



Ultrasonographic examination of equine fetal growth parameters throughout gestation in pony for Equine-Assisted Therapy

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ABSTRACT. Equine-Assisted Therapy (EAT) is gaining popularity. Ultrasound examination is used to decrease the abortion rate in horses. In this study, to monitor fetal well-being throughout the gestation for EAT, we measured fetal heart rate (FHR), fetal eye orbit (FEO), fetal gonad length (FGL), fetal kidney length (FKL), and the combined thickness of the uterus and placenta (CTUP) by ultrasonography in pony mares. Additionally, we measured the plasma progesterone (P_4) and estradiol (E_2) concentrations in pregnant horses using enzyme immunoassay. The FGL peaked at week 32 and then decreased to term, and a strong correlation ($r=0.72$, $P<0.001$) between the FGL and E_2 concentration was observed. A strong correlation with gestational age was detected among the FEO ($r=0.96$, $P<0.001$), FKL ($r=0.85$, $P<0.001$), and CTUP ($r=0.96$, $P<0.001$). The P_4 concentration peaked at week 10, decreased to low levels (below 5 ng/ml), and peaked before parturition. In conclusion, this study provides information on fetal growth throughout gestation in pony mares for EAT. In addition, it revealed the relationship between ultrasonographic profile and plasma hormone concentrations during gestation.

KEY WORDS: fetal growth, Hokkaido native pony, plasma hormone, ultrasound

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Interest in Equine-Assisted Therapy (EAT) and therapeutic horse riding, known as hippotherapy, representing an innovative technique, has been gaining popularity. In the 1950s, EAT was popular in Germany, Austria, and Switzerland; in Japan, interest in EAT has been increasing. As an animal-assisted therapy, EAT has the following benefits: promotes postural control, maintains coordination or balance in children with cerebral palsy [5, 38, 44] or multiple sclerosis [7], and builds the mobility and strength in elderly [3], by utilizing the movement of the horse. In addition, EAT has beneficial effects in psychological therapy, such as rehabilitation for schizophrenia, autism spectrum disorder [6, 20, 42], personality disorders, depression, post-traumatic stress disorder, and receptive or expressive language disorders [36]. The horses chosen for EAT must be calm, even-tempered, gentle, serviceably sound in characteristics, and well-trained both under a saddle and on the ground [36]. Furthermore, horses of height 125–140 cm is considered suitable for riding by children and disabled individuals; however, there is a lack of horses of this height in Japan. Therefore, it is essential to establish complete and efficient standardized criteria for the production of horses suitable for EAT.

Abortion is one of the major causes of low production rate of pony. As one of the effective approaches for the early diagnosis of abortion, ultrasound examination of pregnant mares for equine fetus biophysical profiling was developed by Reef *et al.* [31] in 1996. Fetal heart rate (FHR), aortic diameter, fetal activity level, uteroplacental thickness, uteroplacental contact, and maximal fetal fluid depth were selected to predict the outcome of fetal health. Bucca *et al.* [8] established the most comprehensive biophysical profile of equine fetal well-being from mid-gestation to term in 2005. Murase *et al.* [26] revealed fetal growth throughout gestation in Thoroughbreds in 2014. Vincze *et al.* [41] established a new, rapid examination protocol to evaluate fetal well-being in the clinical field and selected the three most accurate and practical indicators, namely, FHR, fetal aortic diameter, and the combined thickness of the uteroplacental unit (CTUP).

Meanwhile, hormone measurement in pregnant mares is another approach to monitor fetal well-being. In Thoroughbreds,

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Shikichi *et al.* [35] established a cut-off value for detecting an abnormal pregnant mare using maternal plasma hormone concentrations. Additionally, ultrasound monitoring of fetal health and hormonal investigations during the perinatal period can effectively reduce abortion risk and help veterinarians make correct decisions [25].

Therefore, the aims of this study were to (1) establish the biophysical profile of fetus for EAT and the maternal plasma hormone concentrations of pony mares throughout gestation and (2) investigate the correlation between fetal ultrasonographic parameters and maternal plasma hormone concentration.

MATERIALS AND METHODS

Animals

The Animal Experiment Committee of the Obihiro University of Agriculture and Veterinary Medicine, Japan, approved the study. Seven pregnancies (of five mares) were examined from 2018 to 2020. Three of the mares were Hokkaido native ponies and two were crosses between the Hokkaido native pony and Haflinger. The mean age of the mares was 8 ± 2 years (mean \pm SD). All mares were considered normal by reproduction examination, with 341 ± 8 (range 332–359) days of gestational age (GA).

Mares were managed in the paddock of Obihiro University of Agriculture and Veterinary Medicine. In summer and autumn, the mares could obtain fresh forage on the pasture during the day and were supplied 5 kg of hay in a paddock during the night; in winter, the mares were fed 10 kg of hay daily. There were water fountains in the paddock where the mares could drink *ad libitum*. All mares were fed alfalfa hay cubes and combined feeds (Stamm 30; Hallway Feeds, Lexington, KY, USA) daily throughout the year. The body condition score of all mares was approximately six during pregnancy.

Pregnancy in all mares, as recipients, were induced using the embryo transfer technique. In this study, six donors were used. One of the donors, a Hokkaido native pony, was not used as the recipient, the other donors (including 2 crossed breed mares) were used as recipients in this study. The embryos were collected from the donors artificially inseminated with the semen from a Connemara pony. The embryos were randomly transferred to the recipients.

Ultrasound examination

Ultrasound examination was performed both transrectally and transabdominally using a 7.5-MHz linear transducer and a 5-MHz convex transducer (Noblus, Hitachi Aloka Medical, Tokyo, Japan), respectively. For transrectal examination, the transducer was covered with a plastic glove, and ultrasonic coupling gel was used to isolate the air. The operator removed as much feces as possible from the rectum, placed the transducer inside the rectum, and scanned the fetus, uterus, and placenta. Transabdominal examination required abdominal hair shaving of the examined horse. The operator first sprayed alcohol on the examined area, spread the echo jelly in front of the transducer, and scanned the fetus from the mare's mammary gland to the xiphoid process of the sternum along with the abdominal midline.

The examinations were started on day 15 of gestation after confirming the presence of the embryo. Thereafter, the examination was performed weekly. The ultrasound examination was performed in the travis, and each examination event lasted less than 30 min for a mare. No sedatives were administered during the examinations.

All ultrasound examinations were performed by at least two operators, one operator to scan the fetus with the transducer and the other to operate the ultrasound device. The data were stored in an ultrasound device. After the examination, the images were transferred to a computer using a USB device and recorded for the subsequent data analysis.

Fetal growth parameters

Transrectal examination was performed to measure the fetal eye orbits (FEO). The transducer was swept from left to right over the uterus. When the transducer was passed over, the eye image was frozen, and the length of the vitreous body was measured (Fig. 1A) as previously reported [39].

The CTUP was recorded from the ventral aspect of the uterine body using a linear transducer to locate cranially at the cervical–placental junction [33]. Three measurements were taken at each examination, and the mean value was calculated (Fig. 1B).

Fetal gonad length (FGL) was measured using the transrectal and transabdominal methods. The transrectal examination followed the FEO method. For transabdominal examination, the transducer was placed and moved from the mare's udder along the midline of the abdomen. To confirm the fetal position, the fetal ribs are usually used as a landmark of the fetal thorax; the hypoechoic outline of the fetal stomach is a landmark of the fetal abdominal cavity [40]. The fetal gonads were scanned as an oval-shaped homogeneous echo image of the fetal abdominal cavity. The length of the fetal gonads was measured (Fig. 1C).

Fetal kidney length (FKL) was measured only by transabdominal examination. The examination followed the fetal anatomy, with fetal kidneys located dorsally to the fetal gonads. The right kidney is heart-shaped and the left kidney is bean-shaped [8]. The length of the fetal kidneys was measured (Fig. 1D).

The FHR was measured in the power Doppler mode. After confirming the fetal position, the thorax of the fetus was scanned to locate the cardiac region through the fetal ribs, used as a landmark (Fig. 1E). The measurements were taken at least three times when both fetuses and the mares were calm. To reduce the variance caused by fetal movement, the lowest measurement was recorded.

Detection rate

We determined the detection rates for specific organs. When the number of indices that could be measured divided by the total number at each time point was over 50%, the detection was considered positive as previously reported [26].

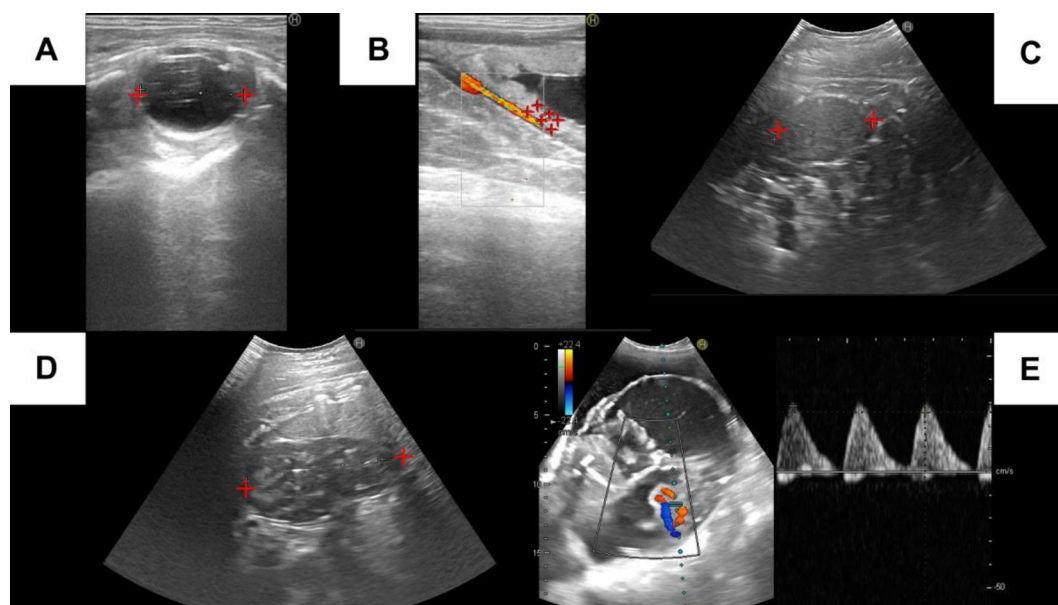


Fig. 1. Measurement of ultrasonographic indices of a pregnant mare. (A) Transrectal image of the fetal eye orbit (FEO). Eye length measurements (+) are shown. Eye length was measured from the maximum length of the inner margin of the vitreous body. (B) Transrectal images of the ventral part of the uterine body, near the cervix. Headers show the combined thickness of the uterus and placenta (CTUP). (C) Transrectal image of the fetal gonad. Fetal gonad length (FGL) measurements (+) are shown. (D) Transabdominal image of the fetal abdomen at the level of the kidney. Fetal kidney length (FKL) measurements (+) are shown. (E) Transrectal image of the fetal heart rate (FHR). Fetal heart rate measurement at the umbilical cord is shown.

Blood sampling and hormone measurement

Blood samples of mares were collected weekly from the day when embryo transfer was performed until foal birth. The blood samples were collected from the jugular vein of pregnant mares into a 10 ml vacuum heparin tube. The blood samples were collected and stored on ice until transfer to the laboratory within 1 hr. The blood samples were centrifuged at 1,500 g for 15 min. Plasma samples were collected instantly for hormone level determination. The remaining plasma samples were stored at -20°C in a freezer for future measurements.

The concentrations of estradiol (E_2) (ST AIA-PACK iE2; Tosoh Bioscience, Inc., San Francisco, CA, USA) and progesterone (P_4) (ST AIA-PACK PROG III; Tosoh Bioscience, Inc.) in the plasma were measured using the enzyme immunoassay (AIA360, Tosoh Bioscience, Tokyo, Japan). The intra- and inter-assay coefficients of variation were under 15%. The specificity of the P_4 kit showed that it has 0.26% cross-reactivity with pregnenolone.

Statistics

Results are expressed as mean \pm standard error of the mean (SEM). Data were analyzed using the one-way ANOVA followed by Tukey's HSD test for multiple comparisons, Pearson's product-moment correlation for correlation analysis, and regression analysis for creating regression curve formulations. Statistical analyses were performed using RStudio ver. 1.4.1103. Significance was set at $P < 0.05$.

RESULTS

The FEO was identified at week 10 of gestation (Fig. 2A). It was scanned and measured only with a linear transducer until term. The FEO displayed a strong correlation ($r=0.96$, $P < 0.001$) with GA and grew from 2.7 mm to 36.1 ± 1.8 mm from week 10 of gestation to term. The optimal growth curve for the fetal orbit was $y = -0.0185x^2 + 1.8479x - 12.2512$, with $r^2=0.98$.

The CTUP was measured from week 13 of gestation until term (Fig. 2B). The CTUP demonstrated a strong correlation ($r=0.96$, $P < 0.001$) with GA and increased from 2.7 mm to 8.6 ± 0.2 mm. No significant difference was observed between weeks 15 and 32 of gestation. The linear regression formula for CTUP was $y = 0.1428x + 0.2129$, with $r^2=0.92$.

The FGL was scanned from week 14 of gestation (Fig. 2C), peaked at week 32 of gestation, and was maintained until week 37 of gestation. The FGL began to decrease with GA from week 37. The detection rate of the FGL decreased during the same period. Transrectal and transabdominal methods were used depending on the fetal position until week 35 of gestation. After week 35 of gestation, only the transabdominal method was used to find the fetal gonads.

FKL was only detected by abdominal ultrasound from week 30 of gestation until term (Fig. 2D). FKL had a strong correlation ($r=0.85$, $P < 0.001$) with the GA. No significant difference in the FKL was observed from week 42 of gestation to the term. The FKL

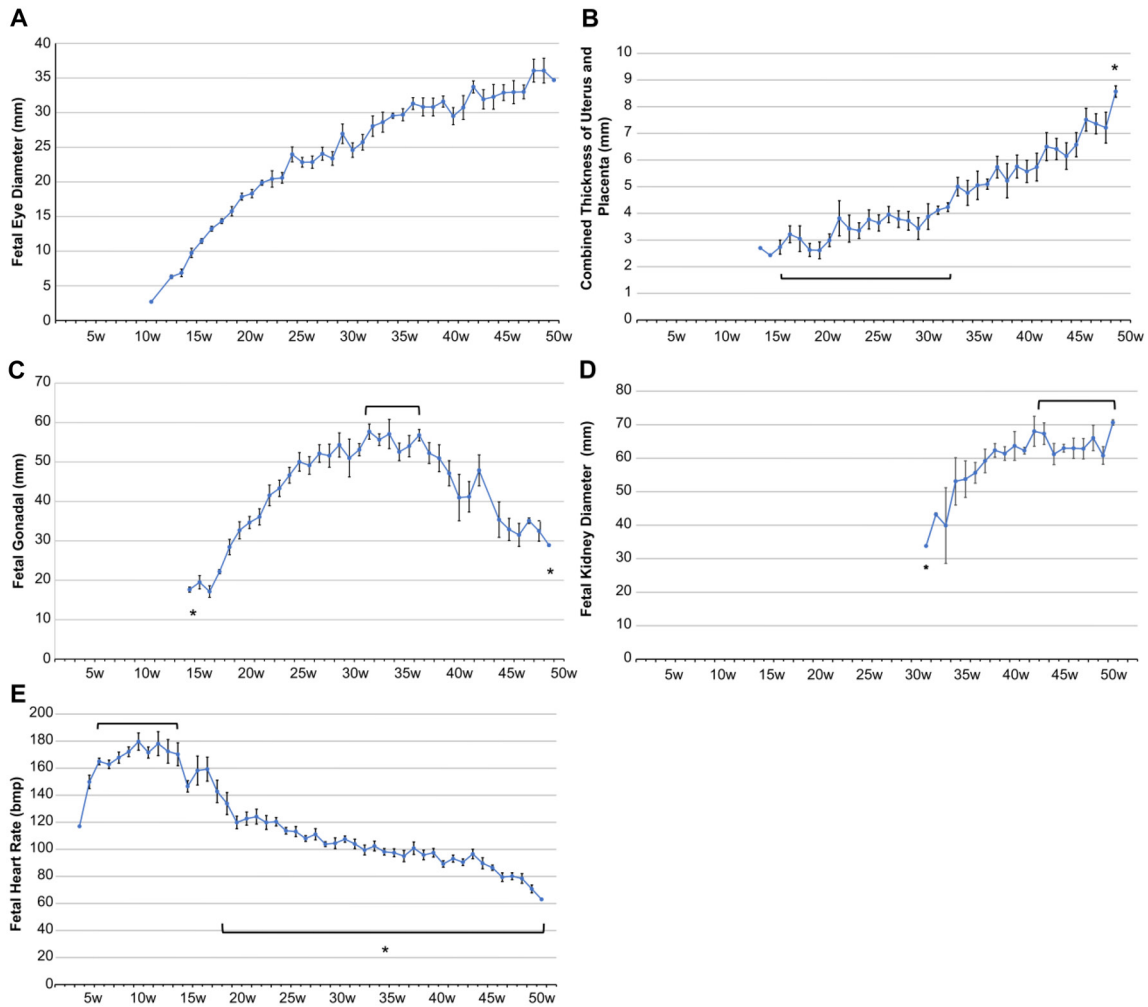


Fig. 2. Changes in FEO (A), CTUP (B), FGL (C), FKL (D), and FHR (E) measured in pregnant mares throughout gestation. The x-axis represents the gestation week. Asterisks show significant differences in CTUP (B) when week 49 was compared with 16–32 weeks, in FGL (C) when week 14 and week 50 were compared with 32–37 weeks, in FKL (D) when week 30 was compared with 41–49 weeks, and in FHR (E) when 19–50 weeks were compared with 5–13 weeks. The error bars represent standard error of the mean (SEM).

increased from 33.8 to 70.7 mm from week 30 of gestation to term. The detection rate of FKL increased with the GA.

The FHR could be measured from day 35 of gestation (Fig. 2E), peaked at week 9 of gestation, at 179.7 ± 6.4 bpm, and was maintained until week 11 of gestation. The FHR declined from week 11 until term. The mean FHR was 70.8 ± 2.9 bpm.

The detection rate of each organ was calculated (Fig. 3). The percentage of positive detection was 85% throughout the gestation period. When the fetal position was dorsoventral or dorsosacral, the fetal gonads and kidneys were difficult to observe.

The concentration of P_4 was maintained at 11 ± 0.9 ng/ml during the first 5 weeks of gestation, and, from week 6, the P_4 concentration started to increase until it peaked at week 10 remained constant until week 14 of gestation. From week 15 of gestation, the concentration of P_4 decreased; from week 23 to week 30, the P_4 concentration remained below 10 ng/ml, and, from week 30 to 45 of gestation, the P_4 concentration remained below 5 ng/ml. The P_4 concentration began to rapidly increase from week 46 of gestation, reached a second peak before term, and then rapidly decreased to less than 1 ng/ml after delivery (Fig. 4).

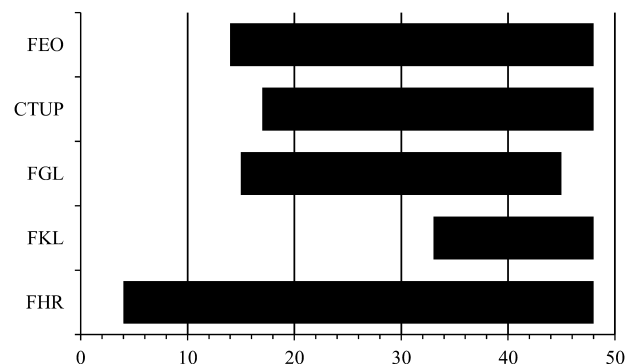


Fig. 3. Detectable periods for each index in pregnant mares. Bars show the detection rates are over 50%. The x-axis represents the gestation week.

E₂ was at a low concentration (<50 pg/ml) until week 20 of gestation. Thereafter, it started to increase by week 39 of gestation; the concentration peaked to 246.6 ± 24.7 pg/ml. At week 44 of gestation, the E₂ concentration began to decline, with a temporary increase at week 49 of gestation; it subsequently declined to 104.6 ± 11.9 pg/ml before delivery. The E₂ concentration decreased to 53.2 ± 6.9 pg/ml immediately following delivery. Additionally, a strong correlation (r=0.72, P<0.001) between the E₂ concentration and FGL was observed (Fig. 5).

DISCUSSION

In the present study, we evaluated the fetal growth parameters FHR, FEO, FGL, FKL, and plasma hormone concentrations in pregnant mares throughout gestation. To the best of our knowledge, this is the first study to investigating fetal well-being

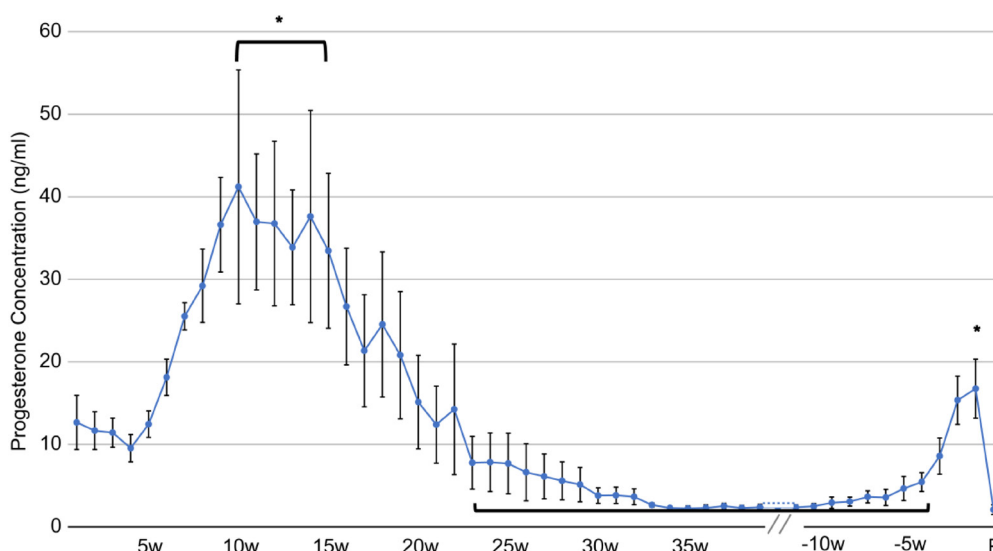


Fig. 4. Maternal circulating progesterone concentrations. Asterisk indicates the concentrations at 10–14 weeks were significantly different from those at 23–39 weeks and the concentrations at –10– –4 weeks and –1 week were significantly different from those at 23–39 weeks and –10– –4 weeks. P is the day after the parturition. The error bars represent standard error of the mean. // separates the figure into two parts. The left part is the progesterone concentration starting from week 0 of gestation. The right part is the progesterone concentration setting the parturition as week 0.

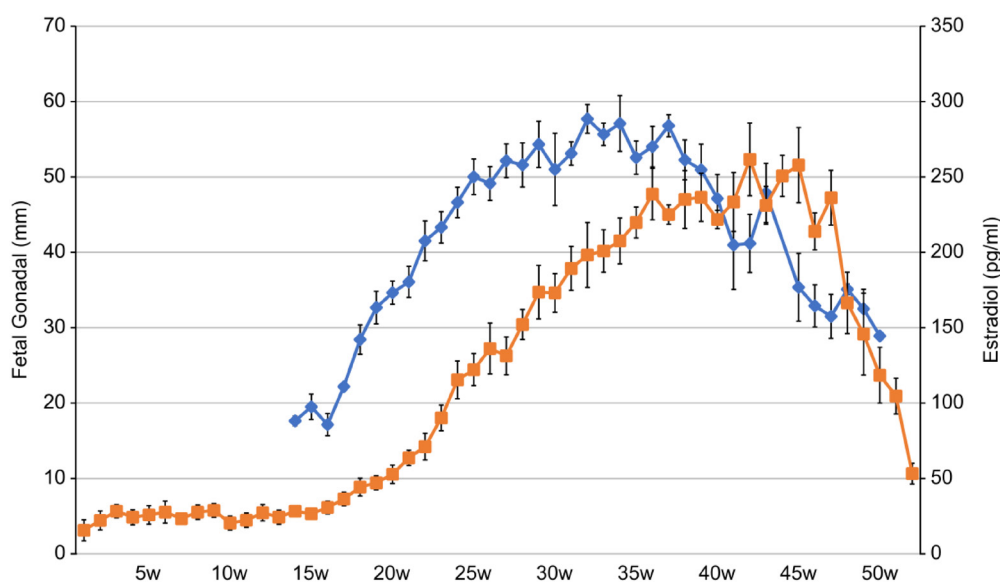


Fig. 5. Maternal circulating estradiol concentrations (■) and fetal gonad length (◆). The x-axis represents the gestation week. The error bars represent standard error of the mean.

parameters and maternal plasma hormone concentrations in pony mares throughout whole gestational period. The findings of this study provide a reference for typical fetal growth values, which can also be used as a tool for the diagnosis of abnormal pregnancy in pony mares. The current research contributes to the production of pony for EAT and provides pregnancy information of pony mares.

Fetal gonad enlargement during pregnancy is a unique feature of horses [11, 17]. The fetal gonads are involved in placental steroid hormone production [23]. In this study, we found a strong correlation between the FGL and E_2 concentration. Because of hypertrophy and hypoplasia of interstitial cells, the gonadal enlargement peak around day 250 of gestation [1, 17]. The size of the fetal gonads can exceed the size of the maternal ovaries [11, 43]. In the present study, gonadal enlargement was observed through gestation. The FGL peaked from weeks 32 to 37 of gestation, at approximately 57 mm. The finding confirmed the enlargement of the gonads during gestation in pony mares like Hokkaido native pony. Pashen *et al.* [30] reported that, after fetal gonadectomy, maternal plasma estrogen concentration decreased. Additionally, Allen *et al.* [2] investigated the estrogen concentration in large Thoroughbreds and small ponies subjected to between-breed embryo transfer. They found that the estrogen concentration correlated with the size of the gonads rather than the size of the placenta or volume of maternal blood. These studies demonstrated that fetal gonads play an important role in estrogen synthesis. In the present study, to confirm the relationship between the fetal gonads and plasma E_2 concentration, the plasma E_2 concentration and ultrasonic profiles of FGL were investigated. We found a strong correlation ($r=0.72$, $P<0.001$) between the FGL and E_2 concentrations in maternal plasma. In addition, we found that the FGL was the maximum at week 32 and was maintained until week 37 of gestation. Maternal estrogen concentration was the maximum at week 39 and was maintained until week 44 of gestation. Notably, the FGL was the highest 6 weeks before the maternal estrogen peak. This interval may be required for the aromatization at the fetoplacental unit of the precursors from the gonads.

Bacterial infection is one of the factors causing abortion and reducing the fertility rate of horses [18, 21, 27, 37]. Ascending placentitis is the most common cause of abortion during the perinatal period. Studies have investigated the optimal premature diagnostic indicator of ascending placentitis [4, 22]. Among them, the CTUP is considered a useful indicator. Although ascending placentitis cannot be diagnosed using the CTUP alone, the CTUP has still been used to monitor fetal well-being. Bucca *et al.* [8] reported methods through which the CTUP can be detected both transrectally and transabdominally. However, placental thickness measurements may vary depending on the assessment area, and the thickness at the cervical star increases in the early stage of ascending placentitis. Therefore, the transrectal CTUP values are preferred for the diagnosis of ascending placentitis. In the present study, the CTUP was only measured transrectally using a linear transducer.

The range of typical CTUP values varies among different breeds of horses. In the present study, the CTUP was between 6 and 9 mm in the last 2 months of gestation. These values are lower than the CTUP reported by Kimura *et al.* [19] in heavy draft horses (approximately 13 mm) and Spanish Purebred mares (10.45 mm) on day 300 of gestation [34]. The CTUP values observed in the present study were close to those in Thoroughbred horses (range 7–10 mm on day 330 of gestation) [12, 33]. Therefore, investigation of the normal range of CTUP at gestation for different breeds of horses can help determine placental abnormalities.

Fetoplacental steroid hormones [10], such as P_4 and E_2 , are considered potential biomarkers for the diagnosis of placentitis, especially during the last third of the gestation period [10, 29]. The concentration of P_4 remained low (under 10 ng/ml) from 7 to 10 months during gestation. In the present study, all mares maintained a low concentration of P_4 during this period without any abnormalities. Shikichi *et al.* [35] reported that the serum progesterin concentration in the abnormal pregnancy group was higher than that in the normal group. In the present study, the concentration of E_2 was maintained at a high level, over 200 pg/ml, from months 8 to 11 of gestation. Compared to P_4 , E_2 has a long half-life in the maternal circulation. Despite this, the concentration of E_2 was thought to possess diagnostic value for fetal loss in the late gestation [13]. Therefore, from months 8 to 11 of gestation, veterinarians often combine clinical symptoms and CTUP and maternal plasma P_4 and E_2 concentrations as criteria to diagnose ascending placentitis in mares [35].

As in the human fetus, the present study results demonstrated a single increase in FHR in the first 3 months during gestation, followed by a decrease until delivery. Ginther [14] and Murase [26] *et al.* demonstrated the same pattern in the first 3 months of gestation for FHR. Under physiological conditions, the FHR of an equine fetus is accelerated by fetal movement; additionally, the FHR is reportedly inhibited by the effects of sedatives, such as detomidine [24]. In pathologic conditions, the FHR is an essential indicator of fetal well-being. Persistent fetal tachycardia, bradycardia, and cardiac arrhythmias are common predictors of fetal abortion or fetal distress [31]. In the current study, no sedative was used in ultrasound examinations, and all results were displayed in the normal pregnant mare. Abnormal FHR was not observed during this study.

In this study, the FEO measurements strongly correlated ($r=0.96$, $P<0.001$) with GA, consistent with the results reported by Turner *et al.* [39] and Renaudin *et al.* [32]. This finding illustrates that the FEO is a valuable indicator for predicting GA when examining pregnant mares with an unknown GA.

In the present study, we also calculated the regression formula for FEO with GA to find the best formula to predict GA. Compared to the linear formula ($y=0.7235x + 2.3565$, $r^2=0.91$), the quadratic equation ($r^2=0.98$) is more descriptive of the FEO growth curve. This trend in FEO growth was also reported by Turner *et al.* [39], and not in the model presented by Renaudin [32]. This result most likely indicates that the fetal orbital growth follows a linear pattern during the early stages of gestation, slows down during the last stages of gestation, and the orbits are fully developed and no longer growing near the delivery date.

We found that the CTUP has a strong correlation ($r=0.96$, $P<0.001$) with GA, demonstrating that the CTUP also has good potential to predict GA in the normal pregnant mares. Conversely, similar to previous studies [26, 34], even the CTUP displayed a strong correlation with GA; the CTUP did not rise significantly in the first trimester and only began to rise significantly from day 270 of gestation. In the present study, we did not observe a significant difference in the CTUP until week 32 of gestation. Because

the curves of FEO and CTUP were different, the FEO and CTUP are suitable for GA prediction in the early and late gestation stages, respectively.

The FKL is considered another suitable parameter to predict GA. It demonstrated a strong correlation with GA in studies conducted by Murase *et al.* [26] and Ferrer *et al.* [16]. In the present study, we observed a strong correlation of FKL with GA. However, no difference was observed from week 42 to the term, demonstrating that the fetal kidney had already developed in Hokkaido native pony during this period. Fetal kidney monitoring still has clinical value for diagnosing fetal abnormalities, such as the prenatal diagnosis of megacystitis and hydronephrosis [15].

Fetal birth weight prediction in horses has rarely been made. Turner *et al.* [33] attempted to determine the relationship between the fetal eye and birth weight. However, they did not find a correlation. Additionally, we did not observe a correlation ($r=0.10$) between fetal birth weight and FEO before delivery in this study. Compared to the Thoroughbred's eye orbital length reported by Murase *et al.* [26], there was no significant difference in the orbital length before delivery in Hokkaido native pony, which indicate that the FEO is not a major determinant of fetal birth weight.

In Japan, height at withers, girth circumference, and cannon bone circumference have been used to predict horse weight as a part of previous horse feeding management [28]. However, for fetuses, these parameters are difficult to measure. A study on baboon fetuses [9] demonstrated that biparietal diameter, fetal femur length, and other indicators are strongly correlated to fetal development. However, no single indicator can be a good predictor of fetal birth weight. This observation implied that the prediction of fetal birth weight usually requires multiple indicators to be measured simultaneously.

In the present study, the fetal ultrasound and hormone profile throughout whole gestational period in a normal pregnancy of pony mares suitable for EAT was demonstrated. Furthermore, a relationship between FGL and E_2 concentrations was revealed. In conclusion, the parameters used in the present study are helpful for fetal growth monitoring. In the future, the findings of the present study can be used to monitor fetus to enhance fertility of pony mares for EAT. Furthermore, these findings can be applied to other breed of pregnant mares to ensure the normal progression of pregnancy.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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