7. General Discussion

In this dissertation, the relationship between lactation and reproductive physiology in dairy was examined based on the comparative studies between lactating and non-lactating cows. First, ovarian dynamics, hormonal profiles of ovarian steroids and LH during the estrous cycle were investigated (Chapter 3). The more detailed examinations of hormonal patterns in relation to time of luteolysis and ovulation were performed, together with the observation of estrous signs (Chapter 4). Further, progesterone concentrations at the secreted and the circulating levels and LH pulsatile pattern were determined during the mid-luteal phase, and the metabolic status of progesterone was assessed with reference to feeding (Chapter 5). Finally, the association of LH pulsatile secretion with progesterone secretion by the functional CL in lactating cows was examined (Chapter 6).

7.1. Luteal development and progesterone concentration in estrous cycles

The results in Chapters 3 and 5 consistently suggested that lactating cows had larger CL and higher progesterone concentrations during mid-luteal phase than non-lactating cows. Development of larger CL in lactating cows than non-lactating cows or heifers has been reported in previous studies (96, 97, 128). Sartori et al. (97) suggested the larger CL in lactating cows might simply result from the ovulation of larger ovulatory follicles compared with non-lactating cows. Development of larger ovulatory follicles was also observed in the case of two follicular waves in the present study (Chapter 3). In addition, the present study found that lactating cows had a higher frequency of LH pulses during the early to mid-luteal phase than non-lactating cows. The findings of Chapter 6 suggest that the higher frequency of LH pulses may have a key role in enhancing luteal development in lactating cows and progesterone secretion by the fully-developed CL.

While progesterone concentrations during the mid-luteal phase in lactating cows was consistently higher than that in non-lactating cows (Chapters 3 and 5), the frequent blood sampling at 15-min intervals detected significantly lower levels of progesterone during the early luteal phase (Days 4 and 6) in lactating cows compared with those in non-lactating cows. Low circulating concentrations of progesterone during early luteal phase are considered as one of major risk factors that can decrease embryo survival rates (41, 49, 59). This is because the timing of changes in the uterine environment, upon which the normal embryonic development dependents, is controlled by the time of the increase in progesterone levels (58, 103). In a recent study on multiparous lactating dairy cows, McNeill et al. (59) found that there was a positive relationship between the concentrations of milk progesterone and the probability of embryo survival during the early luteal phase of Days 4 and 6 after ovulation but not during the mid-luteal phase of Days 7 and 8. Further, in an attempt to improve pregnancy rate in cattle, the effects of progesterone supplementation have been investigated in numerous studies. The results are variable, but a meta-analysis study suggests that if progesterone supplementation is commenced after Day 6 no benefit results while if treatment is commenced before day 6 a 10% improvement in pregnancy results (58). In early luteal phase, developing CL cannot produce as much progesterone as functional CL does. As a specific case in lactating dairy cows, the increased metabolic rate of progesterone (93) could have a great influence on circulating progesterone concentrations during the early luteal phase, which can increase the incidence of embryonic loss . Supplementation of progesterone from early luteal phase may be a successful strategy for improving pregnancy rates after AI in lactating dairy cows.

7.2. Follicular dynamics and preovulatory hormonal patterns of estradiol-17β, progesterone and LH

In Chapter 3, the dominant follicle of the first wave and the ovulatory follicle in the estrous cycles with two follicular waves in lactating cows grew larger than those of non-lactating cows. Increased size of the ovulatory follicle in lactating cows was previously reported in comparisons with non-lactating cows (17, 96, 97) or with nulliparous heifers (128). It has been demonstrated that increased frequency of LH pulses led to continuous growth of the dominant follicles (100), which could result in the development of larger preovulatory follicles in lactating cows. However, further difference was not found between the groups in the duration of the first follicular wave, the days from emergence of the ovulatory follicles to ovulation and the estradiol-17β concentrations in the daily collected blood samples through the estrous cycles (Chapter 3).

Recent studies have found that high variability exists in the timing of estrus and ovulation in modern dairy cows (9, 99, 108). Extended interval from insemination to ovulation resulted in low pregnancy rates (27, 112), probably because of sperm aging and lack of fertilizing ability. The variations in the timing of estrus and ovulation could be relevant to asynchrony of hormonal profiles such as estradiol-17β rise around estrus and timing of LH surge. Bloch et al. (9) reported that about 10% of high-yielding Holstein cows exhibited long estrus to ovulation interval which comprised long intervals both from estrus to LH surge and from LH surge to ovulation. In that study, the extend interval from estrus to ovulation was associated with low preovulatory estradiol concentrations and low amplitude of LH surges (9). When the greater steroid metabolism in lactating dairy cows is taken into consideration, I supposed that some alterations in hormonal profiles should appear by intensive monitoring of follicular phase events in Chapter 4. However, no significant difference was found in the preovulatory dynamics of estradiol-17β during the period from

luteolysis to ovulation, and the amount of LH surge was similar between the lactating and non-lactating cows. These similar hormonal profiles may bring the fewer variations in the time from the estradiol-17β peak to ovulation (range 12 h) and the time from the LH surge to ovulation (range 0 h) in the present study compared with other studies in lactating (9) and non-lactating cows (108).

7.3. Expressions of estrous signs

Estradiol-17β acts on the hypothalamus to induce estrous behavior in most species (2). It is suggested that low expressions of estrus and estrous signs in lactating dairy cows might be attributed to low concentrations of estradiol-17β around estrus. In the present study, however, concentrations of estradiol-17β during the period from luteolysis to ovulation in lactating cows were comparable to those in non-lactating cows, and therefore the appearance of estrous signs in behavior, vulva and vagina might not be different between lactating and non-lactating cows. Nevertheless, the problem of low estrus detection rate already exists as a major concern to dairy farmers, AI technicians and veterinarians. It is likely that other factors rather than estradiol-17β level may be attributed to low expression of estrous behavior in dairy cows. It is commonly known that the housing and type of flooring can influence the intensity of estrous expression in cows. In addition, lameness negatively affected the ability of cows to express estrus, and herds with prevalent lameness experience a decline in the estrus detection rates (106, 118). Effective management strategies and more research efforts are needed to provide an environment that allows cows to maximize their estrus display.

On the other hand, the estrous signs in the vulva and vagina were observed more clearly compared with those related to behavior. It seems to be easy to distinguish the appearance in the vagina around estrus and ovulation from that in luteal phase. This is of significant importance in the practical use of the vaginoscopic examination for the diagnosis of estrus in

cows. A recent study investigated the incidence of error in estrous detection based on the secondary estrous signs such as restlessness and mucus discharge in a tie-stalled dairy herd (84). The study reported that of a total of 68 AI, 13 (19.1%) and 2 (2.9%) were carried out in the luteal phase and during pregnancy, respectively. High incidence of estrus detection error may be partly attributed to poor expression of estrous signs and shorter duration of estrus in modern dairy cows (23, 113, 129). The present basic findings of genital estrous signs related to the timing of luteolysis and ovulation are informative for the practical use of vaginoscopic examination as an aid to detect estrus and determine the timing of AI.

7.4. Progesterone dynamics in the caudal vena cava and the jugular vein with reference to feeding

Progesterone concentration in the circulating blood reflects the balance between secretion and metabolic status of progesterone. I employed catheterization procedure into the caudal vena cava in order to assess the secretion status of progesterone. In agreement with previous studies (75, 119), the present study showed a temporal association between endogenous LH pulses and progesterone pulses in the caudal vena cava both in lactating and non-lactating cows during the mid-luteal phase (Chapter 5). Furthermore, it was experimentally demonstrated by the pulsatile injections of GnRH that luteal response to an LH pulse can be detected as a pulsatile increase in progesterone concentrations in the caudal vena cava (Chapter 6). This confirms that the fluctuation of progesterone concentrations in the caudal vena cava well reflects the LH-promoted progesterone secretion from the CL.

However, I could not detect any difference in the progesterone concentrations in the caudal vena cava between lactating and non-lactating cows, in spite of the higher progesterone concentrations in the circulating blood of the lactating cows (Chapter 5). This is partially due to the wider variations in progesterone concentrations in the caudal vena cava

compared with that in the jugular vein. In addition, the possibility that differences in blood volume or blood flow in the examined animals may influence the progesterone concentrations at the sampling site should be taken into consideration in a further study.

Feeding related changes in progesterone concentrations have been investigated in peripheral blood samples to examine the effect of progesterone metabolism (60, 80, 115). To my knowledge, there is no available information on the feeding-related changes in progesterone dynamics in the caudal vena cava in cows. In the present study, feeding causes a significant increase in the progesterone concentrations in the caudal vena cava in lactating and non-lactating cows, and this occurred without apparent alterations in the LH patterns. This implies that other factors rather than LH could be involved in mediating the feeding-related changes in progesterone concentrations in the caudal vena cava.

On the other hand, feeding 50% of the daily amount of diet decreased progesterone concentrations in the jugular vein in lactating cows. However, such a decrease was not detected in the non-lactating cows in the present study. It has been previously demonstrated by Sangsritavong et al. (93) that feed intake increases liver blood flow and metabolic clearance of progesterone, and thereby decrease the circulating concentrations of progesterone. The post-feeding decrease in the circulating progesterone observed only in the lactating cows is likely the result of elevated steroid metabolism associated with the great feed intake and the elevated liver function required for lactation. Practical strategies, such as frequency of feeding, feed composition, or dietary supplements for alleviating the elevation of progesterone metabolism are needed to be developed to improve fertility of lactating dairy cows.

7.5. Role of LH pulses in luteal function during the mid-luteal phase of lactating dairy cows

Luteinizing hormone is released in a pulsatile manner from the anterior pituitary gland during the estrous cycle, and the frequency of the LH pulses decreased from the early to the mid-luteal phase by the negative feedback effect of progesterone. It is widely accepted that pulsatile LH secretion is necessary for both structural and functional development of the CL in cattle (4, 71, 79). However, the role of pulsatile release of LH in the fully developed CL is considered to be not essential (71) or minor even if not at all (63, 126). This is because large luteal cells that constitute 40% of the CL volume produce at least 80% of the secreted progesterone by the CL independent of LH stimulation (62), while progesterone secretion by small luteal cells is stimulated by LH in a dose-dependent manner (34).

The detailed examinations with frequent blood sampling (Chapters 5) showed a significant difference in the frequency of LH pulses between lactating and non-lactating cows, in which the frequency of LH pulses during the mid-luteal phase in lactating cows (one pulse every 2 h) was around two times higher than that in non-lactating cows (one pulse every 4 h) in the present and earlier studies (82, 119). In addition to the temporal association between LH and progesterone secretion patterns, it was experimentally confirmed by the pulsatile GnRH injections (Chapter 6) that an increase in the frequency of LH pulses leads to increases in the progesterone concentrations both in the caudal vena cava and the jugular vein. These findings suggest a possibility that relatively higher frequency of LH pulses in lactating dairy cows than that in non-lactating cows may have a role in stimulating progesterone secretion by the CL.

7.6. Conditions of examined cows

Cows undergo a normal process of nutrient partitioning and adipose tissue mobilization during early lactation (5). When nutrient requirements for maintenance and lactation exceed the ability of the cow to consume energy in the feed, NEB status should occur and which leads to a significant decrease in BW and BCS. Increased genetic merit for milk yield is often associated with a greater degree of BCS loss in early lactation and less BCS throughout lactation, reflecting a greater degree of NEB (77). When studies are conducted to examine the reproductive function of dairy cows in association with milk production, nutritional or energy status of the examined cows should be also taken into account. This is because these factors have a significant influence on follicular development and luteal activity as well as uterine involution in postpartum period (50, 69). The current decline in fertility of lactating dairy cows may be partly attributed to the difficulty of managing high genetic merit animals appropriately (121).

In the present study, the lactating cows were in mid-lactation period (> 80 days postpartum) and their milk production during the experiment was around 30 kg per day on average. At the herd level, the milk yield per lactation per cow was around 9,000 kg. This production level is not comparable with that in high-producing cows (> 10,000 kg per year or > 40 kg per day) described in other studies (52, 96, 128), but corresponds to the average value of 9,266 kg in dairy herds in 2012 that were registered to Dairy Herd Milk Recording Association, Japan. The lactating and non-lactating cows maintained their BCS and BW within a normal level in all studies in Chapters 3 to 6. Also, all cows used in the present studies had resumed cyclic ovarian activity prior to being subjected the studies. Butler et al. (15) have reported that cows ovulated approximately 10 days after they begin to move towards a positive energy balance. Thus, as far as I observed, the lactating cows as well as the

non-lactating cows appeared to be not experiencing NEB or major nutritional problems throughout the studies.

In addition, from the results of blood chemical examination (Chapter 5), no abnormal values representing nutritional deficiency or impaired hepatic function were detected in all individual cows. The lactating cows showed significantly higher AST values compared with those in non-lactating cows, despite all individual values were within a normal range. In general, the increased metabolic requirements for lactation cause both hypertrophy and hyperplasia of the liver and gastrointestinal organs (33). Such adjustment of liver function for milk production may lead a transient increase in AST value in postpartum lactating dairy cows (36). Also, it should be noted that there was a great difference between lactating and non-lactating cows in the concentrations of total cholesterol (224.5 ± 18.5 and 79.9 ± 3.7 mg/dl). Cholesterol, which can be derived from the diet or be synthesized de novo and transported to the ovaries by lipoproteins, is a common precursor for steroids synthesis (85). The higher concentrations of total cholesterol in the lactating cows compared with those in the non-lactating cows may reflect greater feed intake and alterations in lipid metabolism to support lactation (5). Some studies reported that dietary fat supplements may stimulate follicular and luteal activity by improving energy balance and enhancing utilization of blood cholesterol for progesterone synthesis (32, 122). The high cholesterol concentrations in lactating cows may positively influence luteal function along with the stimulation of LH pulses.

7.7. Conclusions

In this dissertation, the observed differences in reproductive parameters in lactating cows compared with those in non-lactating cows were as follows:

1) Larger CL size and higher plasma progesterone concentration during the mid-luteal phase (Chapters 3 and 5).
2) Lower progesterone concentrations during the early luteal phase (Chapter 3).
3) Post-feeding decrease in progesterone concentration in the circulating blood (Chapter 5).
4) Greater development of dominant follicle (Chapters 3 and 5) and subordinate cohort of follicles (Chapter 3).
5) Higher frequency of LH pulses during early to mid-luteal phase (Chapters 3, 5 and 6).

The low progesterone concentrations during the early luteal phase and decrease in circulating progesterone concentration may arise from elevated metabolic rate of progesterone in the liver and could be a crucial factor that negatively influences the fertility of dairy cows. The milk yield of lactating cows in the present study was not comparable to that of high-producing cows. It is possible that increase in milk yield per cow may extend these negative influences of lactation on fertility of dairy cows. This assumption is supported by the studies showing markedly low conception rates and high incidence of pregnancy loss in high-producing cows (54, 77, 94). To overcome these problems, practical approaches are needed including the use of progesterone supplementation after insemination or feeding management that can alleviate the effect of steroid metabolism.

On the other hand, greater development of the functional CL, higher plasma concentrations of progesterone during the mid-luteal phase, and similar estradiol-17β concentrations throughout the estrous cycles were observed in lactating cows compared with those in in non-lactating cows. Furthermore, lactation did not interfere with the expressions of estrous signs and endocrine profiles from luteolysis to ovulation. This finding suggests that

the problem of low estrus detection rates in modern dairy cows can be partly overcome by developing management techniques to enhance the accuracy and efficiency of estrus detection. It is concluded that when lactating cows are maintained in better nutritional and management condition, they can partly compensate for negative influence of lactation by developing larger CL and follicles and increasing the secretion of ovarian steroids. The greater frequency of LH pulses in lactating cows may have an important role in stimulating follicular and luteal function in dairy cows, together with other hormones and metabolic factors that are related to lactation.

Chapter 8. Summary

Fertility in lactating dairy cows has been declining over the last 50 years simultaneously with a rapid increase in milk yields. Even when lactating cows show normal estrous cycles, the conception rate after breeding is markedly lower than that in heifers, which indicates that reproductive physiology of lactating dairy cows have been altered associated with high milk production. To deal with reproductive problems in modern dairy cows, it is necessary to understand better the influences of lactation on ovarian function and the associated endocrine profiles. Therefore, the objective of this dissertation was to investigate the influence of lactation on the follicular and luteal dynamics and endocrine profiles of ovarian steroids and LH during the estrous cycle in lactating dairy cows by comparing with those in non-lactating ones.

In Chapter 3, the dynamics of ovarian follicle, CL, and peripheral plasma ovarian steroids were compared between lactating and non-lactating cows, and a possible association of pulsatile LH secretion with the dynamics was examined. Ovarian ultrasonography was performed daily throughout two consecutive estrous cycles (Day 0: day of ovulation). Blood samples were collected daily and at 15-min intervals for 8 h on Days 2, 4, 6, 8, and 14 of the second cycle. Lactating cows had larger CL and higher progesterone concentrations during mid-luteal phase compared with non-lactating cows. Maximal diameters of the first wave dominant follicle (17.2 ± 1.8 vs. 15.5 ± 0.8 mm; mean ± SD) and the ovulatory follicle (17.9 ± 1.2 vs. 15.2 ± 0.8 mm) were larger ($P < 0.05$) in lactating cows than in non-lactating cows during the estrous cycles with two follicular waves. Plasma estradiol-17β concentrations did not differ between the groups throughout the experiment. Lactating cows had more LH pulses from Day 2 to 14 than non-lactating cows. These results imply that differences in ovarian

dynamics may exist between lactating and non-lactating cows, for which the increased number of LH pulses detected in lactating cows may have responsibility.

In Chapter 4, profiles of ovarian steroids and LH and the appearance of estrous signs in relation to luteolysis and ovulation were monitored during the first cycle of the study described in Chapter 3. Blood samples were collected at 3-h intervals after luteolysis until ovulation. Estrous signs in terms of behavior, the vulva and the vagina were checked at 8-h intervals after luteolysis until ovulation. Profiles of progesterone, estradiol-17β and LH did not differ between the groups. There were no differences in the interval from luteolysis to ovulation (4.6 ± 0.5 and 4.2 ± 0.8 d) and the interval from the estradiol-17β peak to ovulation (34.2 ± 4.5 and 30.6 ± 3.9 h) between lactating and non-lactating cows. The interval from the peak of the LH surge to ovulation was 27 h in all cows examined. Appearance of estrous signs did not differ between the groups. The vaginal estrous signs were observed conspicuously in all cows examined, but the behavioral signs were not observed in 20.0% of the cows. The duration of behavioral signs (41.3 ± 23.6 h) was shorter ($P < 0.05$) than that of the vagina (68.9 ± 25.4 h). These results imply that lactation might not interfere with the hormonal profiles from luteolysis to ovulation.

In Chapter 5, progesterone profiles at the secreted (caudal vena cava) and circulating levels (jugular vein) and LH secretion pattern were compared between lactating and non-lactating cows, and their metabolic status of progesterone was assessed with reference to feeding. Blood samples were collected simultaneously from the caudal vena cava and the jugular vein at 15-min intervals for 12 h during the mid-luteal phase. Cows were fed 50% of the daily diet 6 h after the start of blood sampling. During the 12-h sampling period, mean progesterone concentrations in the caudal vena cava did not differ between lactating and non-lactating cows (49.0 ± 29.2 and 53.3 ± 51.9 ng/ml), whereas mean progesterone concentrations in the jugular vein in lactating cows were higher than those in non-lactating

cows (6.4 ± 1.8 and 5.6 ± 1.4 ng/ml, $P < 0.001$). Lactating cows had a higher frequency of LH pulses than non-lactating cows (7.0 ± 1.4 and 4.3 ± 1.9 pulses/12 h, $P < 0.05$). Progesterone concentrations in the caudal vena cava increased after feeding in both groups, whereas progesterone concentrations in the jugular vein decreased after feeding in lactating cows but not in non-lactating cows. The post-feeding decrease in the circulating progesterone detected only in the lactating cows is likely the result of elevated steroid metabolism in the liver.

While feeding causes a decrease in the circulating progesterone concentrations in lactating cows, the higher concentration of progesterone through the mid-luteal phase of Days 8 – 14 in lactating cows than in non-lactating cows (Chapters 3 and 5) may suggest that greater amounts of progesterone were secreted from the CL in the lactating cows than in the non-lactating cows. In Chapter 6, it was examined whether increased frequency of LH pulses would influence luteal progesterone secretion during mid-luteal phase, by measuring progesterone concentrations at the secreted level (in the caudal vena cava) as well as at the circulating level (in the jugular vein) in lactating dairy cows. Cows were intravenously administered 2.5 μg GnRH (gonadorelin acetate; GnRH group; n = 4), or 2 ml saline (saline group; n = 3) six times at 1-h intervals during the mid-luteal phase. Blood samples were collected via catheters inserted in the caudal vena cava and the jugular vein at 12-min intervals for 12 h, starting from 6 h before the first GnRH administration. During the pre-treatment 6 h, any difference was not found between the groups in the LH secretion profiles (mean concentrations, basal level, pulse frequency and pulse amplitude) as well as in progesterone profiles in the caudal vena cava and the jugular vein. During the post-treatment 6 h, frequency of LH pulses (5.3 ± 0.5 vs. 3.0 ± 0.0 pulses/ 6 h) and mean LH concentration, but not the amplitude was higher ($P < 0.05$) in GnRH-treated cows than in saline-treated cows. In the caudal vena cava, mean progesterone concentrations and amplitude of progesterone pulse were higher ($P < 0.05$) in GnRH group than in saline group. In the jugular blood, mean

progesterone concentrations during the post-treatment 6 h were also higher in GnRH group than in saline group (7.0 ± 2.0 vs. 5.4 ± 1.4 ng/ml). However, secretion patterns of progesterone in the jugular vein (pulse frequency, pulse amplitude and basal level) did not show any difference among the groups. These results indicate that progesterone secretion from the functional CL can be enhanced by the increased frequency of LH pulses.

All the lactating cows used in the studies were in the mid-lactation period and producing a moderate level of milk yield, and maintained their BW and BCS within normal ranges. No pathological conditions in the liver function and nutritional status were detected from the blood chemical profiles both in the lactating and non-lactating cows in Chapter 5. Taken these findings together, this dissertation concluded that lactating dairy cows with good nutrient status have a capacity to develop CL and follicles to produce sufficient levels of ovarian steroids during the estrous cycles. Further, there was no apparent alteration in the appearance of estrous signs between lactating and non-lactating cows. The negative influence of lactation has presented as the lower progesterone concentration during the early luteal phase and the post-feeding decrease of circulating progesterone. However, lactating dairy cows can compensate for the greater reduction of circulating progesterone by the elevated progesterone metabolism to some extent by developing larger CL and elevating progesterone secretion during the mid-luteal phase. The increased frequency of LH pulses could contribute to the maintenance of the circulating progesterone concentrations during the estrous cycles in lactating cows.

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