Journal of Reproduction and Development, Vol. 55, No. 2, 2009

—Full Paper—

Fetal Age Estimation of Hokkaido Sika Deer (*Cervus nippon yesoensis*) Using Ultrasonography During Early Pregnancy

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Abstract. 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, we concluded that a precise estimation of fetal age in early gestation is best performed using SCRL measurements.

Key words: Fetal age estimation, Fetal development, Hokkaido sika deer (*Cervus nippon yesoensis*), Ultrasonography

(**J. Reprod. Dev. 55:** 143–148, 2009)

Seasonal breeding is a common feature among cervid species in temperate regions. As they are short-day breeders, they mate in autumn [1, 2] and fawn in early summer [3] in Japan. However, a previous study [4] indicated that conception is delayed in young and old females and that lactating adults are likely to conceive later than non-lactating adults. It is also known that delayed conception results in delayed fawning [2]. Delayed fawning negatively influences calf survival and the female's reproductive success since it is difficult for a fawn born late to attain a sufficient nutritional condition to survive the winter due to food limitations and females that deliver late sometimes fail to conceive in the following mating season [5–10]. Furthermore, conception may be influenced by the conditions of the population and habitat since conception delayed in females in poor nutritional condition [11] resulting from poor food resources. Therefore, precise estimation of the conception date allows estimation of the population and habitat quality for this species. It also advances the physiological researches by using both live animals and carcasses obtained in the wild.

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 (*Cervus elaphus*) [12], fallow deer (*Dama dama*) [13, 14] and various other mammals [15]. Likewise, an equation is also available for estimation of fetal age from fetal body weight in Hokkaido sika deer (*Cervus nippon yesoensis*) [1]. 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 [16], sheep [17, 18], goats [19], muskoxen [20] and llamas and alpacas [21]. Ultrasonography has been used for pregnancy diagnosis and fetal measurement in a number of cervid species, such as red deer, fallow deer and reindeer (*Rangifer tarandus*) [22–29]. 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 [24, 27]. 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 intact adult stag at Asahiyama Zoological Park, Hokkaido, Japan (43.8 N, 142.5 E) during the rutting season in 2007. The females included

Accepted for publication: November 12, 2008
Published online in J-STAGE: December 24, 2008
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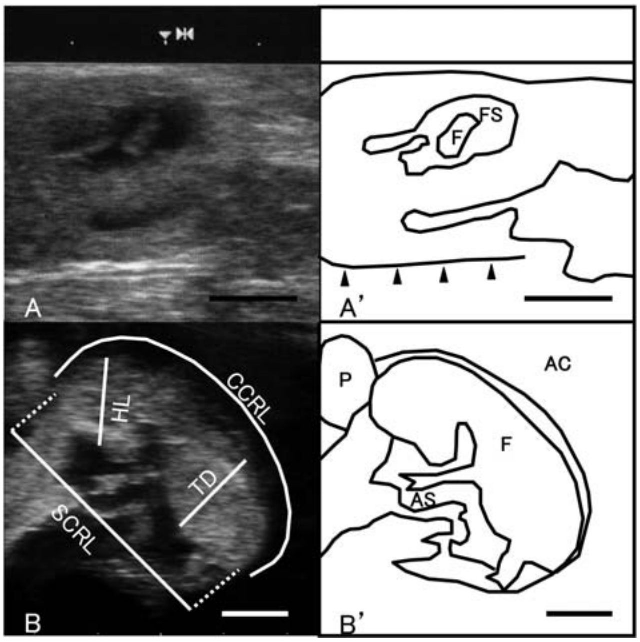

Fig. 1. (A) Ultrasonographic visualization and (A') illustration of a Hokkaido sika deer fetus, first visible on day 25 of gestation. (B) Ultrasonographic visualization and (B') illustration of a Hokkaido sika deer fetus on day 49 of gestation and fetal dimensions recorded from ultrasonograms. Arrowheads 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.

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. An average 1 kg of concentrated ruminant feed was supplied daily to each animal; they were also provided with hay and water *ad libitum*.

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).

Immobilization

The sika deer were immobilized for examination by intramuscular administration of an aqueous mixture containing xylazine-HCl (1 mg/kg; Celactal; Bayer Medical, Tokyo, Japan) and ketamine-HCl (3 mg/kg; Ketalar; Sankyo, Tokyo, Japan) or medetomidine-HCl (60 μg/kg; Domitor; Zenoaq Nippon Zenyaku Kogyo, Fukushima, Japan) and ketamine-HCl (3 mg/kg) in the rump or shoulder area with a blowpipe [30, 31]. 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).

Observation of fetal development

After securely immobilizing them, the sika deer were laid and examined in the recumbent position for fetal observation using ultrasonograph (Diagnostic scanner HS-1500V; Honda Electronics, Toyohashi, Japan) by insertion into the rectum of a linear transducer (HLV-375M; Honda Electronics) 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 finfused into the rectum before insertion. 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 [32], 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. 1B and 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 truncus.

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 [1] ($age=(\sqrt[3]{weight} + 2.73)/0.091$).

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

Table 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

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, Tokyo, Japan).

Results

Structural development

The chronological sequence of the ultrasonographic appearances of various fetal features and uterine structures are presented in Table 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 was 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. 1A and 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 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 2, Figs. 2A and 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 2). HL and TD increased linearly during the observation period; FHR increased linearly throughout the observation period (108–288 beats/min; Fig. 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 Fig. 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 under 40 days based on fetal weight, those of fetuses less than 0.044 g, converged shortly after day 30 (Fig. 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 [24, 27], 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

Table 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)

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

Ln=natural logarithm.

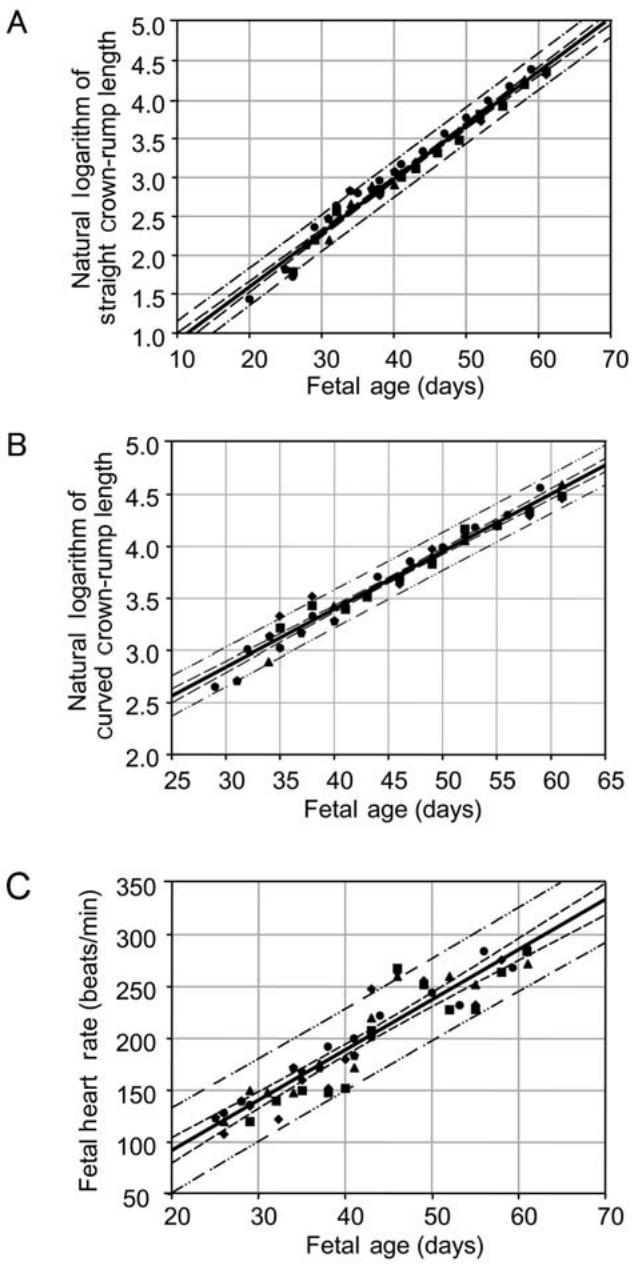

Fig. 2. Scatter diagram of data and linear regression for (A) the natural logarithm of the straight crown-rump length of the fetus, (B) natural logarithm of the curved crown-rump length of the fetus and (C) fetal heart rate against fetal age in Hokkaido sika deer. Data from the same individual but at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

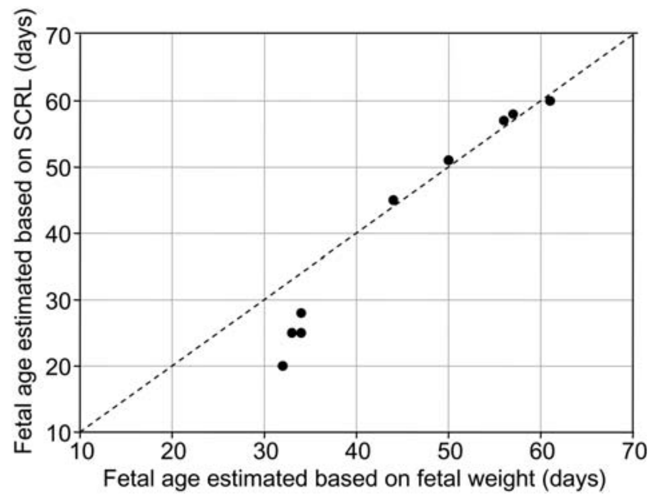

Fig. 3. Scatter diagram of fetal age estimated by SCRL against that estimated by fetal weight for Hokkaido sika deer collected in the wild with the line y=x.

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 [26]. 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 [26].

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 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 [33]. 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 [34]. 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 [34–36], it might not be appropriate for practical use.

The fetal age of sika deer is normally estimated using fetal body weight [1]. 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 [1] 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 [1].

In many studies of domestic animals, including cervid species [37, 38], bone length and the appearance of ossification centers have been used instead of estimations based on fetal weight to estimate fetal age more precisely [39–44]. Kobayashi *et al*. [45] 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 [46, 47]. In poor environments leading to substandard nutritional conditions, both fetal growth rates and birth weights are restricted [6, 48, 49]. 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 [38, 43], 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*. [1], Kobayashi *et al*. [45] 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*. [45], enables precise estimation of fetal age. The equation of Suzuki *et al*. [1] 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.

In the case of evaluating population and habitat quality by conception period, it is practical to use carcasses since many deer are shot in the field. Carcasses also provide valuable data in regard to reproductive physiology at specific gestational ages. To determine the conception dates of individuals, measuring the SCRL or fetal weight, dependent on the size of the fetus, is practical in the field since they are currently easy to measure and precise (though less precise in the case of fetal weight). For more precise population and habitat quality evaluation, further study using fetuses of more advanced age might be needed to extend the effective period of the equation for fetal age estimation based on SCRL. This study enables estimation of a precise conception period in early pregnancy, which reflects the population quality. Therefore, it is expected to be valuable for population dynamics monitoring, which is helpful in planning of appropriate management plans for sika deer.

Acknowledgements

We would like to thank A Ohsawa and S Nakahara for providing animals for this research. We are also grateful for the help of Dr. H Igota and Mr. J Igota for assistance in quarantine of animals and to the members of the Asahiyama Zoological Park for their contributions during the data collection. Finally, we are grateful to our colleagues at the Laboratory of Wildlife Biology in Hokkaido University for their encouragement. This work was supported by a grant from JSPS (no. KAKENHI19688013) and the “Inui Memorial Trust for Research on Animal Science.”

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