Characteristics of reproductive physiology during conception period and maintenance of pregnancy in Hokkaido sika deer (*Cervus nippon yesoensis*)

エゾシカ（*Cervus nippon yesoensis*）の受胎と妊娠維持における繁殖生理学的特徴の解明

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Abbreviation

ANOVA: analysis of variance

BSA: bovine serum albumin

CCRL: curved crown-rump length

CL: corpus luteum

CMO: O-Carboxymethyl Oxime

EIA: enzyme immunoassays

ER: estrogen receptor

FHR: fetal heart rate

FSH: follicle stimulating hormone

GE: glandular epithelium

HL: head length

INFτ: interferon tau

LE: luminal epithelium

LH: luteinizing hormone

MYO: myometrium

OTR: oxytocin receptor

$PGF_{2\alpha}$: prostaglandin F 2 alpha

PR: progesterone receptor

SD: standard deviation

ST: stroma

SCRL: straight crown-rump length

TD: trunk depth

Preface

There are many species of boreal or temperate deer (Lincoln 1992) and they are short-day breeders (Sadleir 1987, Lincoln 1985, Loudon and Brinklow 1992) mating in autumn and fawning in early summer (Kaji 1988, Koizumi 1991, Matsuura et al. 2004a). Their seasonal reproductive pattern ensures that fawns are born at a time of year when food is available for lactating and the weather is favorable for survival of offspring (Loudon and Brinklow 1992). Although most of parturition takes place during period of about one month (Guinness et al. 1978a, Koizumi 1991, Birgersson and Ekvall 1997, Bowyer et al. 1998), range of 83 to 135 days for birth date due to late parturitions were reported in sika deer (Koizumi 1991, Matsuura 2004).

Late conception leads to late parturition (Matsuura et al. 2004a) and late parturition results in decline in reproductive success of females, defined as the number of fawns a mother reared to one year old over a specified period of time (red deer: Clutton-Brock et al. 1982), due to increase in mortality rate of fawn (Guinness et al. 1978b) or decrease in female conception rate in the following mating season (Clutton-Brock et al. 1983). Therefore, conceptions at the appropriate breeding season and subsequent maintenance of pregnancy throughout gestation are required for females to enhance their reproductive success. Since most of female conceive at the first estrus of the season (Matsuura et al. 2004a), the change in physiological condition from the anestrous season to the estrous season is important factors which responsible for determining the time of conception. However, there is no insight into mechanism for the successful conception and physiological factor influences to the conception date, and even basic information about the reproductive physiology such as ovarian dynamics and changes in hormones around the conception are not well known in sika deer. Therefore this study was conducted to clarify the characteristics of reproductive physiology of sika deer with special interest in around conception and gestation period by revealing the changes in

reproductive organs and steroid hormones.

There are two approaches to study about the issue; using carcasses and using live animals. Carcasses are available from nuisance control, sport hunting and hunting for the research purpose, and provide information on morphology and histology of reproductive organs in which animals are needed to be killed. Although abundant carcasses can be obtained, information from carcasses reflects the condition only at the time they were sampled. On the other hand, studying live animals provides the temporal changes in reproductive physiology in animals. However, number of captive deer available for physiological study is limited due to lack of institution holding animals, insufficient equipment, and financial problem in Japan. Therefore, combination of these two approaches is essential for further improvement for understanding reproductive physiology of sika deer.

Carcasses provide the physiological data such as occurrence of ovulation (Suzuki and Ohtaishi 1993) and steroidogenic ability of corpus luteum (CL; Matsuura et al. 2004c). In the study using carcasses, the reproductive status must be estimated from the condition of the female, and when they are pregnant, gestational ages are estimate from fetal weight in sika deer (Suzuki et al. 1996). However, present method of gestational age estimation will contain an intrinsic error at early gestational stage. The accurate estimation of early gestational age of the samples contributes to revealing characteristics of reproductive physiology around early pregnancy.

Study using live animals is advantageous in understanding temporal changes in the individuals and noninvasive examination, such as behavioral observation and fecal progesterone analysis (Matsuura et al. 2004a), provided the important insight in sika deer. However, data of noninvasive examination are limited to indirect changes in reproductive physiology. To know the characteristics around the first estrus and conception more in detail, invasive methods as it has be done in other cervid species, such as blood collection for hormone assay (Plotka et al. 1977, Kelly et al. 1982,

Adam et al. 1985, Garcia et al. 2003) and transrectal ultrasonography for follicular and luteal dynamics (Asher et al. 1997, McCorkell et al. 2004, 2006, 2007) of captive animals under restrain or immobilization are also needed.

In sika deer, there is a unique reproductive characteristics observed from early pregnancy. It is multiple CLs formation in spite of having singleton (Yamauchi et al. 1984, Suzuki et al. 1992, Suzuki and Ohtaishi 1993). Since both CLs have ability to synthesis progesterone, it is assumed that forming surplus CL have an important role for establishment and maintenance of pregnancy (Matsuura et al. 2004c). However, significance and function of surplus CL is not obvious, and even the origin and the timing of formation of surplus CL are not known. For understanding the conception and maintenance of pregnancy in this species, these questions must be revealed.

In present thesis, the author focused on conception and pregnancy period in sika deer. In chapter 1, the temporal ovarian dynamics and changes in steroid hormones were investigated from anestrous to estrous season to know the characteristics during the seasonal transition and to discuss the factor influence to the successful conception. In chapter 2, the temporal ovarian dynamics and changes in steroid hormone during conception and early gestation were investigated and the significance of multiple CLs was discussed. In chapter 3, description of fetal development and estimation of fetal age during early pregnancy was reported. In chapter 4, distribution of steroid hormone receptors in uteri derived from the wild deer were examined to know the steroid hormone action site at the each stage of pregnancy.

Chapter 1

Characteristics of follicular and luteal dynamics and changes in steroid hormones during seasonal transition in Hokkaido sika deer (*Cervus nippon yesoensis*)

Introduction

Since most female Hokkaido sika deer (*Cervus nippon yesoensis*) conceive at the first estrus of the breeding season (Matsuura et al. 2004a), information of reproductive physiology, such as ovarian activity and steroid hormones secretion, during the transition period from anestrous to estrous season is important to understand the physiological factor affecting conception date. However, physiological information is limited to progesterone changes in peripheral blood (Liu et al. 2002) or feces (Yamauchi et al. 1997, Matsuura et al. 2004a) together with behavioral observation in sika deer. Therefore, detailed information of ovarian activity including follicular and luteal dynamics together with steroid hormone changes remain to be studied.

Ultrasonography has been used to monitor the folliclular and luteal dynamics in various domestic animals such as cattle (Ginther et al. 1989, Knopf et al. 1989), horses (Ginther 1993), sheep (Ravindra et al. 1994) and goats (Menchaca and Rubianes 2002). In wapiti (*Cervus elaphus*), follicular and luteal dynamics by ultrasonography together with peripheral hormone concentrations were studied during the anestrous season (McCorkell et al. 2004) and estrous season (McCorkell et al. 2006). Same issues were also examined during the first inter-ovulation period (McCorkell et al. 2007). However, since McCorkell et al. (2007) did not conducted the behavioral observation and silent estrus had been observed in red deer (Asher et al. 2000), the previous study of the first inter-ovulation (McCorkell et al. 2007) assumed to be not representing the characteristics of the first

estrus and first estrous cycle of the season. Further, there was no comparison of follicular wave, corpus luteum (CL) and hormone concentrations between the periods. Therefore, the present study aimed to characterize the reproductive physiology during seasonal transition from anestrous to estrous season by comparing follicular and luteal dynamics and changes in steroid hormone during the first estrous cycle of the season with those during the before and after the first estrous cycle.

In addition, vaginal cytology and crystallization of cervical mucus, those which never examined in cervid species before, were examined during estrous season. The vaginal cytology changes under influences of ovarian steroid hormones and examination of the exfoliative cytology of the vagina is a useful indicator of the estrous cycle in dogs (Schutte 1967), cats (Shille et al. 1979) and ewes (Sanger et al. 1958). Similarly cervical mucus is useful indicator of estrus since it shows a characteristic fern pattern of crystallization on drying during estrus (Alliston et al. 1958, Noonan et al. 1975, Bishonoi et al. 1982, Hafez and Hafez 2000).

Materials and Methods

Animals and behavioral observation

This study was conducted in Asahiyama Zoological Park, Hokkaido, Japan (43º46' N, 142º28' E) for two successive breeding seasons in 2006 and 2007. Behavior of the female sika deer was continuously observed for estrus and copulation with a male by visual observation or video recording. If the copulation was observed on the two successive days, the first day was recorded as a day of estrus.

In first season, behaviors of three captive female deer were observed from September 20, 2006 to February 21, 2007. They were farmed in a pen of about 70 m^2 with a vasectmized stag until December 25, 2006, and then they were with an intact young stag of one year old, thereafter. The estrous behavior and copulation were recorded.

In second season, behaviors of another five captive female deer were observed from September 20, 2007 to November 20, 2007 and the first estrus and copulation of all females were recorded. They were farmed in a pen of about 80 m^2 with an intact adult stag.

An average 1 kg of concentrated ruminant feed was supplied daily to each animal; they were also provided with hay and water *ad libitum*. Detail information of all females is shown in Table 1-1.

Immobilization

The sika deer were immobilized for examination by intramuscular administration of an aqueous mixture containing xylazine-HCl (1 mg/kg; Celactal, Bayer Medical Co., Ltd, Tokyo, Japan) and ketamine-HCl (3 mg/kg; Ketalar, Sankyo Co., Ltd., Tokyo, Japan) or medetomidine-HCl (60 μg/kg; Domitor, Zenoaq Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) and ketamine-HCl (3 mg/kg) in the rump or shoulder area with a blowpipe (Asano et al. 2004, Onuma et al. 2004). After examination by ultrasonography, they were awakened with an intramuscular administration of atipamezole-HCl (0.1-fold volume of xylazine-HCl or 5-fold volume of medetomizine-HCl; Antisedan, Zenoaq Nippon Zenyaku Kogyo).

Ultrasonographic observation of ovaries

After securely immobilizing them, the sika deer were laid and examined in the recumbent position for fetal observation using ultrasonography (Diagnostic scanner HS-1500V, Honda electronics co., Ltd., Aichi, Japan or SSD900SE, Aloka) by insertion into the rectum of a linear transducer attached to a rigid plastic pipe extender (length, 60 cm; diameter, 3.5 cm). The transducer was covered with rubber cover filled with conductivity gel, and conductivity gel was also infused into the rectum before insertion. They were examined for ovarian morphology at two- or three-day intervals from September 23, 2006 to February 21, 2007 in first season, which include both

anestrous and estrus season (examination of the one female started on December 20, at the first estrus), and September 24 to November 20, 2007 in second season, which is until first estrus was recognized. Detailed drawings of the ovaries were made to record the diameter and relative position of follicles and the CL. The drawings were used to tabulate the follicles with >3mm of diameter within the pair of ovaries of each female for each day of the examination and to construct diameter profiles of uniquely identified follicles from their first appearance at 3 mm in diameter, due to the minimum limit size of ultrasonographic observation, until they could no longer be uniquely identified (regressed to ≤3 mm). The definition of incidences occurred in follicular dynamics are shown in Table 1-2.

Blood samples and hormone assays

A blood sample was collected at each examination. Samples were collected from the jugular vein using an 18-gauge, 3.8-cm needle into vacuum tubes loaded with EDTA, and they were centrifuged immediately after sampling. The plasma was removed and stored at -20°C until assayed.

Hormone concentrations were determined using competitive double antibody enzyme immunoassays (EIA). The primary antisera used for EIA for the estradiol-17β and progesterone measurements were anti-estradiol-17β-6-CMO-BSA (Meyer et al. 1990) and anti-progesterone-3-CMO-BSA (7720-0504, Biogenesis Ltd., Poole, UK). The antiserum against estradiol-17β cross-reacted with estrone (1.0%), estradiol-17β (1.0%), estradiol-3-benzoate (50.0%) (Braun et al. 1994). The antiserum against progesterone cross-reacted only with prgnenolone (20.0%). The secondary antiserum was goat anti-rabbit serum (Seikagaku Co., Tokyo, Japan). All samples were assayed in triplicate. The assay sensitivities were 2.8 pg/well for estradiol-17β and 4.3 pg/well for progesterone. The intra- and inter-assay coefficients of variation were 2.6 and 11.6% for estradiol-17β and 3.2 and 7.3% for progesterone, respectively.

Vaginal smear

Cervical mucus was collected with cotton swabs (length: 80 mm, diameter: 4 mm) to smear a drop on a clean slide grass. The slide was allowed to dry in air and crystallization was examined with microscope at ×400 magnification. Crystallization was classified into four scales from 0 to 3 (Bishnoi et al. 1982); 0: no crystal formation; 1: formation of only atypical crystals; 2: formation of both atypical and typical crystals; 3: formation of only typical fern-like crystals.

Cotton swabs moistened with saline were used to obtain material from the vagina for cytological examination. The smear was fixed immediately with 90% methanol for at least 1 min, followed by air-drying. After air-drying, slides were stained with Giemsa solution for 20 min, washed in tap water and allowed to dry, and coverslips were applied. Cells were counted in five different fields at ×200 magnification by microscope (BX50, OLYMPUS, Tokyo) and classified into four types: parabasal, intermediate, superficial (including large pycnotic nuclei cell and cornified cell) and neourophil as described before (Schutte 1967). In addition, each slide was scanned to obtain an impression of the degree of "clearing" on the background. Clearing was defined as the absence of non-cellular debris and of eosinophilic strings of mucus, as well as a lack of coalescence of cells into sheetlike aggregates in previous study (Shille et al. 1979).

Experimental design and data analysis

Summary of which animals were used for each analysis are shown in Table 1-1. All follicular and luteal dynamics data were classified into three periods: before the first estrous cycle, at the first estrous cycle and after the first estrous cycle. For the analysis before the first estrous cycle, the data of ovarian morphology and peripheral steroid hormone fluctuation of seven females (Nos. 1, 2, 4-8) were used. For the analysis of the first estrous cycle and after the first estrous cycle, data of three females (Nos. 1-3) were used. Interwave interval, maximum diameter of dominant follicles of a

wave and duration from wave emergence to day when dominant follicle of a wave attained the maximum diameter for anovulatory and ovulatory waves were compared between the three periods. CL lifespan, maximum diameter of CL and maximum concentrations of progesterone during each period were also compared among the three periods.

The comparison on characteristics of anovulatory follicular wave, ovulatory follicular wave and luteal dynamics among three periods were examined by analysis of variance (ANOVA) using SPSS 11.0.1 J for Windows (SPSS Japan Inc., Tokyo, Japan). Significant increase in progesterone concentrations during anestrous period of each female was examined by ANOVA from ten serial data of progesterone concentrations adjacent to first estrus. For the analysis and illustration of peripheral steroid hormone concentrations, vaginal smear and crystallization of cervical mucus, data were centralized to the day of estrus (Day 0) and mean value were calculated for each day from Day -7 to Day 7. Since majority of number of inter-estrus follicular waves were two and three, ovarian and hormonal data were compared between two and three wave cycles, and extremely short (10 days in female No. 2) and long (35 days in female No. 1) inter-estrus duration was eliminated from analysis as unusual. The duration of inter-estrus in two- and three-wave were compared by Student's t-test. The effect of wave (*i.e.* Wave 1, 2, etc.) and wave pattern (*i.e.* two- and three-wave estrous cycle) to the wave characteristics during estrous cycles were examined by ANOVA. For comparison of day for emergence of wave, Student's t-test was performed for each pair (*i.e.*, corresponding waves between two- and three-wave cycles). All data are presented as the mean ± S.D.

Results

First estruses were observed between October 30 and December 20 (Table 1-1) and 13 inter-estrus periods were observed in the present study (19.2 ± 5.5 days; 10-35 days). Ovarian follicular and luteal dynamics of individuals (Nos. 1-3) after the first estrus are shown in Fig. 1-1.

The numbers of inter-estrus follicular waves during the first three estrous cycles were consistently two and three in females No. 1 and No. 3, respectively. Female No. 2 showed mixed number of inter-estrus follicular waves, one-wave to three-wave. Since majority of number of inter-estrus follicular waves were two and three, ovarian and steroid hormone data were compared between two and three wave cycles. The overall mean estrous cycle duration was 18.6 ± 1.6 days. The duration differed between two-wave (17.5 ± 1.4 days) and three-wave (19.8 ± 0.8 days; Table 1-3). There was no significant difference in day of wave emergence at both first and second wave, interwave interval, maximum diameter of dominant follicles and duration from wave emergence to maximum diameter of follicle between them (P>0.05). Number of days to detect CL after estrus was 3.0 ± 1.0 days after and it does not differ between numbers of wave in cycle (P>0.05).

Ovarian follicular and luteal dynamics before the first estrous cycles are shown in Fig. 1-2. Follicular waves were observed at the beginning of the examination period in all females. At least one (two in female No. 7) ovulatory wave which did not accompany with estrus behavior and copulation (i.e., silent estrus) was found in all females just before the first estrus (Fig. 1-2). CL formed after every ovulation in all females. Lifespan of CL formed before the first estrous cycle (6.3 ± 2.4 days) was significantly shorter than those of the first estrous cycle and thereafter (14.3 ± 2.1 and 16.6 ± 1.8 days respectively; Table 1-4).

The comparison of anovulatory and ovulatory follicular waves among three periods (before, during and after the first estrous cycle) is shown in Table 1-5 and 1-6, respectively. There was no difference in interwave interval, maximum diameter of dominant follicle and duration from wave emergence to maximum diameter of follicle among the three periods (P>0.05). However, maximum diameter of CL before the first estrous cycle (9.9 ± 1.2 mm) was significantly smaller than that after the first estrous cycle (11.5 ± 1.3 mm; Table 1-4). Although increases in progesterone concentration were observed before the first estrous cycle, the maximum concentrations (0.5 ± 0.4 ng/ml) were

significantly low compared to those of the first estrous cycle and thereafter (1.4 ± 0.2 and 2.2 ± 0.6 ng/ml, respectively; Table 1-4). Maximum progesterone concentration of the first estrous cycle tended to be lower than that after the first estrous cycle, though there was no significant difference (P=0.05).

Changes in steroid hormone concentrations and characteristics of vaginal cytology and crystallization of cervical mucus around the estrus were shown in Fig. 1-3. The peak of estradiol concentration (Day -1 and Day 0) and nadir of progesterone concentration (Day -1 to Day 1) were detectable around the estrus. The peak concentrations of estradiol was 21.6 ± 3.6 pg / ml (range: 13.2 - 58.9 pg/ml). The proportion of vaginal cytology did not show a clear change associated with estradiol peaks and estrus behaviors. However, superficial cell showed a trend of increase in its proportion around the estrus. Crystallization score of cervical mucus increased from Day -1 to Day 3 and was highest on Day 0. During the period that crystallization was positive, clearing of the vaginal smear was relatively high.

Discussion

This is the first study which describes ovarian follicular and luteal dynamics in sika deer using transrectal ultrasonography. Although daily examinations provide detailed information on the ovarian activities, daily immobilization for an extended period could be detrimental to animal health and alter physiological function including estrus and ovulation. Therefore interval of examination was determined for two or three days in the present study.

Before the first estrous cycle, at least one ovulation adjacent to the first estrus and subsequent CL formation were observed in all females. However, the CL lifespan was significantly shorter in comparison to that of other periods. Progesterone concentrations were relatively low before the first estrous cycle and this assumed to be due to small size CL in this period. Therefore, subsequent

dominant follicle was capable to ovulate due to lack of progesterone effect to suppress the luteinizing hormone (LH) pulse frequency (Bergfeld et al. 1995, Rawling and Bartlewski 2007, Stevenson 2007) which brings up the dominant follicle to size sufficient for inducing LH surge which results in ovulation (Petersen et al. 2003). In wapiti, most of female had one wave and short luteal phase during first interovulatory interval at seasonal transition (McCorkell et al. 2007). In many other cervid species, first ovulations are not associated with estrus or copulation (Thomas and Cowan 1975, Harder and Moorhead 1980, Asher 1985, Jopson et al. 1990, Asher et al. 2000, Shipka et al. 2007). A transient and low increase of serum progesterone concentration at the seasonal transition was also reported in ewe (Yuthasastrakosol et al. 1975, Walton et al. 1977, I'Anson 1983). The progesterone sensitization induces estrus (Rawlings and Bartlewski 2007) and it also had been shown to be necessary for ensuring the normal length of estrous cycle and normal luteal function to secrete progesterone (McLeod et al. 1982, McLeod and Haresigh 1984, Legan et al. 1985, Legan et al. 1991). Therefore, the transient increase of progesterone in sika deer predicted to induce the first estrus of the season and ensure the normal estrous cycle and CL function as in ewes, and partially determines the conception date since most of female conceive at the first estrus of the season (Matsuura et al. 2004a).

There was no difference in characteristics of anovulatory follicular wave among three periods (before, during and after the first estrous cycle). Similar to the present results, growth and regression of ovarian antral follicles are remarkably similar in before first ovulation and thereafter in ewes (Schrick et al 1993, Ravindra et al. 1994, Ravindra and Rawlings 1997). Meanwhile, it is reported that during anestrous period, follicles that developed in late anestrous season grew to a larger maximum diameter and were uniquely identifiable for a longer interval than those observed during mid-anestrous season in wapiti (McCorkell et al. 2004). Therefore, although there might be difference in maximum diameter of dominant follicle and interwave intervals between earlier season

before the first estrus and after the first estrus, the difference could not be found since the examination was conducted at the period relatively close to the breeding season in this study. The examination in earlier, *i.e.*, during summer or earlier period, may be needed for further discussion.

Similarly to anovulatory wave, there was no difference in characteristics of ovulatory follicular wave among three periods. However, increase of estradiol could not be observed at the time of ovulation without behavioral estrus before the first estrus whereas the peak of estradiol concentration was observed during the first estrus and thereafter together with nadir of progesterone concentration. It is consistent to previous study that estradiol concentration indicate the peak on the day of behavioral estrus in cervid species (Plotka et al. 1980, Bainbridge et al. 1996) and other domestic animals (ewes: Rawlings and Bartlewski 2007, cattle: Stevenson 2007). There was no difference in characteristics of follicular wave, luteal dynamics and pattern of steroid hormone changes between the first estrous cycle and thereafter. However, progesterone concentrations tend to be lower during the first estrous cycle than thereafter. Since intervals between the examinations were two or three days, more frequent examination might be needed to confirm whether there is a difference or not in progesterone concentrations between these periods.

The crystallization of cervical mucus was significantly related to day of estrus. It is similar to the previous findings in cattle that fering of the dried cervical mucus occurred to a greater extent on the day of estrus than that during any other stage of the estrous cycle (Noonan et al. 1975). Since the day of high score of crystallization coincident with estradiol peak, crystallization of cervical mucus may be a useful indicator of determining the peak of estrus in sika deer. Whereas, exfoliative vaginal cytology did not show a clear change depend on the stage of estrous cycle in sika deer and it is similar to cattle in which changes in vaginal cytology between the estrous stage was not apparent (Miroud and Noakes 1990).

The duration of estrous cycle observed in the present study (18.6 ± 1.6 days) was similar to that

reported in Formonsan sika deer (19.3 ± 1.8 days; Liu et al. 2002). The duration of estrous cycle was longer in three-wave estrous cycle, which is similar in cattle (Ginther et al. 1989, Driancourt 2001). The difference in duration of estrous cycle may be due to the number of waves, since day of wave emergence and wave lifespan of the first and second waves were not different.

In the present study, female repeated the estrus even after exchanges to intact male in the first season. Since animal in the pubertal year (i.e., one year old) was used as intact male, the failure of conception might be due to the age and/or lack of the experience. Although copulation was done several times during estrus, the male was able to copulate few times during estrus and sometimes he gave up the copulation even he tried to mount on female. Alternatively, there may be influence of the frequent examination that may alter physiological events even though the interval of examination was set two or three days as discussed above. Conception and subsequent embryo loss could be happened at the extremely long inter-estrus duration observed in female No.1. The interval of examination was mainly three days and females conceived successfully in second season of the present study, while it was two days in first season.

This study confirmed the ovulation before the first estrus and the short luteal phase together with transient increase of progesterone in the seasonal transition. This transient increase assumed to influences to conception date since progesterone induces the estrus and most females conceive at the first estrus of the season. For further investigation, more frequent examinations of follicular and luteal dynamics with additional measurement of factors, such as luteinizing hormone (LH) and follicle stimulating hormone (FSH) are needed for better understanding of physiological characteristics during anestrous and estrous season to reveal the characteristics in the seasonal transition more in detail.

Summary

Although the transition from anestrous to estrous is important since most of sika deer conceive at the first estrus of the season, physiological characteristics during the seasonal transition are not well studied and physiological factor influences to the conception date is unknown in sika deer. This study revealed the follicular and luteal dynamics and changes in steroid hormones concentrations of before, during and after the first estrous cycle and compared among the three periods. Eight captive female Hokkaido sika deer (*Cervus nippon yesoensis*) were observed for estrus and follicular and luteal dynamics were observed at two- or three-day intervals using ultrasonography together with temporal changes in peripheral steroid hormone concentration and characteristics of vaginal smear. Before the first estrus of the season, CL has formed subsequent to dominant follicle ovulation and transient increases of progesterone were detected in all females. Maximum concentrations of progesterone and CL lifespan were significantly low and short before the first estrus compared to those in other periods ($P<0.05$). Also maximum diameter of CL in anestrous period was significantly small in comparison to those after the first estrous cycle ($P<0.05$). Transient increase of progesterone assumed to be important for on set of the first estrus of the season and ensuring the normal estrous cycle and sufficient CL function. Therefore transient increase in progesterone may influence to conception date since most of female conceive at the first estrus of the season.

Table 1-1. Information of females and list of data used for each analysis

ID	Examined year	Age[1)]	Parity	Date of first estrus	Before the first estrous cycle			First estrous cycle			After the first estrous cycle			Vaginal smear
					Anovulatory follicular wave	Ovulatory follicular wave	Luteal parameter[2)]	Anovulatory follicular wave	Ovulatory follicular wave	Luteal parameter[2)]	Anovulatory follicular wave	Ovulatory follicular wave	Luteal parameter[2)]	
No.1	2006	2	Parous	Nov. 16	+	+	+	+	+	+	+	+	+	+
No.2	2006	≥3	Parous	Oct. 31	+	+	+	+	+	+	+	+	+	+
No.3	2006	1	Nulliparous	Dec. 20	-	-	-	+	+	+	+	+	+	+
No.4	2007	≥3	Parous	Oct. 30	+	+	+	-	+	-	-	-	-	-
No.5	2007	≥3	Parous	Oct. 30	+	+	+	-	+	-	-	-	-	-
No.6	2007	1	Nulliparous	Nov. 8	+	+	+	-	+	-	-	-	-	-
No.7	2007	1	Nulliparous	Nov. 20	+	+	+	-	+	-	-	-	-	-
No.8	2007	1	Nulliparous	Nov. 16	+	+	+	-	+	-	-	-	-	-

+, examined; -, not examined

[1)] Animals older than three years old are indistinguishable by age estimation based on replacement of tooth (Koike and Ohtaishi 1985).

[2)] Luteal dynamics and concentrations of progesterone.

Table 1-2. Definition of incidence occur in follicular dynamics.

Incidence	Definition
Ovulation[1)]	Disappearance of a follicle ≥7 mm in diameter identified during the previous examination on the subsequent examination,and was confirmed by detection of a corpus luteum at the same location
Wave	The synchronous growth of a group of small follicles
Dominant follicle	A follicle that attained a diameter ≥7 mm and exceeded the diameter of all others
Day of wave emergence	The day the dominant follicle of a wave was first detected at≥ 3 mm in diameter
Day of wave regression	The day of the dominant follicle could no longer be uniquely identified
Inter-wave interval	Number of days from the day of wave emergence to the day of next wave emergence.

[1)] The threshold was determined as 7 mm since it was a minimum size that the disappearance of dominant follicle observed in this

Table 1-3. Comparison of follicular dynamics in sika deer with two and three waves of follicle development during the estrous cycle

Number of wave	n	Estrous cycle duration (days)	wave No.	Day of wave emergence	Interwave interval (days)	Maximum diameter of follicle (mm)	Duration from wave emergence to maximum diameter of dominant follicle (days)
2	6	17.5 ± 1.4^{a}	1	1.2 ± 1.8	8.0 ± 2.0	9.7 ± 2.3	5.5 ± 2.2
			2	9.3 ± 2.0	8.8 ± 2.9	9.5 ± 1.0	6.7 ± 3.4
3	5	19.8 ± 0.8^{b}	1	2.0 ± 0.7	5.2 ± 1.8	8.2 ± 3.5	4.4 ± 2.4
			2	7.2 ± 2.0	5.6 ± 1.3	7.8 ± 0.8	6.4 ± 1.3
			3	13.6 ± 1.8	7.0 ± 3.0	7.8 ± 0.8	5.6 ± 1.8

Values with different superscripts within a column are different ($P < 0.05$)

Table 1-4. Comparison of luteal dynamics and maximum progesterone concentration in sika deer among periods of before, during and after the first estrous cycle.

Ovarian cycle	CL lifespan (days)	Maximum diameter of CL (mm)	Maximum concentration of progesterone (ng/ml)
Before the first estrous cycle	6.3 ± 2.4^{a}	9.9 ± 1.2^{a}	0.5 ± 0.4^{a}
First estrous cycle	14.3 ± 2.1^{b}	10.7 ± 0.6^{ab}	1.4 ± 0.2^{b}
After the first estrous cycle	16.6 ± 1.8^{b}	11.5 ± 1.3^{b}	2.2 ± 0.6^{b}

Values with different superscripts within a column are different ($P < 0.05$)

CL, corpus luteum

Table 1-5. Comparison of follicular dynamics of anovulatory wave in sika deer among periods of before, during and after the first estrous cycle.

Ovarian cycle	Interwave interval (days)	Maximum diameter of dominant follicle (mm)	Duration from wave emergence to maximum diameter of dominant follicle (days)
Before the first estrous cycle	7.9 ± 3.0	7.8 ± 1.4	5.6 ± 2.3
First estrous cycle	6.5 ± 1.9	7.5 ± 1.3	4.5 ± 1.9
After the first estrous cycle	6.3 ± 2.2	9.0 ± 2.6	5.8 ± 2.1

Table 1-6. Comparison of follicular dynamics of ovulatory wave in sika deer among periods of before, during and after the first estrous cycle.

Ovarian cycle	Interwave interval (days)	Maximum diameter of dominant follicle (mm)	Duration from wave emergence to maximum diameter of dominant follicle (days)
Before the first estrous cycle	10.4 ± 5.0	9.5 ± 2.1	7.5 ± 3.1
First estrous cycle	9.3 ± 3.0	9.3 ± 1.2	6.9 ± 2.3
After the first estrous cycle	8.5 ± 3.3	9.0 ± 1.5	6.6 ± 3.0

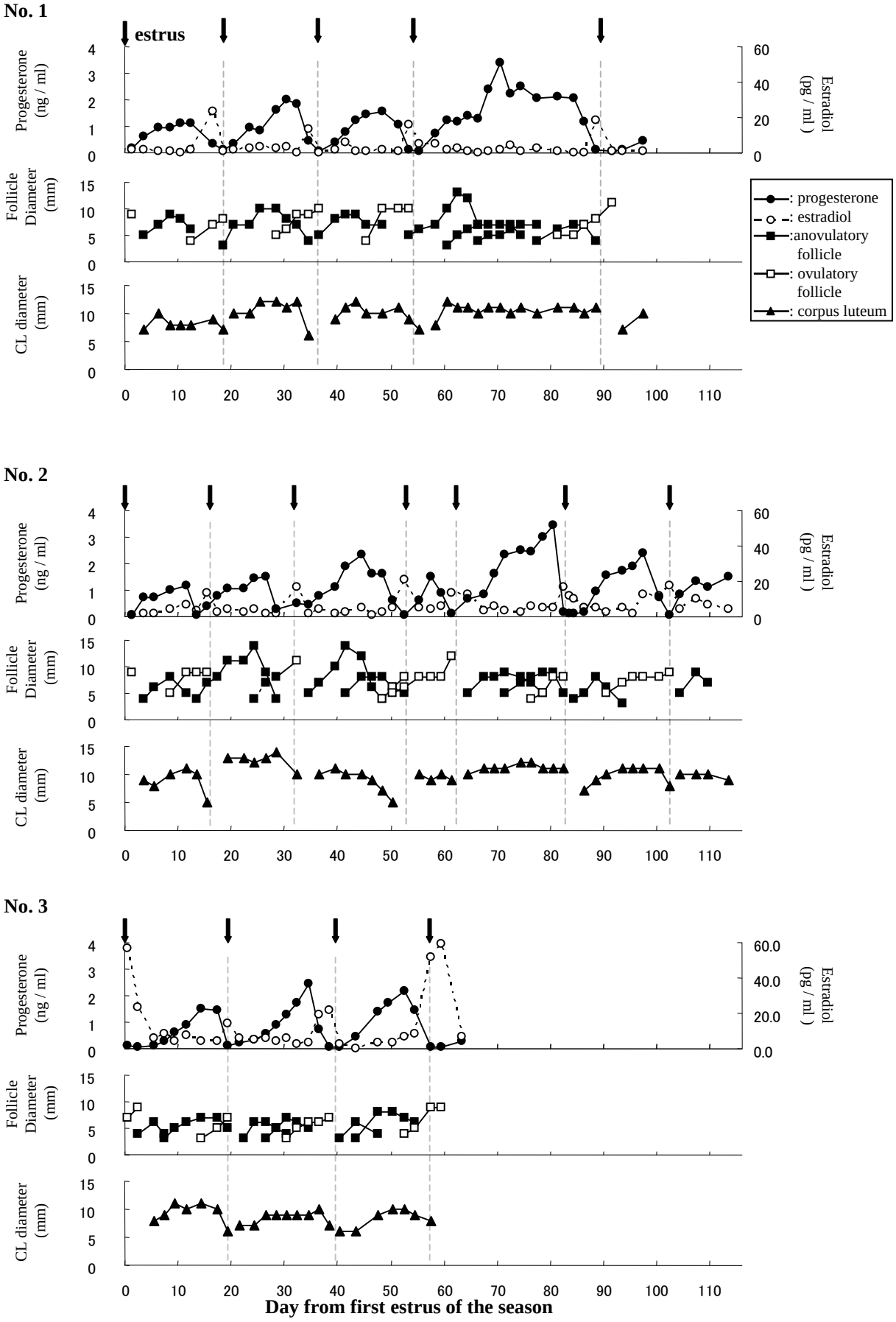

Fig. 1-1. Changes in the ovarian structure and steroid hormone concentrations of females (Nos. 1 –3) after the firstestrus. CL= corpus luteum, Day 0 = day of on set of first estrus of the estrous season.

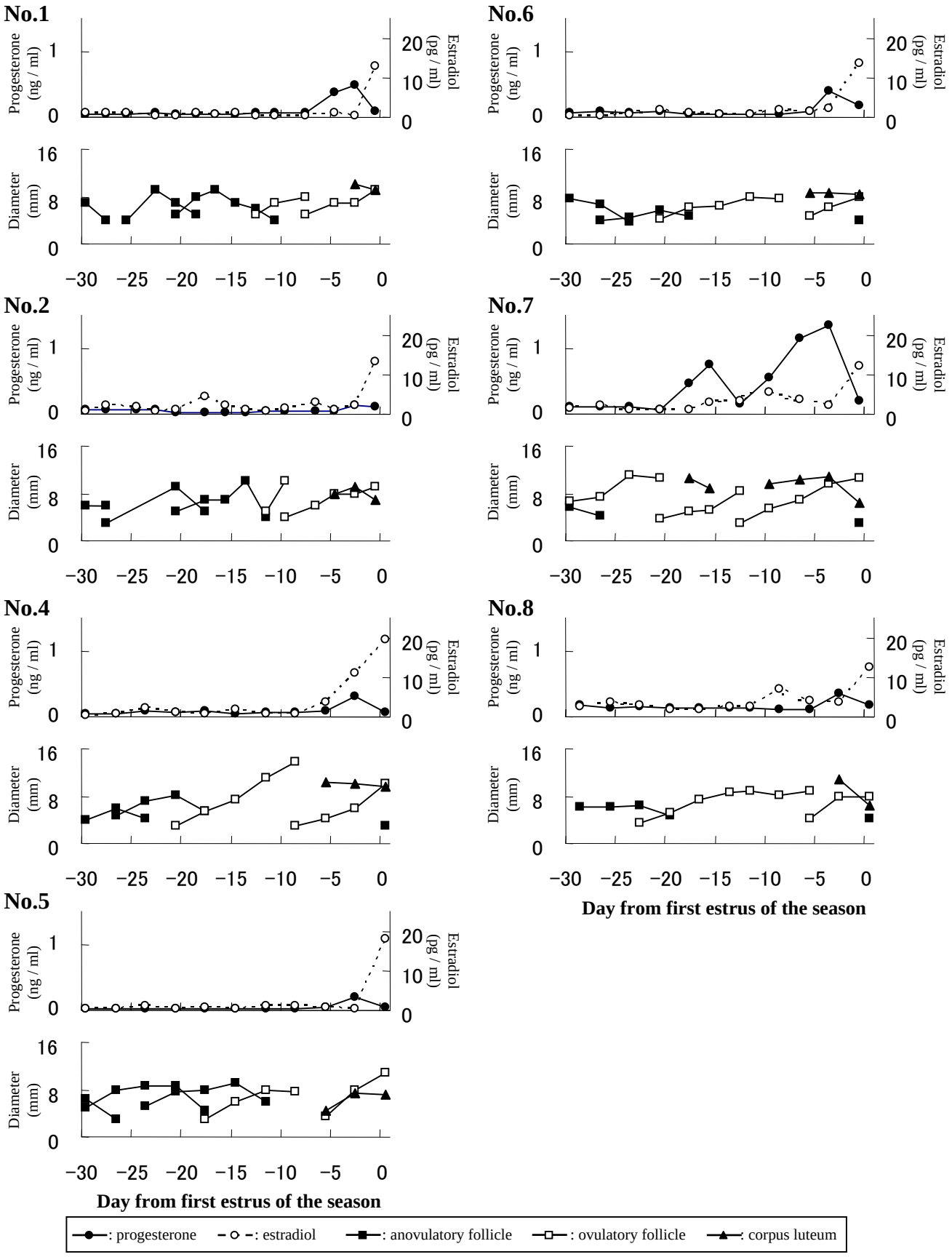

Fig. 1-2. Changes in the ovarian structure and steroid hormone concentrations of females (Nos.1, 2, 4-8) before the first estrus. All females ovulated once before first estrus except female No.7 that ovulated twice. Day 0= day of on set of first estrus of the estrous season.

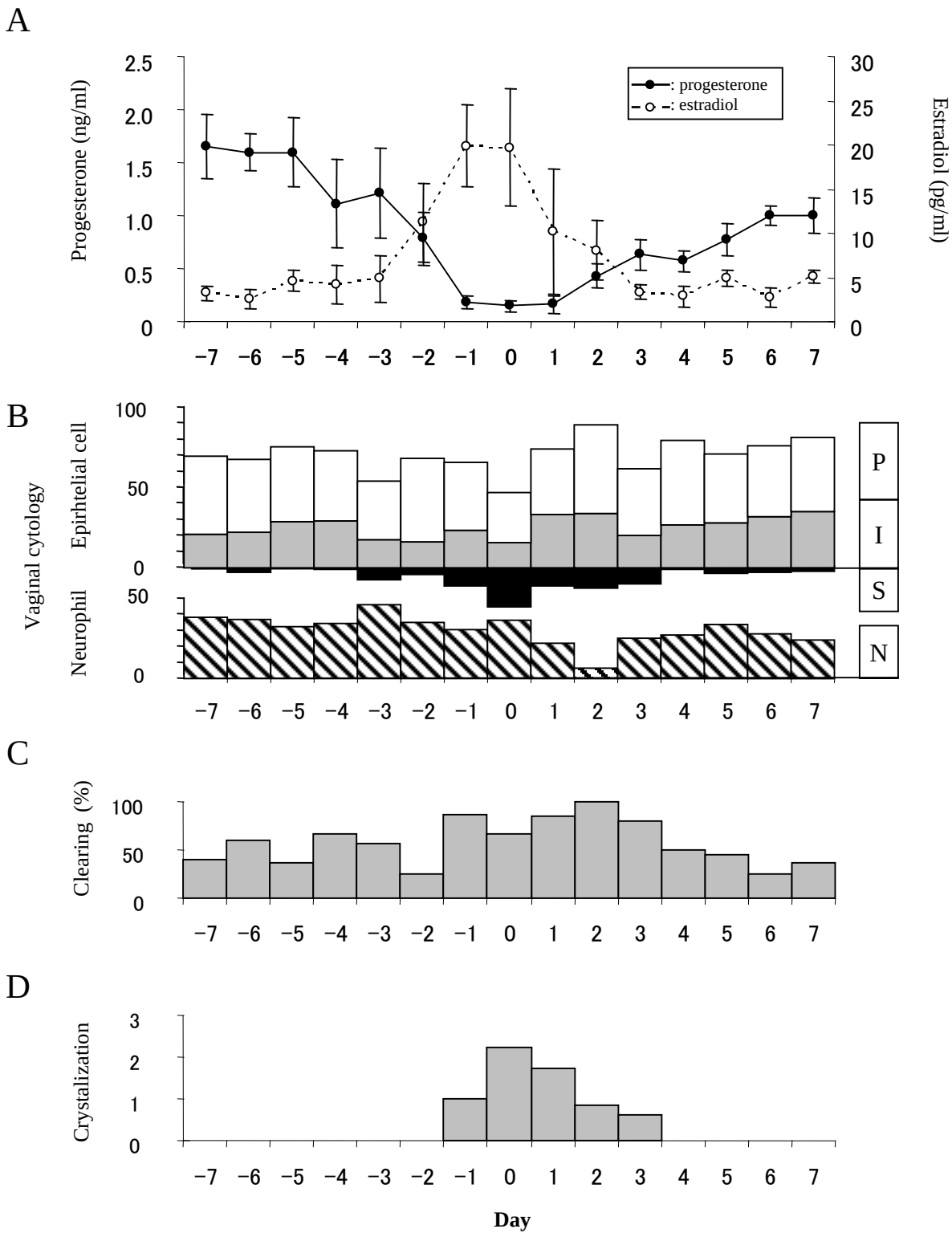

Fig. 1-3. Changes in steroid hormone concentrations and characteristics of vaginal smear before and after estrus. (A) Changes in progesterone and estradiol concentrations. (B) Changes in proportion of the four types of cells in vaginal smear. (C) Changes in impression of clearing of vaginal smear. (D) Changes in mean score of crystalization in cervical mucus. Numbers of samples are not same between the Days. P: parabasal cell, I: intermediate cell, S:superficial cell, N:neurophil. Day 0= day of estrus

Chapter 2

Characteristics of follicular and luteal dynamics and changes in steroid hormones during conception and early pregnancy in Hokkaido sika deer (*Cervus nippon yesoensis*): Origin and timing of formation of multiple corpora lutea.

Introduction

Although sika deer (*Cervus nippon*) usually produce a single fawn, two (rarely three) functional corpora lutera (CLs) were often found during pregnancy (Yamauchi et al. 1984, Suzuki et al. 1992, Suzuki and Ohtaishi 1993) as well as red deer (*Cervus elaphus*; Douglas 1966, Guinness et al. 1971, Kelly and Challies 1978, Kelly et al. 1985). In sika deer, multiple CLs were found in about 80% of pregnant females with a single fetus (Suzuki et al. 1992, Suzuki and Ohtaishi 1993) from early pregnancy throughout gestation period (Suzuki 1993) and each CL is known to develop at different times (Matsuura et al., 2004c). No difference was found between CLs formed at different times in neither histology (Suzuki 1993) nor ability to produce steroid hormones assessed by immunohistochemistry for steroidgenic enzymes (Matsuura et al. 2004c). Together with a high incidence of appearance, this may indicate a physiological role of the second CL in establishment and maintenance of pregnancy in this species. To understand the significance of the second CLs, the time of second CL formation and the changes of steroid hormone concentrations in relation to the second CL formation need to be studied.

Frequent observations of ovaries using ultrasonography have been used extensively in domestic animals to investigate the follicular and luteal dynamics (Driancourt 2001) and also a few in cervid species (McCorkell et al. 2004, 2006, 2007). This methodology, together with measurement of

peripheral steroid hormone concentrations would allow clarifying detailed changes in ovarian activity including formation of the second CL.

The present study was conducted to examine the time of second CL formation in pregnant sika deer by frequent observation of the ovaries using ultrasonography with special interest in follicular and luteal dynamics. Plasma steroid concentrations were also examined to evaluate function of CLs to understand significance of the second CL.

Materials and Methods

Animals and behavioral observation

The present study was conducted in Asahiyama Zoological Park, Hokkaido, Japan (43º46' N, 142º28' E). Behavior of the six female sika deer was continuously observed for estrus and copulation by visual observation or video recording. If the copulation was observed on the two successive days, the first day was recorded as a conception date. Behaviors were observed from September 20, 2007 to January 17, 2008. Three females were at one year old (Table 2-1). The other three were estimated to be three years old or older by tooth replacement (Koike and Ohtaishi 1985). The animals were housed and fed as described in the chapter 1.

Ultrasonographic observation and blood collection

Animals were chemically immobilized as described in the chapter 1. Female sika deer were examined for ovarian morphology using ultrasonography at two- or three-day intervals from September 24, 2007 to January 17, 2008 (four- or five-day intervals for female No.9 until December 15) as described in the chapter 1. The size of dominant follicles and CLs were recorded with their position in the ovary, and follicular wave and ovulation were determined as described in chapter 1. Examination was ceased before the end of study period if a female reached two months of gestation.

A pair of ovaries was collected from a female killed by accident during transportation after the present study (female No. 4).

Blood samples collection and hormone assay were done as described in chapter 1.

Data analysis

Diameters of CLs were compared by Student's t-test. Total amount of progesterone secretion was calculated as area under the curve of the plasma concentration of progesterone. Concentrations and total amount of plasma progesterone were compared by one way analysis of variance (ANOVA). For statistical analysis, SPSS 11.0.1 J for Windows (SPSS Japan Inc., Tokyo, Japan) was used in the present study. All data are presented as mean ± S.D.

Results

Pregnancies were diagnosed by the presence of fetuses with heart beat between Day 25 and 26 of gestation using ultrasonography in all females. The date of conception is defined as day of fertile copulation in the present study (Day 0). Three females conceived at the first estrus of the season and the other three females conceived at the second estrus of the estrous season (Table 2-1).

Formation of the second CL was observed in three females (Nos. 4-6) conceived at the first estrus whereas females conceived at the second estrus (Nos. 7-9) did not form a second CL (Table 2-1). Changes in ovarian structures and steroid hormones in females formed two CLs and one CL after conception are shown in Fig. 2-1 and Fig. 2-2, respectively. In females conceived at the first estrus, the dominant follicles of the first waves that emerged immediately after ovulation for conception (Fig. 2-3 A, A') ovulated under the presence of the first CL. Subsequently, the second CLs formed in the position of the ovulated dominant follicle in the three animals. In the ovary of female No. 4 that was killed by accident, two CLs similar to ultrasonographic images were observed

(Fig. 2-3C). The second CLs first observed between Day 11 and 23, and time of ovulation associated with the second CLs was estimated to occurr between Day 5 and 20 (Table 2-1). The maximum diameters of the first CLs were significantly larger than that of the second CLs (11.8 ± 1.8 mm vs. 9.5 ± 1.2 mm; $P < 0.05$) throughout the study period. The maximum diameters of CLs formed after fertile copulation was similar in animals conceived at the first and second estrus (11.8 ± 1.8 mm vs. 11.6 ± 0.4 mm; $P > 0.05$).

In the females conceived at the first estrus, an increase in plasma estradiol-17β concentrations was observed around the time of the second ovulation and the levels of estradiol-17β concentrations were similar to (Nos. 5, 6) or exceeded (No. 4) those observed at the time of estrus. Peripheral plasma progesterone concentrations during the estimated ovulation of the dominant follicle destined to be the second CL, which might occurred at the peak of estradiol-17β, were between 1.2 and 2.2 ng/ml (Fig. 2-1). The progesterone concentrations were not different in female conceived at the second estrus (Nos. 7-9) at the time when dominant follicle of the first wave attained maximum size both after the first estrus (between 1.0 and 2.1 ng/ml) and second estrus (between 1.6 and 2.2 ng/ml). In females Nos. 7-9, no apparent increase in estradiol-17β concentrations was observed after copulation. There was no difference in the total amount of progesterone secreted between from estrus to the estimated ovulation in females Nos. 4-6 and estrus to the time when dominant follicle of the first wave attained maximum size females Nos. 7-9.

Plasma concentrations of progesterone after formation of second CL in female Nos. 4 and 6 increased to 3.9 and 3.8 ng/ml by Day 17 and 14, respectively, and then decreased to less than 3 ng/ml (Fig. 2-1). Progesterone concentrations started to increase and reached over 3 ng/ml again by Day 40 (No. 4) and Day 23 (No. 6). Progesterone concentrations of female No. 5 maintained between 1 and 3 ng/ml until Day 38 (Fig. 2-1) and the concentrations exceeded 3 ng/ml thereafter. In females Nos. 7-9, plasma progesterone concentrations exceeded 3 ng/ml by Day 10 - 21 (Fig. 2-2).

Discussion

Although the presence of two (rarely three) CLs in pregnant sika deer with single fetus has been described (Yamauchi et al. 1984, Suzuki et al. 1992, Suzuki and Ohtaishi 1993), origin and timing of the second CL formation were not known. In the present study, three out of six females formed two CLs. In all three females, origin of second CLs was the dominant follicle of the follicular wave that emerged immediately after ovulation associated with the first estrus of the season. These dominant follicles ovulated under the presence of first CL.

Progesterone suppresses both the frequency of luteinizing hormone (LH) pulse and concentration of estradiol (Bergfeld et al. 1995, Stevenson 2007) and consequently suppress LH surge since it is induced by the surge of estradiol (Petersen et al. 2003). In goats, progesterone at 3 ng/ml, but not 1 ng/ml suppressed LH pulse frequency significantly (Kim et al. 2003). In the females conceived at the first estrus, progesterone concentrations were low (< 3 ng//ml) at the time when the dominant follicle has been selected after fertile ovulation, thus, LH pulse frequency may be high enough for the dominant follicle to develop to the preovulatory stage. Estrogen secretion from the preovulatory dominant follicle may have triggered the LH surge. However, the first dominant follicle after the first estrus in the other three females did not ovulate although progesterone concentrations were at the similar levels (< 3 ng/ml) and the total amount of progesterone as well. The reason of this inconsistency is not clear, but the levels of progesterone which is effective to suppress LH pulse may influenced by puberty since reproductive abnormality such as short estrus, low fertility and abnormal timing of ovulation due to hormonal disorder are observed in postpubertal animal (Foster 1988). Whether conceived or not is the critical difference after the first estrus between females formed two CL and one CL, the second CL formation might be influenced by interferon tau (INF-τ), known as the factor of maternal recognition of pregnancy and also found in cervid species (Demmers et al.2000) since it involve in regulation of luteolysis (Spencer et al. 1995b).

Furthermore, hypothalamus-pituitary-ovary function around the first estrus of the breeding season may be unstable. In ewes, progesterone concentrations at the first estrus of the season were lower than mid-breeding season (Bartlewski et al. 1999) and similar trend was observed in chapter 1. Also, despite of returning to cyclical activity and ovulation, the pregnancy rate is low in post-partum cattle (Noakes 1997). This may partially explain the different dynamics of the first dominant follicle after the second estrus in female Nos. 7-9. Progesterone concentrations of these females were low (below 3 ng/ml) and total amount of progesterone were not different from female No. 4-6 at the time of dominant follicle of the first follicular wave after fertile conception were maximum in size. However, the dominant follicle did not ovulate. The obvious difference between the former females and the latter females was whether conceived at the first estrus or the second estrus of the season. In sheep, it is known that the progesterone sensitization is necessary to ensure the luteal function to secrete progesterone (McLeod et al. 1982, McLeod and Haresigh 1984, Legan et al. 1985, Legan et al. 1991). Females conceived at the second estrus had sensitized to progesterone during first inter-estrus period while females conceived at the first estrus had been exposed only to a transient and low increase in progesterone before the first estrus which observed in chapter 1, and it may be insufficient to maintain subsequent luteal function. Since female Nos. 7-9 did not form second CL even the progesterone concentrations were as low as level of females conceived at the first estrus, the sensitivity to progesterone was assumed to be different. After sensitization of enough levels of progesterone, LH pulse frequency may be suppressed by relatively low level of progesterone.

After second CL formation, consistent increase in plasma progesterone concentrations was not evident in the present study. This may be incompatible to the expected roles for the second CL that it supports the establishment and maintenance of pregnancy by increasing progesterone concentrations. However, in case of the absence of the second CL, the plasma progesterone concentrations could be even lower than the present study. By forming second CL, the progesterone concentrations assumed

to be kept over the minimum level essential for maintenance of pregnancy.

The second CLs were formed in females conceived at the first estrus of the season, whereas only one CL formed in females conceived at the second estrus of the season. For this reason, the second CL may form in case that female conceives at the first estrus of the season. In cervid species, conception at the early breeding season contributes to their reproductive success. Formation of the second CLs at the first estrus of the season may be advantageous for early conception which leads to high reproductive success. The previous study also supports this hypothesis that pregnant females with multiple CLs have heavier fetuses than pregnant females with single CL (Suzuki et al. 1992). Also the hypothesis is consistent to the interesting correspondence that 10 out of 12 females (about 80%) conceived at the first estrus of the season (Matsuura et al. 2004a) and the rate of multiple CLs found in the wild population is reported as also about 80% (Suzuki et al. 1992, Suzuki and Ohtaishi 1993).

The second CL became detectable as early as the second week of gestation by ultrasonography in present study. Therefore multiple CLs could be found before the fetus reach to the detectable size by autopsy, around three weeks of gestation at the earliest (Yanagawa et al. in press). This is consistent with previous study reported that multiple CLs were found even fetus was not visible (Matsuura et al. 2004c). Since multiple CLs could not be observed during estrous cycle in chapter 1, the existence of multiple CLs will be useful as an indicator of pregnancy.

Present study revealed the origin and timing of formation of the second CL. However, the detail mechanism of formation of the second CLs remained to be studied. As frequency of the LH pulse determines the fate of dominant follicle, measurement of LH pulse frequency are needed to clarify the mechanism of postconception ovulation.

Summary

Multiple corpora lutea (CLs) are unique characteristics of pregnant sika deer (*Cervus nippon*) and may be important for maintenance of pregnancy from early gestation. However little is known about the origin and timing of formation of second CL. Six captive female Hokkaido sika deer (*Cervus nippon yesoensis*) were observed for estrus and investigated the changes in ovarian morphology at two- or three-day intervals using ultrasonography together with temporal changes in peripheral steroid hormones to reveal the source of second CL and timing of its formation and estimate their function. Two CLs formed in three pregnant female conceived at the first estrus of the season. The second CL was formed from ovulated dominant follicle of follicular wave emerged immediately after ovulation associated with estrus. The peripheral progesterone concentrations of females formed two CLs relatively low and result in ovulation. Only one CL was observed in females conceived at second estrus of the season even peripheral progesterone concentration were similar to the female conceived at first estrus. By the second estrus, sensitivity to progesterone assumed to be increased by sensitization of enough progesterone during first estrous cycle and the LH pulse frequency was suppressed. Therefore, it is hypothesized that second CL forms for supporting progesterone secretion which might be insufficient to maintain pregnancy only by first CL in case of females conceived at first estrus of the season.

Table 2-1. The information of basic status of animals and around the second CL formation.

ID	Age[1)]	Parity	Conceived estrus[2)]	Number of CL formed during pregnancy	Estimated time of ovulation associated with the second CL formation	First detection of second CL	Progesterone concentrations during estimated ovulation (ng/ml)
No.4[3)]	≥3	Parous	First	2	Day 8 - 11	Day 11	2.2 - 3.9
No.5	≥3	Parous	First	2	Day 17 - 20	Day 23	1.7
No.6	1	Nulliparous	First	2	Day 5 - 8	Day 11	1.2 - 2.0
No.7	1	Nulliparous	Second	1	—	—	—
No.8	1	Nulliparous	Second	1	—	—	—
No.9	≥3	Parous	Second	1	—	—	—

CL: corpus luteum

[1)] Animals older than three years old are indistinguishable by age estimation based on replacement of tooth (Koike and Ohtaishi 1985).

[2)] First: first estrus of the estrous season, Second: second estrus of the estrous season.

[3)] Killed by accident on Day 85 of pregnancy

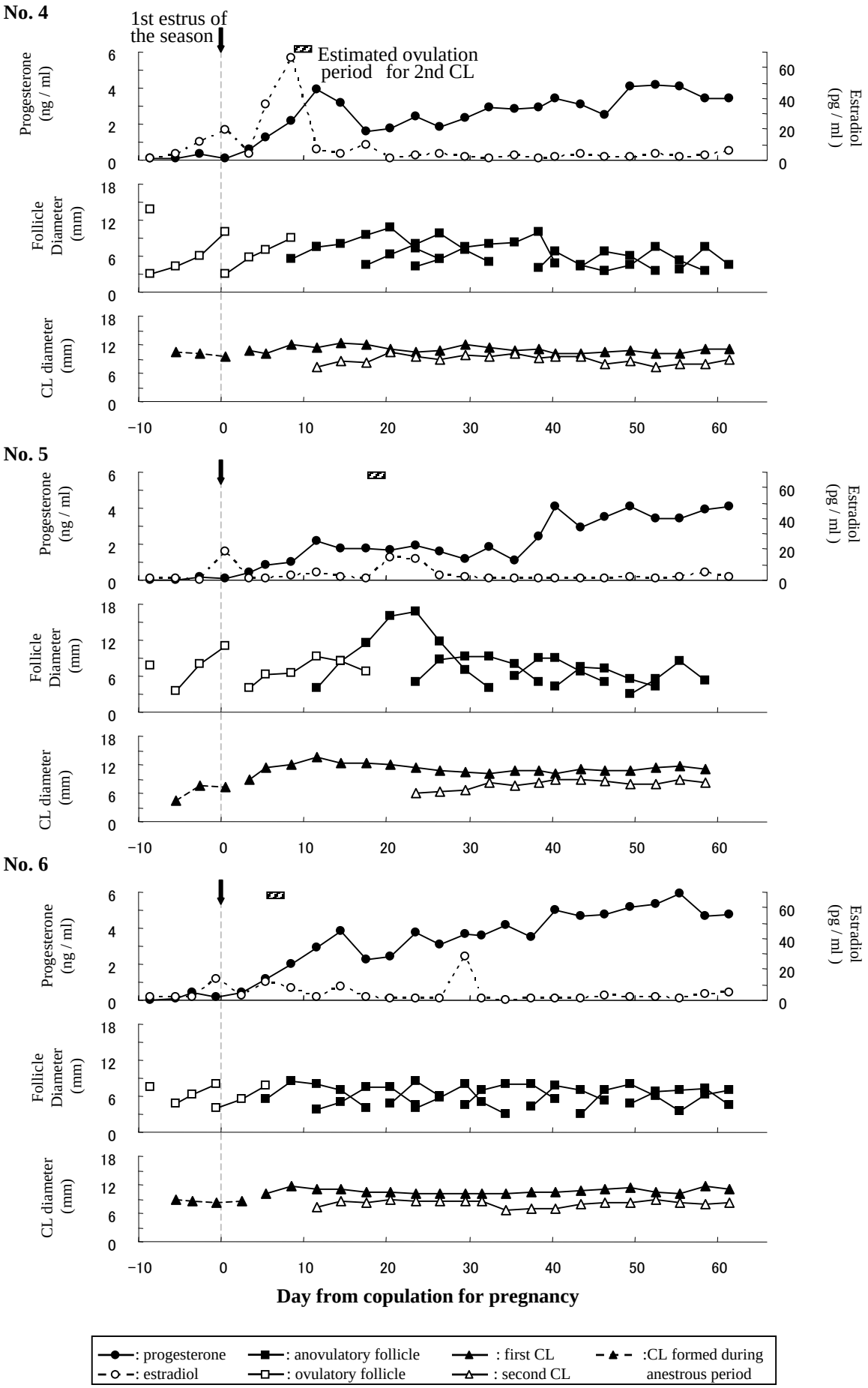

Fig. 2-1. Chages in the ovarian structure and steroid hormone concentrations in pregnant females (Nos. 4–6) formed two corpora lutea (CLs). Day 0=day of copulation for pregnancy.

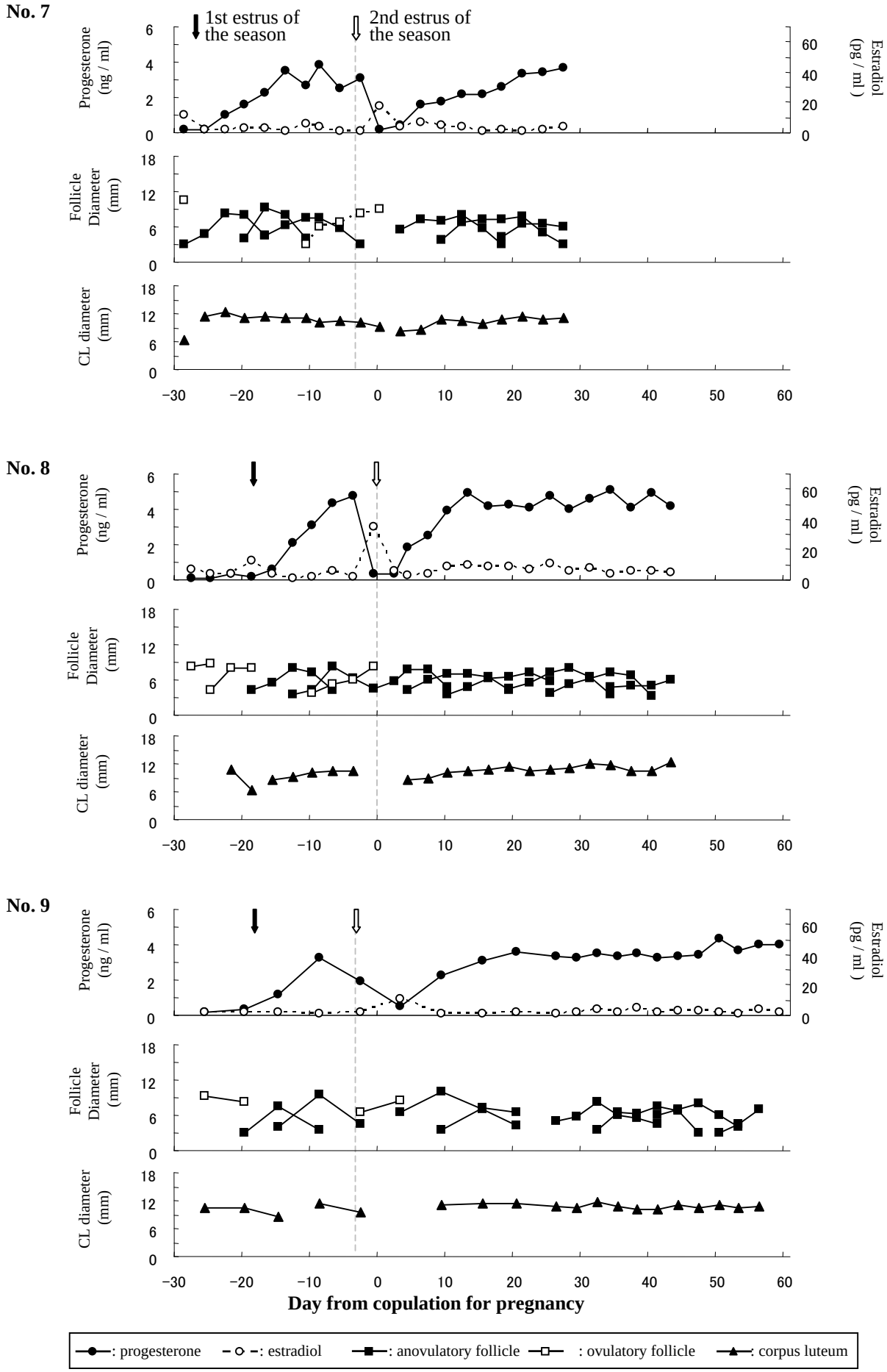

Fig. 2-2. Changes in the ovarian structure and steroid homone concentrations in pregnant female (Nos. 7-9) formed one corpus luteum (CL). Day 0=day of copulation for pregnancy.

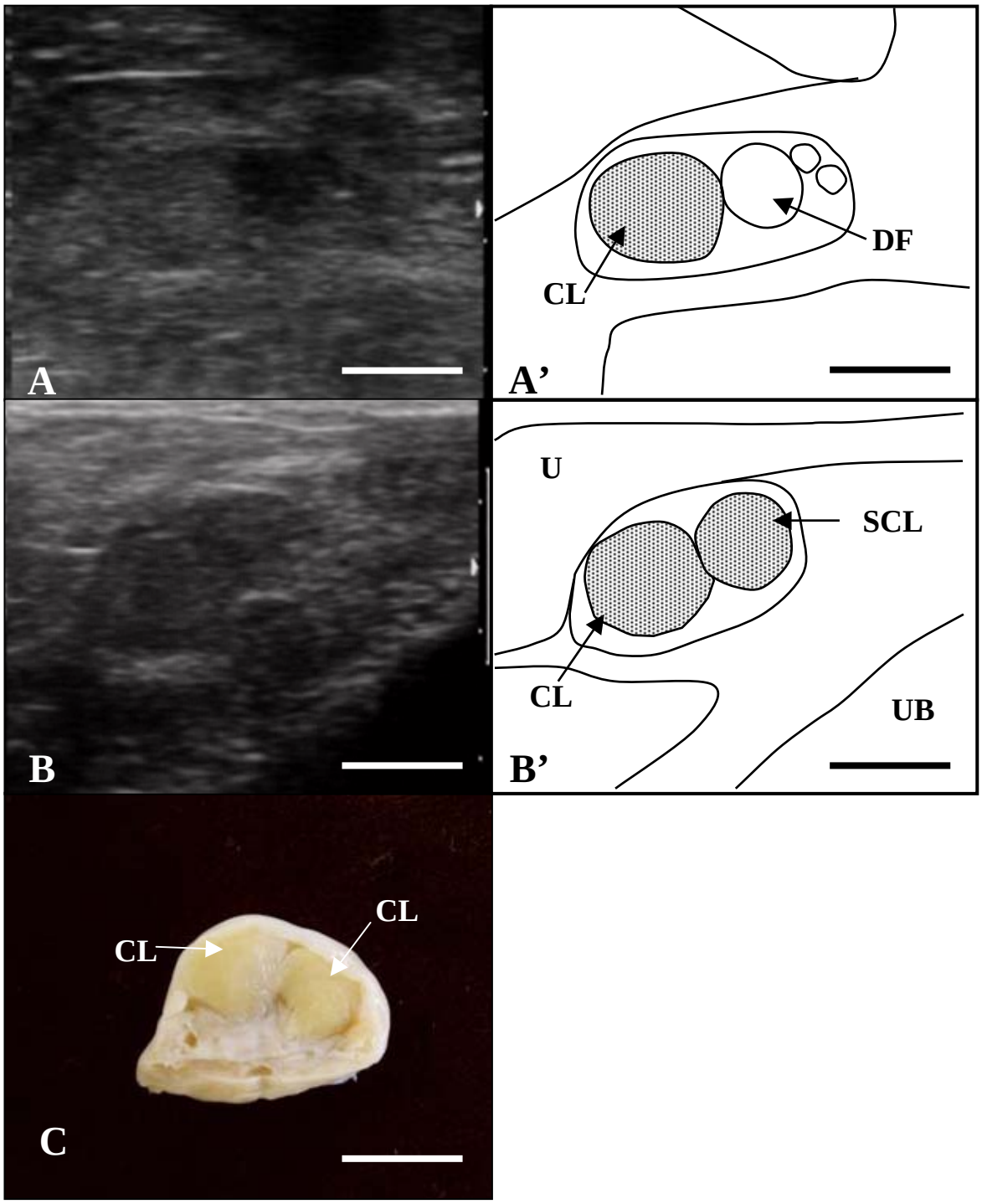

Fig. 2-3. Ultrasonographic and macroscopical images of the ovary in female No. 4 at different period. (A)Ultrasonographic image and (A') illustration of the ovary contains corpus luteum and dominant follicle on Day 8 of gestation. (B) Ultrasonographic image and (B') illustration of the ovary with two corpora lutea on Day 11 of gestation. (C) Macroscopical image of an ovary with two CLs on Day 85 of gestation when animal was killed by accident during trasportation and ovarry was collected on the same day. Bar = 1 cm DF: dominant follicle, CL: corpus luteum, FCL: first corpus luteum, SCL: second corpus luteum , U:uterus, UB: urinary bladder

Chapter 3

Fetal age estimation of Hokkaido sika deer (*Cervus nippon yesoensis*) using ultrasonography during early pregnancy

Introduction

In free-ranging animals, including cervid species, it is impossible to pinpoint exact conception dates by field observations alone. Therefore, they are typically determined by using fetal age, which is normally estimated by fetal weight in animals such as red deer (Mitchell and Lincoln 1973), fallow deer (Armstrong et al. 1969, Chapman and Chapman 1975) and various other mammals (Huggett and Widdas 1951). Likewise, an equation is also available for estimation of fetal age from fetal body weight in Hokkaido sika deer (*Cervus nippon yesoensis*; Suzuki et al. 1996). However, this method contains an intrinsic error in estimating fetal age and is not intended for estimation of fetal age during the early pregnancy period. Therefore, a new method of fetal age estimation is needed.

Estimation of fetal age by observation using ultrasonography is well established in cattle (White et al. 1985), sheep (Sergeev et al. 1990, Aiumlamai et al. 1992), goats (Lee et al. 2005), muskoxen (Pharr et al. 1994) and llamas and alpacas (Gazitua et al. 2001). Ultrasonography has been used for pregnancy diagnosis and fetal measurement in a number of cervid species, such as red deer, fallow deer and reindeer (Mulley et al. 1987, White et al. 1989, Bingham et al. 1990, Wilson and Bingham 1990, Revol and Wilson 1991a,b, Lenz et al. 1993, Vahtiala et al. 2004). In red deer, regression lines are available for fetal age estimation that were obtained by measuring various dimensions of fetuses of known age using ultrasonography as early as Day 24 of pregnancy (Bingham et al. 1990, Revol and Wilson 1991b). However, there is currently no reports available describing detail fetal growth of

sika deer in early pregnancy using ultrasonography. Therefore, the present study was conducted to describe the development of sika deer fetuses and to establish a reliable method for fetal age estimation in early pregnancy based on fetuses of known age.

Materials and Methods

Animals

Five captive female Hokkaido sika deer were kept with an adult stag at Asahiyama Zoological Park, Hokkaido, Japan (43º46' N, 142º28' E) during the breeding season in 2007. The females included two that were one year old and three that were more than three years old. They were kept in an approximately 80-m^2 pen and had partial shade. The animals were fed as described in the chapter 1.

Behavioral observation

The behavior of the five female sika deer was continuously monitored for estrus and copulation with the intact male by visual observation or video recording from September 21, 2007, which was prior to estrous season, to January 17, 2008, which was the day on which all the females were confirmed to be pregnant by the presence of a fetus with a heart beat. If copulation was observed on two successive days, the first day was recorded as the conception date (Day 0).

Observation of fetal development

Animals were chemically immobilized and the fetal developments were examined by transrectal ultrasound using ultrasonography as described in chapter 1. The deer were examined at two- or three-day intervals from September 24, 2007 to January 17, 2008. Observation of each fetus ceased at 59 to 61 days of gestation, except for one fetus in which observations ceased at 43 days of

gestation. Since the influence of maternal age on fetal growth occurs in the last one third of the gestation period (Eckstein and Kelly 1977), the ages of the females were not taken into consideration because the examinations were performed during early gestation in this study. The following dimensions were measured during each scan (Fig. 3-1 B, B').

(1) Straight crown-rump length (SCRL): A linear measurement from the top of the skull to the caudal edge of the perineum.

(2) Curved crown-rump length (CCRL): A measurement of the dorsal line from the top of the skull to the caudal edge of the perineum.

(3) Head length (HL): Distance from the caudal part of the skull to the basal point of the nose.

(4) Trunk depth (TD): Maximum length from the dorsal to ventral lines of the trunks.

Ultrasonographic images were recorded on videotape, and the fetal heart rate (FHR) was determined by counting the number of heartbeats while timing the duration of the video recording with a stopwatch.

Application to wild sika deer samples

To compare the fetal ages estimated by SCRL with those estimated by fetal weight, uteri containing placentae and fetuses were collected from nine pregnant wild sika deer. The animals were shot legally in Nishiokoppe Village on the island of Hokkaido, Japan, between November 2007 and January 2008. Fetal weight was measured with an electronic balance, and SCRL was measured with a caliper. Fetal age was estimated both by SCRL, according to the equation derived in the present study, and fetal weight, according to the equation derived in a previous study (Suzuki et al. 1996; $age = (\sqrt[3]{weight} + 2.73) / 0.091$).

Data analysis

Linear regression analysis using the least-squares principle was applied to derive equations relating each dimension, taken as the independent variable, to fetal age. Analyses were conducted after transformation to natural logarithms for SCRL and CCRL. The equation with the low standard error and high coefficient of determination was chosen as suitable for age prediction based on age as the dependent variable. In addition, a qualitative description of each structure was recorded at each scan, and a chronological record of fetal development was constructed.

In order to compare the fetal ages estimated by SCRL with those estimated by fetal weight, a Pearson correlation analysis was used to examine the correlation of fetal ages.

All statistical analyses were conducted using the Base System and Regression Models of SPSS 11.0.1 J for Windows (SPSS Japan Inc., Tokyo, Japan).

Results

Structural development

The chronological sequence of the ultrasonographic appearances of various fetal features and uterine structures are presented in Table 3-1. A fetus was detected in a cavity in the uterus by 20 days after conception in one female sika deer; this was the earliest conception detected. The cavities in the uteri which contained no fetuses were detected in two other female sika deer by Day 23. Fetuses were visible in cavity by Day 25 or 26 in the four remaining sika deer. The fetus first appeared as an elongated mass, with the heart beat also visible by Day 25 or 26 (Fig. 3-1 A, A'). During the period of Days 20 to 26, cavity in the uterus could be recognized only in the pregnant horn. By Day 29, the cavity elongated to the non-pregnant horn, and the placentome could be observed. By Day 32, the umbilical cord was evident. The aminiotic sac and allantoic cavity were clearly distinguishable, and

the fetal limb buds were visible by Day 34 or 35. Between Days 35 and 38, the fetal head and trunk differentiated. By 41 days after conception, the nose, mouth and tail could be seen. Principal organs could not be differentiated until Days 49 to 52. By Day 55, the orbits, ribs and other skeletal structures were observed.

Linear regression analysis of measurements

Table 3-2 presents linear regression equations for each measurement, the standard error of the regressions and the range of fetal ages and measurements for which the equations are valid. Each regression equation is highly significant ($P<0.001$). Accurate age prediction in early gestation can be achieved by measurement of SCRL or CCRL (Table 3-2, Figs. 3-2A, B). The most appropriate regression equations for SCRL and CCRL were found after transformation to natural logarithms. As its validity range of days started at Day 20, SCRL can estimate the fetal age earlier than CCRL (Table 3-2). HL and TD increased linearly during the observation period; FHR increased linearly throughout the observation period (108 – 288 beats/min; Fig. 3-2C).

Application to wild sika deer samples

The fetal SCRLs and fetal weights of the wild deer ranged between 4.7 and 80 mm and 0.006 and 27 g, and their estimated fetal ages based on these two measurements were from 20 to 60 days and 32 to 63 days old, respectively. A scatter diagram of the fetal ages estimated based on SCRL against those estimated based on the fetal weights of the wild deer is shown in Figure 3-3. For fetuses over 40 days old that had an SCRL of more than 28.55 mm and a fetal weight of more than 2 g ($n=5$), the fetal ages estimated based on SCRL and fetal weight correlated significantly ($P<0.01$, $r=0.993$). However, for fetuses under 40 days old that had an SCRL of less than 8.4 mm and a fetal weight of less than 0.044 g, the plots departed from the y=x line, and they did not correlate

significantly. Fetal age estimated as less than 40 days based on fetal weight, those of fetuses less than 0.044 g, converged shortly after Day 30 (Fig. 3-3).

Discussion

To our knowledge, this is the first report on the monitoring of fetal development in sika deer by ultrasonography. Although the number of animals used in this study was less than those used in previous studies in red deer (Bingham et al. 1990, Revol and Wilson 1991b), we obtained data more frequently to illustrate the fetal growth more precisely. During early pregnancy, ultrasonography enabled a precise monitoring of fetal development. Although a fetus was visualized on Day 20 in one female, fetuses first became visible on Day 25 or 26 in most of the others, a finding similar to that reported in red deer, where fetuses could be observed by Day 24 (Revol and Wilson 1991a). As they also reported observing the chorionic vesicle even earlier (from Day 14), we were not surprised to detect a fetus on Day 20 and cavity in uterus on Day 23 in the present study; structural fetal developments did not differ much from those of red deer (Revol and Wilson 1991a).

Measurement of SCRL is the most suitable marker for estimating fetal age. Both age estimation equations derived from SCRL and CCRL had low rates of standard error and high coefficients of determination (Table 3-2). However, SCRL could be measured from very early gestation, thus yielding fetal age estimates much earlier than CCRL (Day 20 vs. 29). Furthermore, it is much easier to measure SCRL than CCRL, especially when applied to fetuses from carcasses obtained in the field.

The fetal head and body were indistinguishable until Day 32, indicating that neither HL nor TD is particularly useful for estimating fetal age in very early pregnancy. On the other hand, for a fetus older than 60 days, SCRL and CCRL cannot be measured because the size of the fetus normally exceeds the measurement limits of the transducer (80 mm), whereas HL and TD can still be

measured beyond Day 60, making them useful for estimating the fetal ages of older live animals using ultrasonography.

In addition, this is the first report to count the FHR in cervid species of a known fetal age. In the present study, FHRs were measured and were found to increase linearly from 25 - 26 to 59 - 61 days of pregnancy. In a previous study, FHR decreased from 68 days to 226 days of estimated fetal age measured using ultrasonography in sika deer (Hama 1990). In cattle, FHR increases soon after the first fetal detection, shows a peak at about Day 60 of gestation and subsequently declines by Day 100 (Breukelman et al. 2004). There is a similar tendency in sika deer, in which FHR increases until about Day 60 and declines thereafter. It is therefore difficult to estimate fetal age by FHR alone because there might be two estimated fetal ages for a certain rate. Moreover, since FHR can only be used for live animals and since significant interobserver differences have been reported among FHR counts in cattle (Curran et al. 1986, Kastelic et al. 1988, Breukelman et al. 2004), it might not be appropriate for practical use.

The fetal age of sika deer is normally estimated using fetal body weight (Suzuki et al. 1996). Fetal ages estimated based on SCRL and fetal weight in wild sika deer correlated well in fetuses over 40 days old that weighed more than 2 g and had an SCRL of more than 28.55 mm. Therefore, it can be expected that fetal age estimations from fetal weight will not deviate much from actual fetal growth, at least in fetal weights ranging between 2 and 22 g (Days 45 and 60 as estimated by SCRL). However, ages estimated by fetal body weight may deviate from true fetal age below that range. This divergence is inevitable since the fetal weight method (Suzuki et al. 1996) establishes the body weight on Day 30 as zero grams. Thus, for estimation of conception dates in early pregnancy, it is undoubtedly much more appropriate to use the SCRL equation described here than the fetal weight equation (Suzuki et al. 1996).

In many studies of domestic animals, including cervid species (Roine et al. 1982, Wenham et al.

1986), bone length and the appearance of ossification centers have been used instead of estimations based on fetal weight to estimate fetal age more precisely (Gjesdal 1969, Wenham et al. 1969, Richardson et al. 1976, McDonald et al. 1977, Wenham 1981, Richardson et al. 1990). Kobayashi et al. (2004) estimated the fetal age of Hokkaido sika deer by ossification of fetuses using soft x-ray equipment. Although this offers a more precise fetal age estimation equation than estimation based on fetal weight, it is only effective at more than 100 days after conception when bones start to ossify. Moreover, it is troublesome and requires a trained radiographer.

Nutritional condition is involved in conception, and conception is delayed in females in poor nutritional condition (Kohlmann 1999, Noyes et al. 2002). In poor environments leading to substandard nutritional conditions, both fetal growth rates and birth weights are restricted (Verme 1965, Blaxter 1980, Skogland 1984). Consequently, estimating fetal age by fetal weight needs to take it into account that the birth weight might be different dependent on the nutritional status of the population. Furthermore, because poor nutrition does not severely affect skeletal growth and development (Wenham 1981, Wenham et al. 1986), the fetal physique is also unaffected by the nutritional condition of the mother. Therefore, measurement of fetal dimensions is very useful since there is no need to be overly concerned about population quality. As this method enables precise fetal age estimations in early pregnancy, it is now possible to figure out the conception period of a population that reflects that population's quality in more detail.

Although the present study provides precise fetal-age estimations in early pregnancy, measurement are only available until about two months of gestation because of the limits in measuring fetal dimensions using ultrasonography. Therefore, for practical fetal age estimations throughout the entire gestation period, the combination of the three estimates for sika deer as reported by Suzuki et al. (1996), Kobayashi et al. (2004) and the present study might be appropriate. We have provided an appropriate equation valid until 60 days of gestation which, together with an

equation from Kobayashi et al. (2004), enables precise estimation of fetal age. The equation of Suzuki et al. (1996) may be somewhat useful in estimating fetal age (though less precisely) in the mid- to late-gestation period. Given that there is a time period during which precise estimation is not possible, i.e., Day 60 to Day 100, further detailed study is needed to enable far more precise fetal age estimation in sika deer.

Summary

In sika deer, the normal method of estimating fetal age, based on fetal weight, is not applicable during the early pregnancy period. The objective of the present study was to describe the growth and development of sika deer fetuses and to establish a method for fetal age estimation during early pregnancy using ultrasonography. Five captive female Hokkaido sika deer (*Cervus nippon yesoensis*) were observed for estrus and mated (Day 0) with an intact male. At two- or three-day intervals, fetuses were observed by rectal ultrasonographic scans until 59 - 61 days of gestation. The straight crown-rump length (SCRL), curved crown-rump length (CCRL), head length (HL), trunk depth (TD) and heart rate (HR) of the fetuses were measured. Linear regression equations were computed for each measurement together with fetal age. Analyses were conducted after transformation to a natural logarithm for SCRL and CCRL. All equations were significant ($P<0.001$), with SCRL becoming measurable earlier (Day 20) than the others and yielding the best correlation (Days= -2.08+14.15 LnX : X=SCRL, Ln=natural logarithm). Therefore, the author concluded that a precise estimation of fetal age in early gestation is best performed using SCRL measurements.

Table 3-1. Chronological sequence of appearance of the ultrasonographic features of the developing fetus and uterine structures

Fetal age (days)	Features becoming apparent
20 - 25	Fetal sac
20 - 26	Fetus, heart beat, cavity in the pregnant horn
26 - 29	Placentome, cavity fills both uterine horns
31 - 32	Umbilical cord
32 - 35	Limb buds
34 - 35	Differentiation of the aminiotic sac and allantoic cavity
35 - 38	Differentiation of the head and truncus
38 - 41	Nose, mouth, tail
43 - 44	Heart chamber
49 - 52	Liver, lung
50 - 55	Orbits, ribs, vertebrae, diaphragm

Table 3-2. Age prediction equations derived from ultrasonographic measurements of fetal dimensions from 20 to 61 days of pregnancy in Hokkaido sika deer (*Cervus nippon yesoensis*; $P<0.001$ for all equations)

				Range of validity	
Dimension (*X*)	Age-prediction equation	R^2	SE (days)	Days	Measurements
Straight Crown-rump length (mm)	Days= - 2.08+14.15 Ln*X*	0.98	1.66	20 - 61	4.2 – 80.6
Curved Crown-rump length (mm)	Days= - 19.18+17.48 Ln*X*	0.97	1.61	29 - 61	14.2 - 99.2
Head length (mm)	Days=25.35+1.67*X*	0.93	1.99	35 - 61	6.6 – 21.8
Trunk depth (mm)	Days=21.94+1.99*X*	0.91	2.34	35 - 61	7.5 – 20.2
Fetal heart rate (beats / min)	Days=6.07+0.18*X*	0.87	3.79	25 - 61	108 – 288

Ln=natural logarithm.

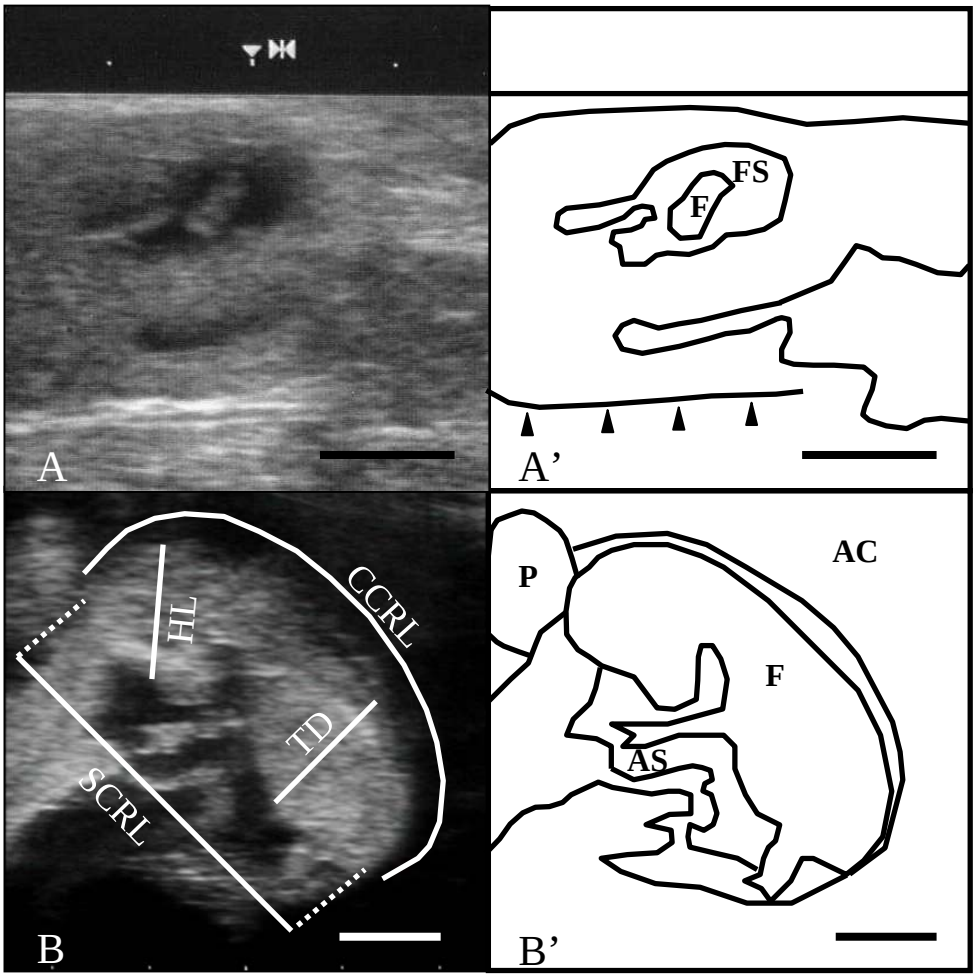

Fig. 3-1. (A) Ultrasonographic visualization and (A') illustration of a Hokkaido sika deer fetus, first visible on day 25 of gestation. (B) Ultrasonographic visualization and (B') illustration of a Hokkaido sika deer fetus on day 49 of gestation and fetal dimensions recorded from ultrasonograms. Arrowheads indicate the border of the uterus. F, fetus; FS, fetal sac; AS, aminiotic sac; AC, allantoic cavity; P, placentome; SCRL, straight crown-rump length; CCRL, curved crown-rump length; HL, head length; TD, trunk depth. Bar=1 cm.

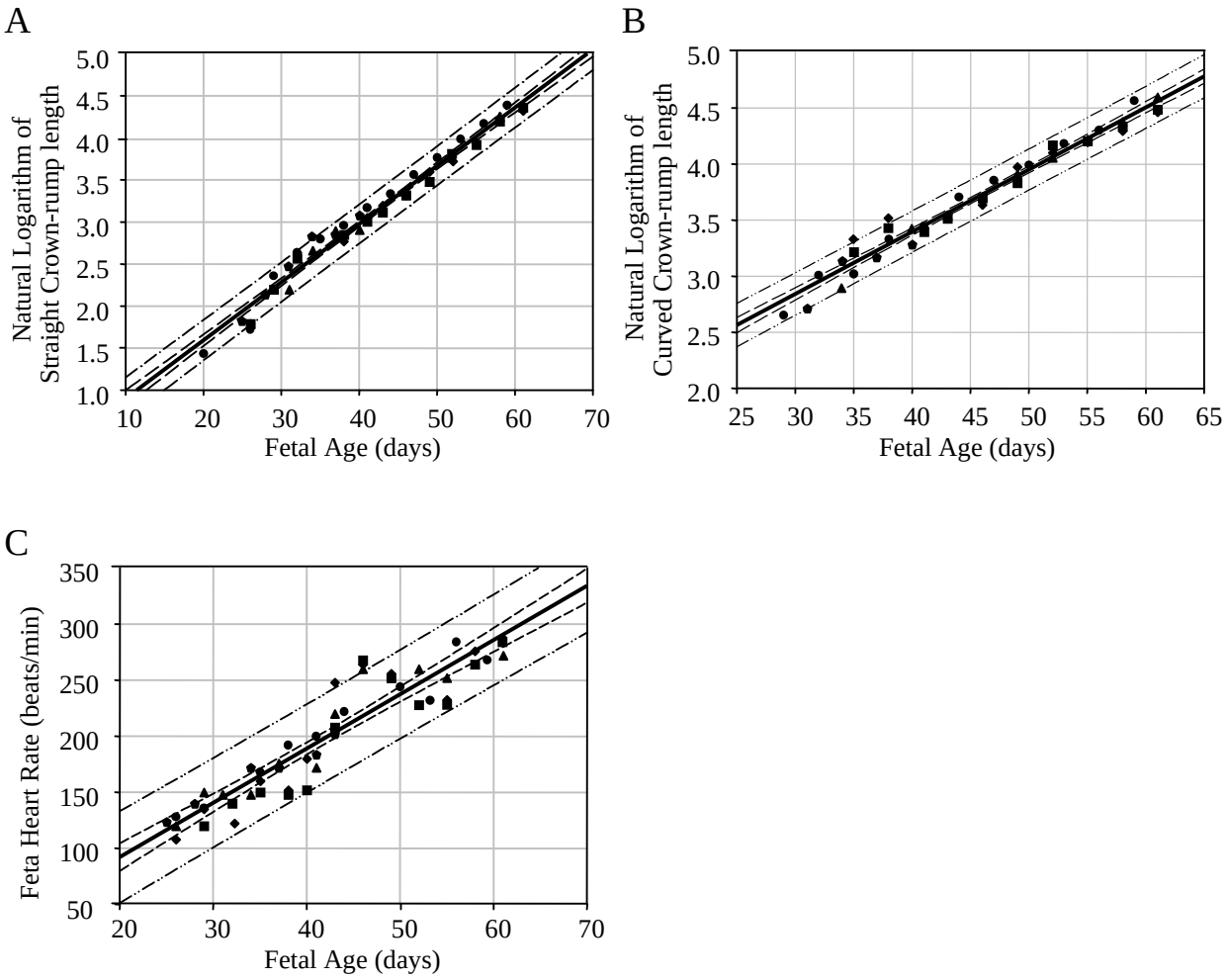

Fig. 3-2. Scatter diagram of data and linear regression for (A) the natural logarithm of the straight crown-rump length of the fetus, (B) natural logarithm of the curved crown-rump length of the fetus and (C) fetal heart rate against fetal age in Hokkaido sika deer. Data from the same individual but at a different fetal age are indicated using the same symbols. Dashed line, 95% confidence intervals; Dashed-dotted line, 95% prediction intervals.

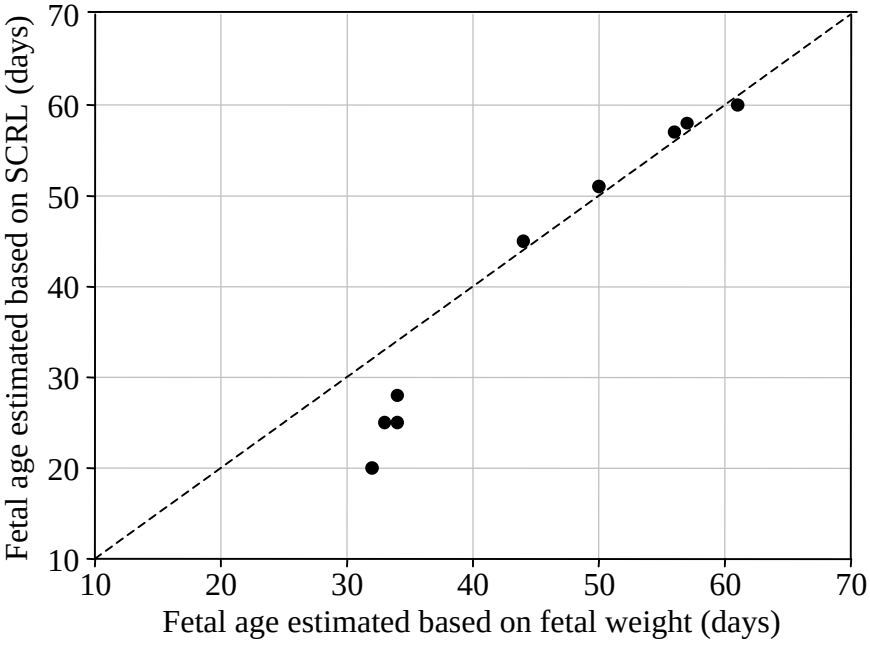

Fig. 3-3. Scatter diagram of fetal age estimated by SCRL against that estimated by fetal weight for Hokkaido sika deer collected in the wild with the line y=x.

Chapter 4

Immunohistochemical localization of the estrogen receptor alpha (ERα) and progesterone receptor (PR) in the uterus of Hokkaido sika deer (*Cervus nippon yesoensis*) during pregnancy

Introduction

Sika deer (*Cervus nippon*) is a seasonal breeder, mating in autumn and fawning in early summer (Koizumi 1991, Matsuura et al. 2004a) after 224 – 230 days' gestation (Matsuura et al. 2004a, b). Ovarian steroid hormones should play a central role in the regulation of uterine function to establish and maintain pregnancy. Information on the steroid hormone receptor distribution in the uterus is, thus, essential to understand when and where steroid hormones (i.e., estrogen and progestin) are required in the uterus during the estrous cycle and pregnancy (Meikle et al. 2004). Spatio-temporal distribution of estrogen receptor (ER) and progesterone receptor (PR) has been investigated in various domestic species, such as sheep (Spencer and Bazer 1995), cattle (Kimmins and MacLaren 2001), pigs (Geisert et al. 1993, 1994) and horses (Hartt et al. 2005), and has provided insights into uterine physiology. In ruminants, including cervid species, luteolysis, which is triggered by secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus, must be prevented to establish pregnancy (Godkin et al. 1997, Martal et al. 1997). As $PGF_{2\alpha}$ secretion is triggered by oxytocin through an up-regulation of oxytocin receptor (OTR) which has been up-regulated by estrogen, the distribution pattern of ER is thought to be important in the onset of $PGF_{2\alpha}$ secretion in sheep (Spencer and Bazer 1995, Spencer et al. 1995a, 1999). On the other hand, progesterone, the hormone of pregnancy, is essential to create a uterine environment that supports the development of the conceptus to term (Spencer and Bazer 2002). It also influences uterine histotroph secretion, presumably aiding embryo survival to maintain

pregnancy in sheep (Spencer et al. 1995b). However, no study has documented the distribution of steroid hormone receptors in the uterus of cervid species.

This study was conducted to characterize the spatio-temporal distribution of endometrial and myometrial ER alpha (ERα) and PR protein during the gestation period to understand the physiological roles of estrogen and progesterone. It is known that over 80% of pregnant sika deer possess two steroidogenic corpora lutea (CLs), known to be formed at a different period, even though they have a single fetus (Suzuki et al. 1992, Suzuki 1993, Suzuki and Ohtaishi 1993, Matsuura et al. 2004c). Therefore, uterine samples from sika deer with two CLs and single fetus were exclusively used in this study except for a single sample (No. 1; Table 4-1), which represents the time between two ovulations.

Materials and Methods

Animals and uterine samples

Ovaries and uteri were collected in the wild from 21 pregnant sika deer (No. 4-24; Table 4-1) and three additional females whose gestational status could not be determined (No. 1-3; Table 4-1). The animals were shot legally on the island of Hokkaido, Japan, in January 2004, November 2005, January to May 2006 and November 2007 to January 2008. All pregnant females (Nos. 4-24) had a single fetus and two CLs in the ovaries. Among three additional females (No. 1-3), female No. 1 had only one developing CL with a cavity and clot in it, indicating that she was most likely within 4 days after estrus (metestrus; Ireland et al. 1980). Females Nos. 2 and 3 had two CLs, one fully developed and the other at the developing stage. They were assumed to be 7 days after estrus or later (diestrus), since development of CL takes about 7 days in cattle and sheep (Robertson 1977, Noakes 1997). Since the author failed to recover an embryo from these three animals, they were thus considered to be non-pregnant in this study. In sheep, it is known that there is no difference in uterine steroid

hormone receptor distribution between pregnant and non-pregnant animals until Day 11 (Spencer and Bazer 1995). However it started to be different on Day 13: the period corresponded to the time of interferon tau (INFτ), the factor of maternal recognition of pregnancy, secretion by an embryo (Godkin et al. 1982). Since INFτ is known to be secreted by the embryo from Day 14 (Demmers et al. 2000) in cervid species, uterine steroid hormone receptor distribution is assumed to be the same until Day 14 regardless of the gestational status. Therefore, three non-pregnant animals are used to estimate the steroid hormone receptor distribution in early pregnancy.

Estimation of fetal age

Fetal weights were measured with an electronic balance or spring scale, and crown-rump lengths were measured with a caliper. Fetal age was estimated by straight crown-rump length (SCRL) up to 60 days (≒ SCRL 80.6 mm) of pregnancy according to chapter 3 ($age = -2.08 + 14.15LnX$, X=SCRL) and fetuses over 60 days old (CRL > 80.6 mm) were estimated by fetal weight according to Suzuki et al. (1996) ($age = (\sqrt[3]{X} + 2.73)/0.091$, X= fetal body weight). The fetal ages ranged from Day 20 to day 207. Since the gestational length of sika deer is reported to be about 224 - 230 days (Matsuura et al. 2004a, b), samples covered almost the whole range of gestation periods.

Immunohistochemistry

Uteri were fixed within one hour after killing and preserved in 10% phosphate-buffered formalin for 3 - 18 months before embedding. Fixed tissues from the dorsal part of uterine horn in which the fetus was present were used as the sample for immunohistochemistry. In three animals (No. 1-3), in which the presence of an embryo was not confirmed, tissue samples were obtained from the uterine horns ipsilateral to the ovary with the CL. After fixation, the specimens were dehydrated in an

ethanol series and embedded in paraffin. Tissues were cut into 5-μm-thick paraffin sections and mounted on silane-coated slide glass (MAS, S9226, Matsunami, Osaka, Japan).

To reveal the presence and distribution of ERα and PR in the uteri, the avidin-biotin immunoperoxidase technique (Vectastain® ABC kit; Vector Laboratories, Inc., Burlingame, UK) was used. After deparaffinization through an ethanol gradient and rehydration in phosphate-buffered salines, the sections were incubated briefly in an antigen-unmasking solution, Target Retrieval Solution (Dako Cytomation, Carpinteria, CA, USA) for 40 min at 99°C. The sections were then incubated in 3% hydrogen peroxide/methanol solution for 10 min to block the endogenous peroxidase activity, and treatment with blocking serum was applied to each section to prevent nonspecific reactions. Primary antibodies were added to sections overnight at 4°C for incubation. The rabbit polyclonal antibody (PA1-309, Affinity Bioreagents, Golden, CO, USA) to the synthetic peptide corresponds to amino acid residues 21-32 from human ERα (Cardenas and Pope 2004) which were completely conserved in several species, including the cervid species (accession X98007) and cross-react with human, porcine, and rat ERα was used as primary antibody to ERα. Also the mouse monoclonal antibody (Ab-8, Neo Markers, Fremont, CA, USA) to human PR (Gray and Satyaswaroop 1988) which cross-reacts with human, horse, sheep and pig PR was used as primary antibody to PR. Negative controls were treated with normal rabbit serum or normal mouse serum. Colorization was performed by 3, 3'-diaminobenzidine-H_2O_2 solution (0.02% w/v) for 5 to 10 min. Steroid receptor staining intensities were scored visually referring to Spencer and Bazer (1995) (- absent, ± scattering, + weak, ++ moderate, or +++ strong) in the luminal epithelium (LE), glandular epithelium (GE), endometrial stroma (ST) and myometrium (MYO). In addition, GE and ST were distinguished into shallow and deep parts when there was a difference in staining. The author classified the area adjacent to epithelium as shallow and the area adjacent to myometrium as deep region.

Results

Estrogen receptor localization

Immunoreactive ERα were detected predominantly in the nuclei of uterine cells. Results of ERα staining in uteri are summarized in Table 4-1. In the uterus of No. 1 (≤Day 4), strong staining of ERα was observed in the deep GE, ST, and MYO, while the LE and shallow GE showed moderate staining (Fig. 4-1A, B). In deep GE, ST and MYO of uteri of female No. 2 and 3, ERα staining was similar to that in uterus of No. 1 (Fig. 4-1D), while the staining in the LE and shallow GE was weaker in No. 2 than in No. 1 (Fig. 4-1C). Staining of ERα in LE became undetectable by Day 20 (No. 4), and remained undetectable up to Day 207 (No. 24). In the deep GE, ST and MYO, weak to moderate ERα staining was observed on Day 20 (No.4, Fig. 4-1E), but the staining became undetectable by Day 28 (No.7) and thereafter (Fig. 4-1F). The staining became detectable during late pregnancy again (Day 164~, Fig. 4-1G). No appreciable staining was detected in uterine tissue sections incubated with normal rabbit serum substituted for primary antibody (Fig. 4-1H).

Progesterone receptor localization

Immunostaining for PR was localized predominantly in the nuclei of uterine cell. Results of PR staining in uteri are summarized in Table 4-1. The staining of PR in LE was only detected in No. 1 (≤Day 4: Fig. 4-2A) and No. 4 (Day 20). In the GE, the staining of PR was detectable in No. 1-3 (Fig. 4-2A-D) and No. 4 (Day 20: Fig. 4-2E), and became undetectable by Day 25 (No. 6). In contrast, moderate to strong staining was detectable in the ST and MYO in all samples (Fig. 4-2F). From Day 164 to Day 207 (No. 21-24), they were consistently detected as strong (Fig. 4-2G). No appreciable staining was observed in sections incubated with normal mouse serum in place of primary antibody (Fig. 4-2H).

Discussion

This is the first study to describe the distribution of steroid hormone receptors in the uterus of cervid species. The results indicate that distribution of uterine ERα and PR is differentially regulated in both a spatial and temporal manner during pregnancy. The distribution pattern of ERα and PR differed between the endometrium and myometrium, and also among cell types in the endometrium.

In the uterus with one developing CL assumed to be within several days after estrus (No. 1), the staining of ERα and PR was strong or moderate in all cell types. This is similar to that of a cyclic ruminant (Spencer and Bazer 1995, Kimmins and MacLaren 2001). The strong or moderate staining of ERα and PR may be due to the influence of a high concentration of circulating estrogen from the Graafian follicle at the estrus, since estrogen is known to up-regulate the ER and PR expression (Wathes and Hamon 1993).

Although the present results show a trend in which ER staining decreases with days after estrus is similar to the change of ER in sheep, a domestic species in which steroid receptor distribution has been well documented, the time and extent of decline may differ between deer and sheep. In sheep, staining of ER in all cell types decreased dramatically by Day 11 of the estrous cycle or pregnancy and remained weak thereafter, when animals are pregnant, while staining of ERα delayed to decrease in sika deer and was detectable as late as Day 25 in the ST. The difference in the time of ER suppression between sheep and deer could be attributed to estrogen secretion by the follicle which is maturing and ovulating after formation of the first CL (>Day 7). This hypothesis could be verified when ER staining in pregnant deer, which does not form a second CL, decreased around Day 11 as in sheep. Alternatively, the delay in suppression of ER staining in shallow GE, ST and MYO up to Day 25 may suggest a potential role of estrogen in these cell types during early pregnancy in this species.

Lower staining of ERα in the LE and shallow GE than in the other part of the uterus is

comparable to sheep. Since OTR appearance is regulated by estrogen, down-regulation of ER may have the advantage of preventing the luteolytic mechanism via OTR down-regulation as oxytocin stimulates the synthesis of luteolytic $PGF_{2\alpha}$ secretion. Down-regulation of ER in LE and shallow GE may be important since $PGF_{2\alpha}$ is thought to be secreted from LE and shallow GE because of the specific localization of cyclooxygenase-2, an important enzyme for $PGF_{2\alpha}$ synthesis in these cell types in sheep (Charpigny et al. 1997). The mechanism of ER appearance which is regulated differently between cell types is not clear, but down-regulation of ER in LE and shallow GE might be due to the effect of INFτ. INFτ produced by the conceptus and involved in the maternal pregnancy recognition (Bazer et al. 1994, Spencer et al. 1995a, b, Spencer et al. 2004a) is known to act in a paracrine fashion on LE and shallow GE to suppress transcription of ER and OTR genes (Mirando et al. 1993, Spencer and Bazer 1996, Fleming et al. 2001), thereby preventing development of the endometrial luteolytic mechanism in sheep (Spencer and Bazer 1995, Spencer et al. 1995a). In red deer, INFτ is reportedly produced by conceptus, and is known to have an anti-luteolytic effect (Bainbridge and Jabbour 1999, Demmers et al. 1999, 2000). Therefore, INFτ may have been responsible for the reduced staining of ERα in LE and GE, which was similar to that in sheep, although the gestational status (i.e., the presence of an embryo) of No. 2 and 3 was unknown.

Staining of PR in uteri of No. 2 and 3 was relatively low in LE. Moreover, staining in LE and GE declined after Day 20 and became undetectable by Day 25. Decline of PR in LE and GE may be due to decline of PR up-regulation by estrogen since ERα appearance was suppressed at this period, or to prolonged exposure to progesterone action since progesterone has negative effects on PR appearance (Zelinski et al. 1980, Clarke 1990, Spencer et al. 1995a). On the other hand, high staining of PR in ST and MYO was detected during early pregnancy (Day 20 - 25) and might be important to establish and maintain pregnancy.

During Day 28 to Day 127 (No. 7 - 20), ERα staining diminished in all cell types, possibly due to a high level of circulating progesterone as it down-regulated ER appearance (Evans et al. 1980, Spencer et al. 1995a, b) and to prevent $PGF_{2\alpha}$ secretion and myometrial contraction (Challis et al. 2000) for the maintenance of pregnancy. On the other hand, the reactivity of PR remained high in ST and MYO, consistent with that observed in sheep and cattle (Spencer et al. 2004a, b, Boos et al. 2006). The PR in ST may be required for proliferation of the endometrium which is stimulated by stromal cell-derived growth factors induced by progesterone (Spencer and Bazer 2002). Presence of PR in MYO throughout pregnancy might also indicate a direct influence of progesterone on contractile activity of this layer, finally resulting in myometrial quiescence (Challis et al. 2000).

In late pregnancy, from Day 164 and later (No. 21-24), ERα staining was detected weakly in all uterine cell types except for LE. It may be due to secretion of estrogen toward parturition as reported in domestic animals (Jenkin and Young 2004). Increase in estrogen secretion toward term is also reported in cervid species; red deer (Kelly et al. 1982), reindeer (Ropstad et al. 2005, Shipka et al. 2007) and white-tail deer (Plotka et al. 1977). The presence of ERα in LE and GE is crucial in late pregnancy since ER up-regulation may increase OTR appearance (Beard et al. 1994, Fuchs et al. 1992) which stimulates production of $PGF_{2\alpha}$ (Spencer and Bazer 1995, Spencer et al. 1995a, 1999), a luteolytic factor. Also, the presence of ERα in ST might play an important role in luteolysis since estrogen induces epithelial proliferation through ER-positive ST (Cooke et al. 1997). The presence of ER in MYO may be important because oxytocin, which promotes myometrial contractility, coacted with $PGF_{2\alpha}$ during late pregnancy and parturition (Chard 1989, Challis et al. 2000). Interestingly, the timing of ERα up-regulation, which may be caused by estrogen, was about eight weeks prepartum in sika deer while estrogen secretion has been reported to increase about six weeks prepartum in other cervid species (Plotka et al. 1977, Kelly et al. 1982, Ropstad et al. 2005, Shipka et al. 2007). Paired samples of uteri and peripheral blood of sika deer of known gestational age are

needed to confirm the timing in increase of estrogen and up-regulation of ER in sika deer.

Summary

Information on steroid hormone receptor distribution in the uterus is essential to understand the roles of their ligands in pregnancy. This study examined the spatio-temporal localization of estrogen receptor alpha (ERα) and progesterone receptor (PR) in the uterus of sika deer (*Cervus nippon*) to determine the estrogen and progesterone action site during pregnancy. Ovaries and uteri were collected from 21 pregnant sika deer with single fetus and two corpora lutea, ranging from Day 20 to Day 207 of pregnancy. In addition, genital organs were also collected from three sika deer whose gestational status was unknown: one female had only one developing corpus luteum: ≤Day 4 (metestrus) and two females had two corpora lutea, one of which was at the developing stage equivalent to diestrus or early pregnancy: > Day 7 (diestrus). Staining of ERα and PR was clear in all cell types during metestrus. During diestrus, the presence of ERα was also clear in deep glandular epithelium, stroma and myometrium, whereas it was suppressed in luminal epithelium and shallow glandular epithelium. Staining of PR was suppressed in luminal epithelium but was detectable in other cell types. Staining of ERα in all cell types and PR in luminal epithelium and glandular epithelium became undetectable by Day 28. PR was presented in stroma and myometrium throughout pregnancy. The distribution pattern of ERα and PR was different during diestrus from that in a ruminant. This could be attributed to estrogen secretion from the maturing and ovulating follicles in the presence of developed corpus luteum.

Table 4-1. Summary of immunolocalization of steroid hormone receptors in the sika deer uterus

ID	Status	Estimated days of gestation[1)]	ERα						PR					
				GE		ST				GE		ST		
			LE	Shallow	Deep	Shallow	Deep	MYO	LE	Shallow	Deep	Shallow	Deep	MYO
No.1	One CL	<4	++	++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
No.2	Two CLs	>7	+	++	+++	+++	+++	++	-	+++	+++	++	++	+++
No.3	Two CLs	>7	±	+	+++	+++	+++	+++	-	±	++	++	+++	+++
No.4	Pregnant	20	-	+	+++	++	+	+	+	++	+++	++	++	+++
No.5	Pregnant	23	-	±	±	+	+	+	-	±	-	++	++	+++
No.6	Pregnant	25	-	-	-	+	-	-	-	-	-	++	++	++
No.7	Pregnant	28	-	-	-	-	-	-	-	-	-	++	++	++
No.8	Pregnant	45	-	-	-	-	-	-	-	-	-	++	++	++
No.9	Pregnant	50	-	-	-	-	-	-	-	-	-	++	++	++
No.10	Pregnant	51	-	-	-	-	-	-	-	-	-	++	++	++
No.11	Pregnant	57	-	-	-	-	-	-	-	-	-	++	++	++
No.12	Pregnant	58	-	-	-	-	-	-	-	-	-	+++	++	+++
No.13	Pregnant	60	-	-	-	-	-	-	-	-	-	+++	++	++
No.14	Pregnant	77	-	-	-	-	-	-	-	-	-	++	++	++
No.15	Pregnant	108	-	-	-	-	-	-	-	-	-	++	++	++
No.16	Pregnant	115	-	-	-	-	-	-	-	-	-	++	++	++
No.17	Pregnant	115	-	-	-	-	-	-	-	-	-	++	+++	+++
No.18	Pregnant	118	-	-	-	-	-	-	-	-	-	++	+++	+++
No.19	Pregnant	126	-	-	-	-	-	-	-	-	-	++	++	++
No.20	Pregnant	127	-	-	-	-	-	-	-	-	-	++	++	++
No.21	Pregnant	164	-	+	+	+	+	+	-	-	-	+++	+++	+++
No.22	Pregnant	197	-	±	+	+	+	+	-	-	-	+++	+++	+++
No.23	Pregnant	197	-	±	+	+	+	+	-	-	-	+++	+++	+++
No.24	Pregnant	207	-	+	+	+	+	+	-	-	-	+++	+++	++

ERα, estrogen receptor alpha; PR, progesterone receptor; LE, luminal epithelium; GE, glandular epithelium; ST, stroma; MYO, myometrium; CL, corpus luteum.

[1)] Fetal ages were estimated by straight crown-rump length up to 60 days (chapter 3), and by fetal body weight thereafter (Suzuki et al. 1996). Days for No.1 - 3 are estimated days after estrus.

-, absent; ±, scattering; +, weak; ++, moderate; +++, strong

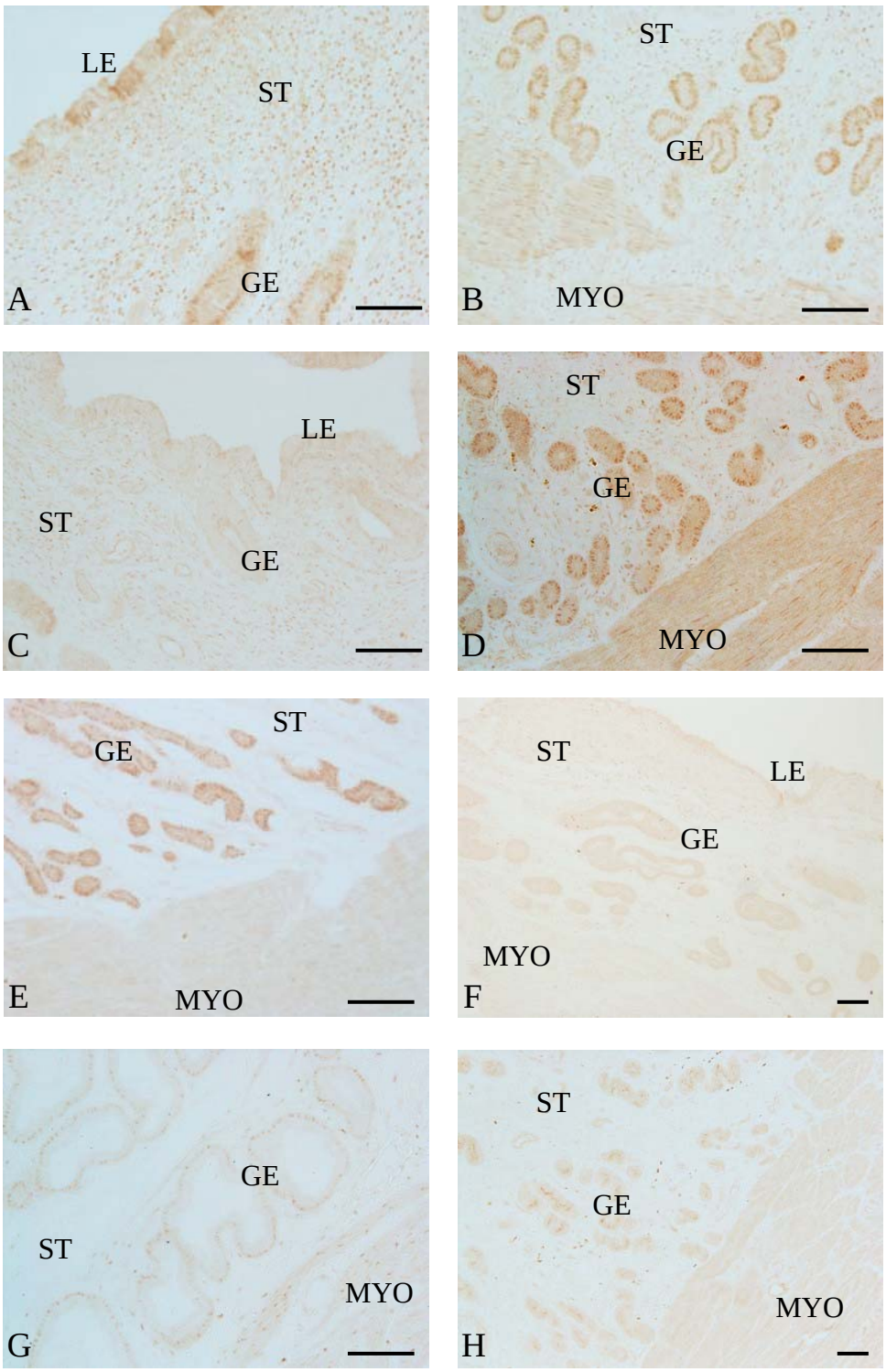

Fig. 4-1. Immunohistochemical localization of estrogen receptor alpha in the uterus of sika deer. (A) Endometrium of female with one CL (No. 1). (B) Myometrium of female with one CL (No. 1). (C) Endometrium of female with two CLs, developed and developing CL (No. 3). (D) Myometrium of female with two CLs, developed and developing CL (No. 3). (E) Myometrium of female on Day 20 of pregnancy (No. 4). (F) Endometrium and myometrium of female in mid-pregnancy (No. 12). (G) Myometrium of female in late pregnancy (No. 21). (H) Negative control. LE, luminal epithelium; GE, glandular epithelium; ST, stroma; MYO, myometrium. Scale bar = 100 μm

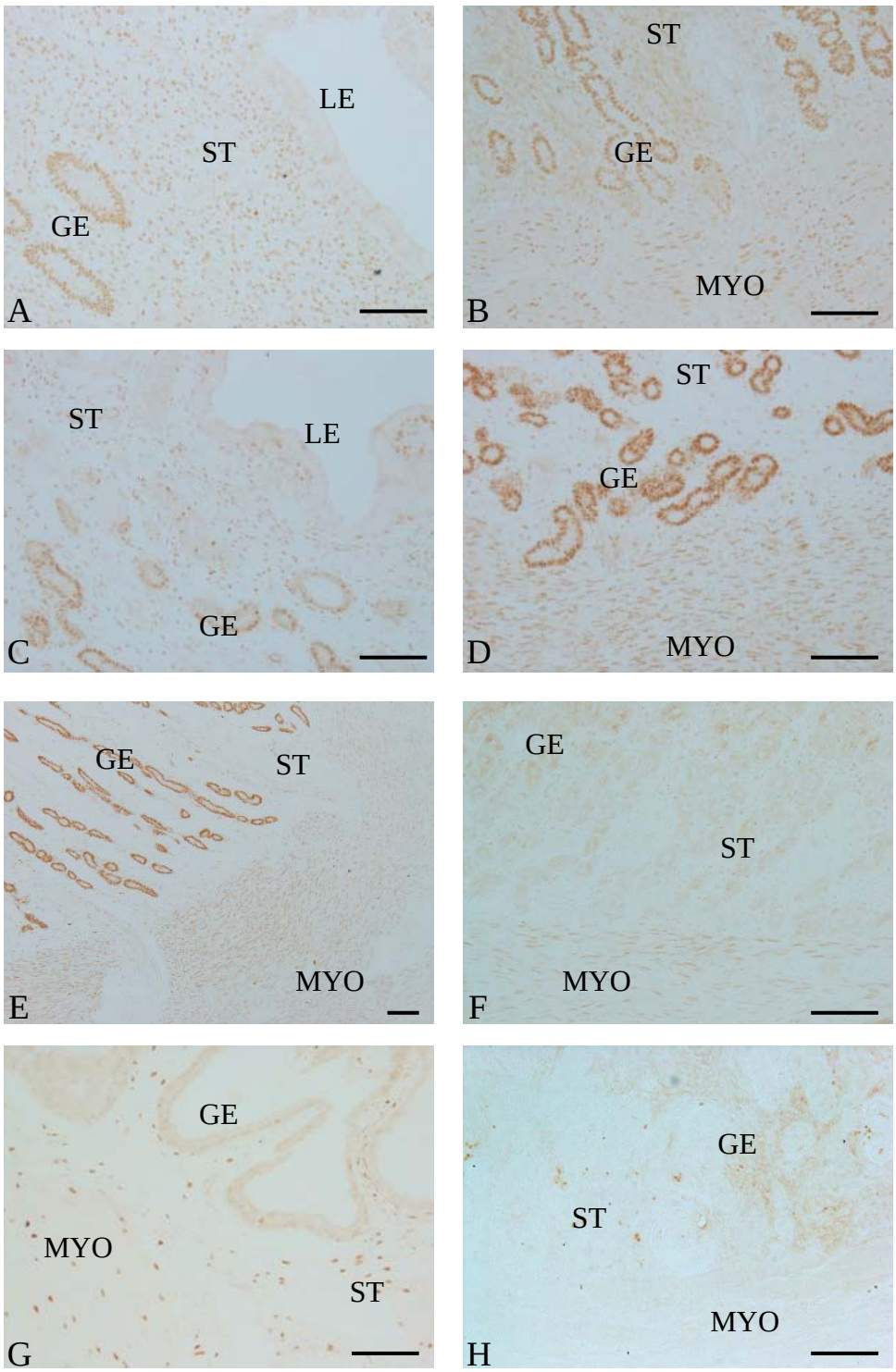

Fig. 4-2. Immunohistochemical localization of progesterone receptor in the uterus of sika deer. (A) Endometrium of female with one CL (No. 1). (B) Myometrium of female with one CL (No. 1). (C) Endometrium of female with two CLs, developed and developing CL (No. 3). (D) Myometrium of female with two CLs, developed and developing CL (No. 3). (E) Myometrium of female Day 20 of pregnancy (No. 4). (F) Myometrium of female in mid-pregnancy (No. 16). (G) Myometrium of female in late pregnancy (No. 21). (H) Negative control. LE, luminal epithelium; GE, glandular epithelium; ST, stroma; MYO, myometrium. Scale bar = 100μm

Conclusion

In the present thesis, the author focused on the period during conception and maintenance of pregnancy in Hokkaido sika deer. There was no insight into physiological factor influences to the conception date and basic reproductive physiology such as ovarian dynamics and hormonal changes were also unclear in sika deer. Therefore author conducted the study to understand basic reproductive physiology around the period of conception and pregnancy.

In chapter 1, the objective was to characterize ovarian dynamics and hormonal changes during seasonal transition. The follicular and luteal dynamics and changes in steroid hormones of eight captive female Hokkaido sika deer were examined and those were compared between the three periods: before, during and after the first estrous cycle. There was at least one ovulation without estrus and subsequently transient formation of corpus luteum (CL) and low increases in progesterone were observed just before the first estrus of the estrous season in all females. Since progesterone is known to induces estrus, transient increase in progesterone may involved in the on set of estrous season. Majority of number of inter-estrus follicular waves were two and three during estrous season. The crystallization of cervical mucus suggested being a indicator of determining the peak of estrus since it was related to day of estrus.

In chapter 2, the objective was to characterize the ovarian dynamics and hormonal changes during conception and early gestation, and reveal the origin and timing of formation of multiple CLs, a unique characteristic of pregnant sika deer, to discuss its significance. Follicular and luteal dynamics together with changes in steroid hormones of six captive female Hokkaido sika deer were examined. Two CLs formed in three females conceived at the first estrus of the estrous season. Second CL was formed from ovulated dominant follicle of follicular wave emerged soon after ovulation associated to the first estrus of the estrous season. On the other hand, first dominant

follicle did not ovulate and only one CL formed in three females conceived at ht second estrus of the estrous season. Therefore, it was suggested that second CL forms in case that female conceive at the first estrus of the season. Since progesterone concentrations did not differ dependent on number of CL, second CL was assumed to support the first CL by secreting progesterone otherwise insufficient for the maintenance of pregnancy.

In chapter 3, the objective was to characterize the early fetal development and produce the formula for precise fetal age estimation during early pregnancy. Fetal growth in five captive female Hokkaido sika deer was examined by transrectal ultrasonography until about 59 - 61 days of pregnancy. Fetus could be detected by Day 20-26 in all females. Straight crown-rump length (SCRL), curved crown-rump length (CCRL), head length (HL), trunk depth (TD) and heart rate (HR) of fetus were measured each examination and those increased linearly as pregnancy progress. By regression analysis, it was concluded that precise estimation of fetal age in early gestation can be done best by measurement of SCRL since it was highly correlated and measurable earlier than others.

In chapter 4, the objective was to characterize the action site of progesterone and estrogen during pregnancy. The spatio-temporal localization of estrogen receptor alpha (ERα) and progesterone receptor (PR) in the uteri of 24 Hokkaido sika deer derived from wild were examined immunohistochemically. Fetal ages of early pregnancy period were estimated based on chapter 3. Distribution of ERα and PR could be observed until Day 25 of pregnancy in sika deer and it was relatively long time compared to dominated ruminant. Therefore, not only progesterone but estrogen may secrete and act until Day 25 of pregnancy.

During seasonal transition, transient increase in progesterone concentration just before the first estrus of the season was observed and this is considered to be important for the onset of estrous season. In addition, the origin and timing of formation of second CL in pregnant females reveal and it was hypothesized that second CL forms in case that female conceived at the first estrus of the

season. All take into consideration, ovulation is likely the key incident which involve in determining conception date. Therefore, the hypothalamic-pituitary-gonadal axis must be investigated for further understanding of detail physiological factor influences conception date in sika deer.

The present study provided the useful method for utilizing carcasses. Therefore, many carcasses are available for studying the physiological changes during early pregnancy more in detail than before. Owing to the method, involvement of estrogen in early pregnancy was suggested. Together with steroid hormone receptor distribution, peripheral steroid hormone concentration must be measured for understanding relationship between them. Further, examinations of other factors which may involve in pregnancy such as interferon tau and the distribution of oxytocin receptor in uterus are needed for understanding the mechanism for successful conception in sika deer.

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Summary in Japanese

受胎日の遅延はシカ類の繁殖成功度に悪影響を与える。そのため、適切な時期に受胎し、妊娠を維持することは繁殖成功を高める重要な要因となる。そこで本研究では、受胎時期と妊娠維持の時期に注目した。ニホンジカにおいては、卵巣動態やホルモン濃度の変化などの基本的な繁殖生理学的情報が欠けているため、どのような生理的要因が受胎時期に影響を与えているかは不明である。そこで本研究では、受胎時期と妊娠維持の時期周辺の基礎的な生理状態および生理機構の特徴について検討した。

第1章では、非発情期から発情期への移行期における卵巣動態とホルモン濃度変化の特徴を明らかにすることを目的とした。8頭の飼育個体を用い、卵胞や黄体の動態、ステロイドホルモン濃度の変化を初回発情周期中とその前後の期間で比較した。発情開始以前から初回発情までは7個体を、初回発情から発情周期3～6回分の期間は3個体を実験に用いた。2～3日間隔で経直腸による超音波画像診断と血中プロジェステロンおよびエストラジオール17β濃度測定を行った。その結果、全ての個体において初回の発情以前に発情を伴わない排卵がみられた。また、それに続く一過性の黄体形成と、低濃度のプロジェステロン濃度の上昇が1回以上観察された。プロジェステロンは発情を引き起こす因子であるため、一過性のプロジェステロンによる感作は、発情期の開始に寄与していると推察された。また、発情周期中には主に2ないし3回の卵胞発育波が観察された。さらに、発情時には子宮頸管粘液の結晶化が観察され、発情の指標となることが示唆された。

第2章では、受胎時期と妊娠初期における卵巣動態とホルモン濃度変化の特徴を明らかにし、妊娠しているニホンジカにおいて特徴的に見られる複数の黄体の起源と形成時期を明らかにし、その存在意義を検討することを目的とした。6頭の飼育個体を用いて受胎から妊娠初期までの卵胞と黄体の動態およびステロイドホルモン濃度の変化を2～3日間隔で調べた。その結果、初回の発情で妊娠した個体3頭において2つの黄体が形成された。2つ目の黄

体は、発情に伴う排卵の後に出現した最初の卵胞発育波の主席卵胞が起源であり、これがエストロジェンの上昇と共に排卵し、黄体を形成した。一方、2回目の発情で妊娠した個体３頭では、発情後の主席卵胞は排卵せず、黄体は1つしか形成されなかった。そのため、2つ目の黄体は発情期初回の発情で受胎した場合に形成されることが示唆された。また、黄体の数によってプロジェステロン濃度に差が認められなかったため、2つ目の黄体は1つ目の黄体のみでは不十分なプロジェステロンの分泌を補助することで、初回発情での妊娠の維持に寄与していると推察された。

第3章では、妊娠初期における胎子の成長を明らかにし、正確な胎齢推定を可能にすることを目的とした。5頭の飼育個体を用い、妊娠59～61日まで2～3日間隔で経直腸超音波画像診断により胎子の成長を観察した。妊娠20～26日までに、全ての個体において胎子が確認された。胎子の直頭殿長、曲頭殿長、頭長、胸深、心拍数を計測した結果、両頭殿長の対数を取った値とその他の計測値は直線的な増加を示した。胎齢推定式を算出した結果、直頭殿長が最も早期から計測可能で、高い相関を示すことが明らかとなった。したがって、直頭殿長を計測することで、正確に妊娠初期の胎齢が推定できることが判明した。

第4章では、妊娠期間中の子宮におけるプロジェステロンとエストロジェンの作用部位の変化を推定することを目的とした。複数黄体を持つ24の死体から得られた子宮組織を用いて、発情直後から妊娠末期までのエストロジェン受容体α (ERα)とプロジェステロン受容体(PR)の発現を免疫組織化学的に調べた。胎齢は第3章の結果と既存の方法をもとに推定した。その結果、ERαおよびPRが妊娠25日まで確認され、他の家畜反芻動物と比較すると、受胎後も遅い時期まで発現していることが明らかとなった。したがって、ニホンジカでは妊娠25日程度まで、プロジェステロンのみならずエストロジェンが作用していることが示唆された。

本研究では、非発情期から発情期、受胎から妊娠初期までの卵胞や黄体の動態と血中ステロイドホルモン変化、さらに妊娠個体の子宮におけるステロイドホルモン受容体の分布

から、これまで情報の少なかったニホンジカの発情期への移行期、受胎時期や妊娠初期における繁殖生理学的特徴を明らかにした。また、妊娠初期の正確な胎齢推定の方法を確立したことにより、今後野外で得られたサンプルの有効活用を可能にした。