Evaluation of Fetal Growth and Estimation of Fetal Age Based on Skeletal Growth in Hokkaido Sika Deer (Cervus nippon yesoensis Heude, 1884)

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ABSTRACT: We investigated fetal development and the estimation of fetal age of 127 Hokkaido sika deer fetuses, categorizing them into three groups according to the nutritional condition of populations. The order and time of the appearance of ossification centers were clarified, and fetal age was determined based on bone length and the appearance of ossification centers. Then we observed the differences in fetal growth among three populations, and discussed the effect of poor nutrition on the fetal growth. The results suggest that fetal diaphyseal length of the femur was affected very little by nutritional conditions, whereas conception dates were delayed and fetal weight was restricted as the nutritional condition became poorer. Although it is impossible to know the exact accurate fetal age in wild populations, it was possible to create a standard to estimate fetal age more precisely by the method described in this study. Both the bone length and the appearance of ossification centers are reliable indices to estimate fetal age precisely in measurements available from fetuses of unknown age, and can be applied to estimate the fetal age of other populations of sika deer, whereas estimation of fetal age based on weight is prone to great errors.

KEY WORDS: conception date, fetal age, Hokkaido sika deer, poor nutritional condition, skeletal growth.

In the cervid species, conception dates and fetal development are greatly influenced by geographical and ecological factors such as latitude, climate, maternal age, population density, and the nutritional condition of the female [5, 7, 8, 19, 21, 26, 29]. Calving dates, which are directly dependent on the conception date, and growth in the fetal period influence fawn mortality, even later adult body size and their future reproductive success [1, 4, 7, 8, 22, 39]. It has been reported that ovulation, estrus or conception dates are delayed in poor nutritional conditions of the female, due to a poor environment [3, 29, 38, 39], and the growth rate of the fetus and the birth weight are restricted [8, 29]. Therefore the parameters of fetal growth are suggested to be useful indices to evaluate the status of populations and habitat [19], and it is important to verify the difference in fetal growth and conception dates among populations to investigate any difference in status.

To evaluate fetal growth and conception dates, it is necessary to estimate fetal age. In many wild populations, fetal age and conception dates have been estimated by fetal weight [2, 6, 13, 21, 33], including Hokkaido sika deer (Cervus nippon yesoensis Heude, 1884). Nevertheless, it is difficult to find a general method of fetal age estimation for wild populations if data on fetal weight are used because they vary greatly with the environment [8, 29]. To precisely estimate fetal age, from bone length and the appearance of ossification centers is more suitable than fetal weight, as done in many studies of domestic animals [10, 11, 20, 23–25, 28, 42, 43], because poor nutrition does not severely affect skeletal growth and development [40, 41].

In the present study, we clarified the order and time of the appearance of ossification centers, and determined fetal age based on bone length and the appearance of ossification centers in Hokkaido sika deer. In addition, we discussed the effect of poor nutrition on fetal growth by observing differences in the fetal growth of three populations.

MATERIALS AND METHODS

Study areas and samples: We used 127 fetuses, which were divided into three groups, A, B, and C, according to the nutritional condition of each population. Group A (70 fetuses) were collected in the Ashoro, Onbetsu and Shari Districts of eastern Hokkaido. These were extracted from females killed for pest control, research or in traffic accidents from 1990 to 1992. Dates of kill ranged from February to May, being equivalent to the middle to latter gestational term. In those years deer populations in this area were in excellent nutritional condition with abundant body fat [44–46], and there was a very high reproductive performance [17, 31, 32]. Group B (52 fetuses) were sampled from the Akan and Shiranuka Districts. These were extracted from females killed for pest control from 1998 to 1999. Dates of kill ranged from March to May (the middle to latter term of gestation). Group C (5 fetuses) were collected in the Akan District from winter-killed females in 1996. The estimated dates of death ranged from 15 April to 26 April. The mean kidney fat mass (KFM) of adult females in Groups A, B and C in April of each sampling year, repre-
sentative of indices for nutritional condition, were 77.8 g, 41.0 g, and 6.9 g, respectively [47, 48]. They were significantly different (p<0.001), therefore fetuses in Group A, B and C were categorized as high quality, poor nutritional condition and critical nutritional condition, respectively.

Fetuses had been preserved in 10% formalin, 10% neutral buffered formalin or ethyl alcohol.

Radiographic technique and measurement: The fetuses were photographed in the lateral position by means of soft x-ray equipment (Softex co., Ltd, Tokyo, Japan) to measure the diaphyseal length of the femur and observe ossification centers. They were laid in a plane parallel to the film, although their postures were changed during preservation. To easily lay fetuses parallel, the limbs were removed and then photographs of trunks and limbs were taken separately in large fetuses. Antero-posterior views of the radius were also taken when needed for anatomical clarification. The diaphyseal length of the femur was measured on the radiographs between the proximal and diaphyseal extremities along the middle of the shaft with a vernier micrometer accurate to 0.01 mm. It was difficult to take photographs of the neck parallel to the film because of the changed postures, so they were serially sectioned by means of a computed tomography scanner (Hitachi Medical, Tokyo, Japan) in a transverse plane with a thickness of 2 mm with no inter-slice gaps.

Estimation of fetal age and conception date: The fetal age of Group A was estimated on the basis of research by Wenhams et al. [42]. They reported that in the fetuses of some ungulates with a difference in size, weight and shape, the estimated diaphyseal length of the femur taken from the Gompertz regressions attained a given fraction, 1/4, 1/2 or 3/4 of their size at birth, at remarkably similar percentages of their respective gestational periods. In red deer, percentages of gestation required to reach 1/4, 1/2 and 3/4 of their size at birth were 51, 67 and 82 %, respectively [42]. Suzuki [30] reported the time when the three largest fetuses in Group A were sampled (May 23, 23 and 25) corresponded to the birth period of Hokkaido sika deer (from late May to early July, average mid-June) [14, 18], and they were verified as being nearly at full-term. So the mean value of their femur length was used as the full-term value, and we derived femur length at given percentages of 231 days, the median gestational period for sika deer [12]. Using these values, the Gompertz equation was fitted to relate the diaphyseal length of the femur to fetal age. The fetal age of Group A was estimated with this equation. To create a standard for estimation of fetal age and growth, we derived the equation to estimate fetal age by ossification scores of the fetuses in Group A. This equation was derived from data on males and females together, according to Wenhams et al. [42]. The Gompertz equation based on the diaphyseal length of the femur could not be applied to deer in Groups B and C because there were no full-term fetuses. To make sure that the equation to estimate fetal age by ossification scores the fetuses in Group A was able to be used for Groups B and C, the equation for the linear relationship between ossification scores and the diaphyseal length of the femur of each population were calculated, and we made sure that the time of the appearance of the ossification centers in relation to diaphyseal length of the femur did not change with poor nutritional condition. They were highly correlated in each (coefficient of determination of Group A, 0.943, B, 0.849, Group C, 0.874), and there were no significant differences among populations (ANCOVA: F=0.605, P=0.05). Therefore the time of the appearance of the ossification centers in relation to the diaphyseal length of the femur had not changed among populations, and thus the fetal ages of Groups B and C were then derived by using the equation for estimation of fetal age by ossification scores of the fetuses in Group A. Conception dates were calculated with the estimated fetal age to count back from the date of kill.

Growth curves: The growth of the diaphyseal length of the femur and weight were characterized by the Gompertz equation with SPSS 11.0 J for Windows (SPSS Japan Inc., Japan) from data on males and females together, according to Wenhams et al. [42]. The curve was \( y = a \left[ \exp \left( -e^{k(t-t_o)} \right) \right] \) (Gompertz equation), where \( y \) is the mean length (mm) or weight (g) at age \( t \), \( a \) is asymptotic length (mm) or weight (g), \( K \) is the growth rate constant (day\(^{-1}\)), \( t \) is the fetal age (days), and \( I \) is the age at the inflection point (days). We did not apply the Gompertz equation to fetuses in Group C because of the small number of subjects and the similarity in fetal age. Fetal weight was quoted from Suzuki [30] and Uno et al. [30, 35].

RESULTS

Observation of ossification and the relationship between ossification score and fetal age in Group A: We showed the fetal age at which each secondary and later developing primary ossification centers became radiographically detectable was recorded in the fetuses in Group A (Fig.1). In secondary centers, the dens of the axis and the spinous process of the fourth and fifth cervical vertebrae were seen at the start, here 101 days, therefore these centers would certainly have been present some days before. All of the secondary centers, except those early ones just noted, appeared after 101 days. Some of the primary centers, the atlas body, carpus, tarsus, and phalanges, appeared after 101 days. The ossification score, which was the total number of some of the primary ossification centers (the atlas body, carpus, tarsus and phalanges) and all the secondary centers, was linearly related to the fetal age of the fetuses in Group A. The regression equation is expressed as follows:

\[ y = 2.237x + 100.640 \quad (R^2 = 0.938) \]

\( x \) is the ossification score and \( y \) is fetal age (Fig. 2). Fetal ages of Group B and C were derived with this equation.

Variation in conception dates: Figure 3 shows the frequency distribution of conception dates in the three populations. The estimated conception dates of Group A ranged from October 4 to January 1 and occurred most frequently late in October. The estimated conception dates of Group B and C ranged from October 17 to November 28, and from
late in October to November 26, respectively. Comparing conception dates among populations, Group B was significantly later than those in Group A, Group C was significantly later than Groups A and B (Steel-Dwass test, p<0.05). The median conception dates of Groups A, B and C were October 28, November 3 and November 21, respectively.

**Growth curves:** Fitted curves for the two measurements are shown in Figs. 4 and 5. Diaphyseal lengths of the femur of the fetuses in Groups A and B at 231 days, the full-term of gestation, were 109.90 mm and 108.16 mm, respectively, and the percentage of the difference in values between populations against the values for the fetuses in Group A was 1.58%. The weight of fetuses in Groups A and B at 231 days were 5441.16 g and 4994.29 g, respectively, and the percentage of difference between the values of populations against the value for the fetuses in Group A was 9.87%.

In comparing the diaphyseal lengths of the femurs, Group C showed similar values to those of the other groups at the same fetal age. In contrast, the weight of fetuses in Group C tended to be smaller than those of the other groups.

**DISCUSSION**

**Observation of ossification centers and estimation of fetal age:** In this study, some ossification centers were found to have a variable appearance with fetal age. For example, the distal metacarpal epiphyses were first seen at 166 days, but were undetected in other fetuses at 187 days, but other centers such as the spinous process of the second, third and sixth cervical vertebrae were seen at 106 days and were always present from then on. Wenham et al. [40] reported similar variations in the appearances of ossification centers in fetal sheep of known age; so the variations in this study can be considered normal. Figure 1 can be used as an easy method to determine fetal age between 101 and 231 days. For example, if the second and fifth distal but not the second and fifth proximal phalanges of a forelimb appeared in a fetus, its fetal age would probably be between 106 and 118 days.

**The differences in conception date and fetal growth between populations:** The conception dates of Groups B and C (Akan population) were significantly later than those of Group A. The poor nutritional condition in the Akan area caused a delay in the conception date; delays in the conception date from poor nutrition have been reported in many animals [3, 29, 38, 39]. The conception date of Group C was significantly later than those of Group B as a result of the difference in nutritional condition between Groups B and C. It was reported that poor nutrition brought on the delay in the birth date resulting from delay in the conception date [27, 29]. In addition, the delay in the birth date increased the over-winter calf mortality at high population density [8]. Actually in 1995/96, when Group C was sampled, many fawns and adult females died in the severe Akan winter. But in 1996/97, many fawns (n=84) died in spite of the weather being milder than in the 1995/96 winter [37]. This happened in the Akan area, the same as in the above studies [8, 27, 29]. Poor nutrition in the fetal period and delay of the birth date resulting from delay in the conception date in 1995/96 resulted in insufficient growth and storage of body fat for wintering after birth. Thus there was a high over-winter fawn mortality in 1996/97.

There was a small difference in the diaphyseal length of the femur between Groups A and B at 231 days and between Group C and the other groups (Fig. 4). These results suggest that the effect of poor nutrition on the diaphyseal length of the femur was small, but other bone lengths were not analyzed in this study. Comparison of lamb fetuses of the same age but in different nutritional condition showed that the difference in femur length was greater than many other bone lengths [41], so the effect of poor nutrition on other bone lengths was also considered to be small.

On the other hand, there was a great difference in fetal weight between Groups A and B at 231 days and between Group C and the other groups (Fig. 5). The poor nutritional condition severely affected fetal weight, especially in winter-killed fetuses. In the Gompertz regression curve of fetal weight, some of the values for Group B were larger than for Group A (Fig.5); this probably was due to the lack of samples for Group A from day 190 to day 227.

From these results, it was verified that poor nutritional condition restricted fetal weight more severely than skeletal growth in wild Hokkaido sika deer. In a wild population, fetal weight has been reported to be severely affected by habitat [8, 29], but the effect on skeletal growth has not been evidenced before. This study clarifies that the effect of restriction of fetal skeletal growth is small whereas the restriction of fetal weight is great, as suggested in domestic ewes by Wenham [41]. Everitt [9] stated that under severe nutritional stress the ovine fetus attempts to augment its nutrient supply by compensatory development of placental components. Whether this is responsible for the effect on the bones is not known, but there are considered to be some differences between the degrees of compensatory development of placental components to fetal weight and bone growth.

The Akan-Shiranuka area is a major wintering area for sika deer [34], and in winter the population density was high (20–90 deer/km²) and the quality and the availability of food resources were low in those years [15, 16, 36]. In particular, in the very severe winter of 1995/96 when the fetuses in Group C were sampled there was a longer snow duration than in normal years [37]. The availability of food resources decreased and many fawns and adult females died in 1996/97. It was considered that a change for the worse of nutritional conditions might be due to a decrease in the availability of food resources depending on a change for the worse of habitat, and poor habitat might bring a delay in the conception date and restriction of fetal weight.

The distribution of conception dates of Group A with Suzuki et al. [33] was significantly different from those of this study (Mann-Whitney’s U-test, p<0.01), and the median conception date (October 25) was earlier. This is considered to be because of the difference in the equation for fetal age
(a) Axial skeleton

- Ages at the center are present in some fetuses but not in all
- Ages at the center are invariably seen

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FETAL GROWTH IN SIKA DEER

(c) Hindlimb

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Fig. 1. Age of appearance of ossification centers in (a) the axial skeleton, (b) the forelimb, and (c) the hindlimb of fetuses in Group A. Ages at the center are present in some fetuses but not in all; ■, ages when the center is invariably seen.

![Graph](image1)

Fig. 2. Relationship between the ossification score and fetal age. 
\[ y=2.237x + 100.640, R^2=0.938. \]

Fig. 3. Frequency distribution of conception dates. [ ], Group A (n=70); [■], Group B (n=52); [●], Group C (n=5).

In the method using weight in Suzuki et al. [33], fetal ages were estimated as older than those of this study using bone length in all fetuses up to 4,400 g. Therefore their conception dates were earlier than those of this study.

We could not analysis the relationship between the conception date and maternal age because of the small number of fetuses at each maternal age, and it is subject for future study to investigate the effect on conception and fetal growth of maternal age.
Utility of the method of estimating fetal age based on skeletal growth: Although it is impossible to know exact fetal age in wild populations, it was possible to create a standard to estimate fetal age more precisely by utilizing the method described in this study. The method of estimating fetal age by bone length is reasonably precise and relatively easy, but cannot be used unless the value at full-term is known. The appearance of ossification centers is also effective in estimating fetal age, and can be applied even if the value at full-term is not available. Nevertheless, both the bone length and the appearance of ossification centers can be said to be reliable indices available from fetuses of unknown age to estimate fetal age as a quantitative and qualitative measurement, respectively. The estimation of fetal age based on weight has a wide margin of error, whereas the method used in this study based on skeletal growth was able to estimate fetal age with little error, and furthermore it should be applicable to other sika deer populations.

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