Elsevier Editorial System(tm) for Theriogenology
Manuscript Draft

Manuscript Number: THERIO-D-18-00243R2

Title: FIELD ULTRASOUND EVALUATION OF SOME GESTATIONAL PARAMETERS IN JENNIES

Article Type: Original Research Article

Keywords: Jenny; Donkey; Pregnancy; Ultrasonography; Fetus; Embryo

Corresponding Author: Dr. Alessia Bertero, Ph.D

Corresponding Author's Institution: University of Milan

First Author: Tiziana Nervo, Ph.D

Order of Authors: Tiziana Nervo, Ph.D; Alessia Bertero, Ph.D; Mariagrazia Poletto, Dr.; Paola Pregel, Ph.D; Roberta Leone, Dr.; Valentina Toffoli, Dr.; Leila Vincenti, Ph.D

Abstract: The aim of this study was to collect and analyze ultrasound measurements of fetal-maternal structures during normal and pathological pregnancies in jennies, a livestock species of growing interest. For two breeding seasons, 38 jennies of different breeds and crossbreeds aged between 3 and 18 years were monitored weekly by transrectal examination using a portable Esaote ultrasound (MyLab™ 30 GOLD VET) with a 5-7.5 MHz probe. The jennies were divided into two groups, < 250 kg and > 250 kg body weight, and the dates of conception and parturition/abortion were recorded to calculate pregnancy length. Descriptive statistics were performed for the following variables: pregnancy length and maternal-fetal parameters (measurements of the orbit, gastric bubble, thorax, abdomen, gonads, heart rate, umbilical artery velocimetry, and combined utero-placental thickness). A total of 68 pregnancies were studied, 36 of which ended during the study period. The average pregnancy length was 370.82 ± 16.6 days for full-term pregnancies (N = 28, 77.8%) and 316.13 ± 36.6 days for abortions (N = 8, 22.2%). The season of conception and fetal gender did not affect the pregnancy length. Pregnancy examination can reasonably be performed by two weeks after last service if ovulation date is not known. The orbital diameter was the most reliable parameter for monitoring the physiological development of the embryo and fetus, and it was strongly related to the gestational age. No differences in fetal development were observed in relation to the mother's body weight. The combined utero-placental thickness was not associated with the gestational age and thickening and edema, frequently observed, were not associated with fetal pathologies.

REVISED HIGHLIGHTED

FIELD ULTRASOUND EVALUATION OF SOME GESTATIONAL PARAMETERS IN JENNIES

Tiziana Nervo[1], Alessia Bertero[2], Mariagrazia Poletto[1], Paola Pregel[1], Roberta Leone[3], Valentina Toffoli[4], Leila Vincenti[1]

1 Department of Veterinary Science, University of Torino, Largo Braccini 2/4, 10095 Grugliasco (TO) - Italy

2 Department of Veterinary Science, University of Milan, Via Celoria 10, 20133 Milano - Italy

3 Private practice, roby.leon.rl@gmail.com

4 Private practice, valentaine.d@hotmail.it

Corresponding author: alessia.bertero@unimi.it

Abstract

The aim of this study was to collect and analyze ultrasound measurements of fetal-maternal structures during normal and pathological pregnancies in jennies, a livestock species of growing interest. For two breeding seasons, 38 jennies of different breeds and crossbreeds aged between 3 and 18 years were monitored weekly by transrectal examination using a portable Esaote ultrasound (MyLab™ 30 GOLD VET) with a 5-7.5 MHz probe. The jennies were divided into two groups, ~~those jennies weighing less than 250 kg and those weighing more than 250 kg,~~ < 250 kg and > 250 kg body weight, and the dates of conception and parturition/abortion were recorded to calculate pregnancy length. Descriptive statistics were performed~~, and the mean, median, standard deviation and range were calculated~~ for the following variables: pregnancy length ~~(in relation to the season in which conception occurred)~~ and maternal-fetal parameters~~, including~~ (measurements of the orbit, gastric bubble, thorax, abdomen, gonads, heart rate, umbilical artery velocimetry, and combined utero-placental thickness) ~~(CUPT)~~. A total of 68 pregnancies were studied, 36 of which ended during the study period. ~~In agreement with~~

~~the literature,~~ The average pregnancy length was 370.82 ± 16.6 days for full-term pregnancies (N = 28, 77.8%) and 316.13 ± 36.6 days for abortions (N = 8, 22.2%). The season of conception and fetal gender did not affect the pregnancy length. ~~In agreement with other authors, pregnancy diagnosis could be made beginning on day 12. We observed that pregnancy diagnosis can be determined on day 12 forward.~~ Pregnancy examination can reasonably be performed by two weeks after last service if ovulation date is not known. The orbital diameter was the most reliable parameter for monitoring the physiological development of the embryo and fetus, and it was strongly related to the gestational age. ~~Measurements of the gastric bubble, thorax, abdomen and fetal gonads showed lower accuracy because these structures were more difficult to measure.~~ No differences in fetal development were observed in relation to the mother's body weight. The combined utero-placental thickness ~~CUPT~~ was not associated with the gestational age and thickening and edema, frequently observed, were not associated with fetal pathologies. ~~but monitoring of this parameter may be useful for the diagnosis of placental diseases. However, unlike in mares, in jennies, it was not possible to diagnose placentitis on the basis of the ultrasonographic appearance of the placenta.~~

Keywords: Jenny, Donkey, Pregnancy, Ultrasonography, Fetus, Embryo

1. Introduction

~~In addition to its cosmetic uses, donkey's milk is achieving resounding success in human nutrition, especially as a substitute for breast milk in newborns and for people with intolerance to cow's milk and multiple food allergies [1,2]. Therefore, the demand for donkey's milk is rising constantly, despite the presence of a flourishing industry of reconstituted milk and hydrolysates [2]. Moreover, the use of these animals in pet therapy~~

and onotherapy should not be forgotten [3]. Although donkeys have been used as working animals for thousands of years, interest in this animal have arisen in various branches of the veterinary medicine very recently. For a long time, donkeys have been considered “little horses” and have played second fiddle to horses, but now the increase in knowledge is revealing significant differences between these two species. In recent years, due to the rising economic interest in donkeys, the reproductive physiology of donkeys has drawn the attention of researchers and veterinary clinicians [1]. The most important goal of researchers in this field is. to improve the reproductive performance of these animals based on a Today, the donkey is used as a pet, for onotherapy, for hiking in the mountains and, above all, for milk production. The use of the donkey for milk production in particular, gave this species value as livestock and shifted the attitudes of breeders and veterinarians. Nevertheless, to date the literature is still very scarce, and the lack of knowledge appears to be much wider than expected. Detailed knowledge of the typical reproductive patterns of the species [2] allows to improve the reproductive performance of these animals and increases the economic returns for breeders. Transrectal ultrasonography is a technique that has been used since 1980, and it has revolutionized the study of early pregnancy in mares [3,4]. The monitoring of the pregnancy in jennies was previously unnecessary because donkeys were mainly used for work (especially in developing countries), and in industrialized countries, their presence and use did not justify the application of such powerful and expensive technology. So far, very few studies have been performed on donkeys, and most of them only address the early stages of pregnancy. The aim of this work was to collect and process data and measurements, obtained by transrectal ultrasonography, regarding the maternal-fetal structures throughout donkey gestation. to understand the following: a) whether it is possible to determine the gestational age of the fetus on the basis of fetal measurements, b) if there is a correlation between the size of the mother and the development of the fetus, and c) whether it is possible to establish physiological growth parameters for one or more fetal structures during pregnancy. Moreover it has been evaluated the presence of a possible relationship between the body weight of the mother and fetal dimensions during growth.

2. Materials and methods

2.1. Animals

This field study was carried out over a period of 14 months, from February 2014 to March 2015, on a semi-extensive family-managed farm located near Turin, Italy, that breeds donkeys to produce milk for human consumption and for the production of cosmetics. The jennies were Provencal, Martina Franca or Ragusa breeds, but most of them were crossbreeds. The age range was 3-18 years.

All the pregnant jennies were examined and subjected to transrectal ultrasonography on a weekly basis. Monitoring of the animals started at the reproductive stage at which they were in at that moment. Data were recorded and statistically analyzed. The animals were fed hay and grass in stables or pastures, depending on weather conditions. No feed supplements or concentrates were used. Thirty-eight jennies in good health were enrolled in the trial, during which each animal was subjected to clinical and ultrasound examinations of the reproductive system on a weekly basis for one or more pregnancies, for a total of 68 monitored pregnancies. Thus, some of the animals were first examined from the beginning of a pregnancy to the end of that pregnancy and again for the length of the following pregnancy, while other animals were first examined at a more advanced stage of the first pregnancy, but the subsequent pregnancy was fully monitored. During the study 5 animals were eliminated from the trial due to concurrent diseases (33 subjects remaining). For each jenny, the thorax circumference and the length from the olecranon to the ischial tuberosity were measured to estimate weight according to the guidelines previously published by Pearson R.A. and Ouassat M. [5]: Live weight (kg) = (circumference thoracic $^{2.12}$) x (olecranon-ischial tuberosity length (cm) $^{0.688}$) / 3801. The jennies were then divided into two groups: animals weighing less than 250 kg (11 subjects) and those weighing more than 250 kg (22 subjects).

2.2. Reproductive Management

The two stallions of proven fertility used for natural service at the farm were of the Ragusana breed. The mating occurred under farmer supervision, and the female was left in contact with the male just for the time required for copulation. Mating was repeated every other day until the female did not show any further signs of estrus. The aim was to create the ideal conditions for the animals to show the typical behavioral patterns of the species for animal welfare reasons. ~~By following this procedure, it was possible to know the exact day of mating and avoid excessive stress to the jenny caused by the aggressiveness of the stallions.~~

After parturition, the jennies were left with the foals for approximately one month without being milked. After this period, the jennies were considered in lactation for the subsequent six months.

When the foals reached 6 months of age and the mothers were carrying new pregnancies, it was possible to wean the foals and to dry off the mothers. If the jennies became pregnant at the foal heat, the dry period lasted for approximately four months, and if they did not become pregnant, then it lasted longer. During lactation, approximately one liter of milk per jenny per day was produced. The milk production at the farm was constant because the herd included both females in early lactation that produced approximately 1.4

liters per animal and females at the end of lactation that produced no more than 0.7-0.8 liters per animal.

2.3. Equipment and technique

For the ultrasound examination, a portable ultrasound machine (Esaote MyLab ™ 30 GOLD VET) was used. To perform serial scans of the reproductive tract and of the fetus, a linear endorectal probe with a frequency of 5-7.5 MHz was used.

2.3. Animals and Preparation of animals and ultrasound examination procedure.

The jennies were restrained in stocks. No sedation was used to examine the animals. The rectal ampulla was emptied using a lubricated gloved hand; then, the transrectal linear probe was inserted, and the ultrasound examination was carried out. All the examinations were carried out by the same skilled practitioner. Ultrasound examination was performed using a portable ultrasound machine (Esaote MyLab ™ 30 GOLD VET). To perform serial scans of the reproductive tract and of the fetus, a linear endorectal probe with a frequency of 5-7.5 MHz was used. Doppler ultrasonography has been used for fetal sexing, for the detection of the heartbeat and for the evaluation of the corpora lutea (data not reported).

2.4. Examined parameters Embryo and fetal evaluation parameters

2.5.1. Pregnancy length

During the study, mating dates, birth/abortion dates and fetal or newborn sex were recorded to evaluate pregnancy length.

~~2.5.2. Maternal-fetal parameters~~

~~The collected data~~ Maternal-fetal parameter data were organized into different groups based on the gestational month in which they were recorded, as indicated in Table 1.

- ~~first month (M1): from 0 to 31 days;~~
- ~~second month (M2): from 32 to 62 days;~~
- ~~third month (M3): from 63 to 93 days;~~
- ~~fourth month (M4): from 94 to 124 days;~~
- ~~fifth month (M5): from 125 to 155 days;~~
- ~~sixth month (M6): from 156 to 186 days;~~
- ~~seventh month (M7): from 187 to 217 days;~~
- ~~eighth month (M8): from 218 to 248 days;~~
- ~~ninth month (M9): from 249 to 279 days;~~
- ~~tenth month (M10): from 280 to 310 days;~~
- ~~eleventh month (M11): from 311 to 341 days.~~

~~2.5.2.1. Embryonic vesicle~~

The maximum diameter of the embryonic vesicle was measured from the time of pregnancy diagnosis until the third month. During the first month of pregnancy, the loss of sphericity of the embryonic vesicle was also recorded.

~~2.5.2.2. Loss of sphericity of the embryonic vesicle~~

~~During the first month of pregnancy, the day at which loss of sphericity of the embryonic vesicle was observed was also recorded.~~

~~2.5.2.3. Embryo~~

The day of pregnancy at which the embryo proper became visible within the embryonic vesicle was recorded, and from that time until the 3rd month of gestation, the embryo length was measured along the major axis.

~~2.5.2.4. Embryo Depolarization~~

~~Initially, the embryo was located in an antimesometrial position, quite central within the vesicle, but then, it became more and more eccentric, reaching the ventral wall of the vesicle.~~ The gestational age when it was possible to observe migration of the embryo within the vesicle was recorded.

~~2.5.2.5. Orbit~~

The orbit of the fetus was measured along its major axis from ~~the first trimester~~ day 70 to the end of gestation.

~~2.5.2.6. Gastric bubble~~

The fetal stomach, that ultrasonographically looks like a mobile, anechoic, bean-shaped structure due to its gaseous contents, was measured along its long axis from the 2nd until the 7th month of gestation.

2.5.2.7. Chest

The thorax was identified by visualization of the hyperechoic ribs and by the presence of cardiac motion. The measurement was performed at the point of maximal amplitude of the thorax from the 2nd to the 6th month of gestation.

2.5.2.8. Abdomen

The landmarks used to identify the abdomen were the gastric bubble and the intestines. The abdominal diameter was taken at the point of maximum amplitude, just caudal to the gastric bubble, from the 2nd to the 6th month of gestation.

2.5.2.9. Gonads and fetal sexing

The fetal gonads appeared as oval structures in the ventral and caudal portions of the abdomen near the kidneys. Once identified, the fetal gonads were measured along their long axis, and then, color Doppler was used to visualize the blood supply to the gonads to facilitate sexing of the fetus. The measurements of the gonads and sex determination were performed from the 4th to the 7th month of gestation.

2.5.2.10. Fetal heart rate

After the identification of the heart in the thorax, Doppler echocardiography was used to precisely determine the heart rate in beats per minute (BPM). It was possible to measure the BPM in the second month of gestation and then from the 4th to the 7th month of gestation.

2.5.2.11. Umbilical artery

To locate the vascular triad at the level of the umbilical cord, we tried to follow the course of the umbilical cord in a short-axis view, in which it was distinguishable by four defined circular structures: the two veins, the umbilical artery and the urachus. Then, continuous-wave Doppler velocimetry was performed to obtain the numerical value for the umbilical artery velocimetry. These data were recorded between the 5th and 7th months of gestation.

2.4.1. Combined utero-placental thickness (CUPT) evaluation

The ultrasound measurement of the CUPT was recorded from the 4th month of gestation onwards. It was performed in the caudal portion of the uterine body, close to the cervical star, as described by Renaudin C.D. et al. [6,7], using the uterine artery as a landmark position (Figure 1).

2.4.2. Echogenicity of the fetal fluids

For each ultrasound examination of each jenny, the echogenicity of the amniotic and allantoic fluids was evaluated.

The different echogenicity levels were classified as reported below:

- 0: presence of widespread anechogenicity
- 1: presence of some particles that made the fetal fluids more turbid and then slightly more echogenic
- 2: echogenicity much greater than in condition 0

2.4.3. Fetal motility

For each ultrasound examination of each jenny, fetal motility was evaluated. The evaluation was subjective and was based on the ultrasound images and on the physical/tactile perception of the operator.

The fetal motility was classified into 3 groups:

- 1: the fetus was sleeping; it did not move or made extremely limited movements.
- 2: the fetus showed good motility but was still enough to allow the operator to perform a thorough ultrasound examination and measure the fetal structures.
- 3: the fetus moved excessively to the extent that measurement of the fetal structures became extremely difficult.

2.5. 2.15. Fetal presentation

From the 6th month of gestation onwards, on the basis of the observed structures, the fetal presentation (anterior or posterior) was evaluated.

2.4.6. Mammary gland development

From the 8th month of gestation onwards, during the ultrasound examination, the mammary glands were evaluated to detect signs of development preceding parturition.

The evaluation was based on a visual and tactile examination of the mammary glands, which allowed observation of thickening and increased mammary engorgement and

~~temperature. If deemed necessary, the presence of watery (serous) or colostrum secretions from the nipples were checked.~~

2.5. Statistical analysis

Pregnancies were divided into 4 groups depending on the season in which mating occurred (spring, summer, autumn, winter). The jennies were divided into 2 categories (A and B) based on their weight, with a cut-off value of 250 kg, because if the standard cut-off described in the literature had been used [8,9], almost all the jennies would have been included in the same category. Descriptive statistics (mean, median, standard deviation, range) were performed for the following:

- pregnancy length (parturition and abortion);
- pregnancy length for jennies at term, in relation to the season in which conception occurred;
- maternal-fetal parameters: measurements of the orbit, gastric bubble, thorax, abdomen, gonads, fetal heart rate, umbilical artery velocimetry, and CUPT.

The normality of the distributions was assessed by means of the Kolmogorov and Smirnov test. The differences in pregnancy length in relation to the season when conception occurred were analyzed by means of analysis of variance (ANOVA).

Fisher's test was used to verify the presence of a possible statistical association between the sex of the newborn and pregnancy outcome, the loss of sphericity of the embryonic vesicle and the day of gestation if changes were observed, and between the fetal presentation and the trimester of pregnancy.

The correlation between the orbital diameter and gestational age, and between CUPT and gestational age was evaluated with Spearman's test.

The chi-square test was used to detect possible associations between the echogenicity of the fetal fluids and the trimester of pregnancy, fetal motility and the trimester of pregnancy, and the ability to determine fetal sex and the month of gestation.

Differences in the duration of pregnancy in relation to the fetal sex were assessed by means of Student's t test for unpaired samples.

Linear regression analysis was performed including the days of pregnancy and the following parameters:

- dimensions of the embryonic vesicle, embryo length, measurements of orbit, gastric bubble, thorax, abdomen, gonads, fetal heart rate, and umbilical artery velocimetry;
- dimensions of the orbit from 100th day of pregnancy onwards, comparing jennies in groups A and B.

In all the analyses, differences were considered statistically significant when $P<0.05$.

A statistical analysis similar to that retrieved from the literature [10-12] was performed in order to compare data in the most appropriate way.

3. Results.

3.1 Pregnancy

The study was performed on 68 pregnancies, 36 of which came to term during the trial; of these, 28 ended with the birth of alive and vital foals (77.80%), while 8 ended with abortion (22.20%). The mean pregnancy length was 370.82 ± 16.60 days (range: 342-402 days) for pregnancies that came to term and 316.13 ± 36.60 days (range: 236-356 days) for abortions, but a 48 h-error should be considered, since the exact moment of ovulation is unknown.

Among the 28 pregnancies that came to term, 16 newborns were females (57.10%), and 12 were males (42.90%). Among the 8 abortions, 4 fetuses were male (50.00%), and 4 were female (50.00%).

A statistically significant association between the sex of the newborn and the outcome of pregnancy was not demonstrated, and no significant association between the sex of the newborn and the pregnancy length was found (p-value= 0.58). Nevertheless, we observed a longer duration of pregnancy for males (372.8 ± 16.8 days; range: 351-402 days) and a shorter duration for females (369.3 ± 16.8 days; range: 342-395 days).

A statistically significant association between the season when conception occurred and pregnancy length was not observed, even though pregnancy length seemed to be longer when conception occurred in autumn (374.43 ± 19.62 days; range: 354-395 days) or in winter (375.83 ± 22.95 days; range: 342-402 days) compared to conception that occurred in spring (369.50 ± 13.67 days; range: 349-389 days) or in summer (362.40 ± 7.20 days; range: 353-371 days), independent of the sex of the newborn foal.

Regarding the 8 abortions, in 7 cases, conception occurred in spring (87.00%), and in only one case, it occurred in winter (13.00%). A higher percentage of abortions was found in winter (3/8, 37.00%) and spring (4/8, 50.00%) ~~than in~~ compared to autumn (1/8, 13.99%) and summer (no cases). However, no statistically significant association was observed between the season of conception and abortions.

3.2 Maternal-fetal parameters

The collected parameters, grouped by gestational month, are reported in Table ~~1~~ 2.

3.2.1. Embryo growth parameters

In addition, embryo growth parameters measured during the first three months of gestation are showed in Table ~~2~~ 3. ~~The embryo growth parameters, measured during the first 3 months of gestation, are summarized in Table 2.~~

~~3.2.1.1 Embryonic vesicle~~

The embryonic vesicle was detected for the first time on day 12 of gestation (with a variability up to 48 hours linked to assisted natural mating and to the non-identification of the exact moment of the ovulation).

The mean vesicle diameter between days 16 and 18 (days from the last mating) was 23.4 ± 6.8 mm.

~~In graph 1, the~~ Linear regression between the vesicle diameter and the day of gestation calculated until day 90 is depicted ($y = 0.884x + 7.128$; $R^2 = 0.8304$; p-value < 0.001; Figure 2).

~~3.2.1.2 Loss of sphericity of the embryonic vesicle~~

Loss of sphericity of the embryonic vesicle was observed from the 21st day (± 48 h) of gestation. An extremely significant association (p-value < 0.001) was demonstrated between the loss of sphericity of the embryonic vesicle and the third week ~~of the first month~~ of pregnancy.

~~3.2.1.3. Embryo~~

The embryo proper was observed for the first time on day 23 of pregnancy (with a variability up to 48 hours linked to assisted natural mating and to the non-identification of the exact moment of the ovulation).

A positive linear regression between the longitudinal dimension of the embryo and day of gestation (± 48 h) was calculated until day 90 and is depicted in ~~graph 2~~ Figure 3 ($y = 0.0708x - 0.8413$; $R^2 = 0.7519$; $p\text{-value} < 0.001$).

~~3.2.1.4. Embryo Depolarization~~

Initially, the embryo was located in an antimesometrial position, quite central within the vesicle, but then, it became more and more eccentric, reaching the ventral wall of the vesicle. This embryo depolarization within the vesicle was observed from day 32 (± 48 h) of gestation.

3.2.2. Fetal growth parameters

The fetal growth parameters, measured from the 100th day (± 48 h) of gestation onwards, are summarized in Table ~~3~~ 4.

~~3.2.2.1. Orbit~~

The orbit of the fetus (Figure 4) was detected and measured for the first time on day 70 (± 48 h) of pregnancy.

A statistically significant correlation between orbital diameter and day of gestation (± 48 h) was demonstrated (y = 0.0071x - 0.5951; $R^2 = 0.7861$; p-value <0.001).

A positive linear relationship between the orbital diameter and the day of gestation (± 48 h) was demonstrated (Figure 5).

~~3.2.2.2. Gastric bubble~~

The gastric bubble (Figure 6) was identified for the first time in our study on day 51 (± 48 h) of gestation.

A positive linear relationship between the diameter of the gastric bubble and the day of gestation (± 48 h) was demonstrated (p-value < 0.001).

~~3.2.2.3. Chest~~

The thorax was detected for the first time on day 52 (± 48 h) of gestation.

A positive linear relationship between the diameter of the thorax and the day of gestation (± 48 h) was demonstrated (p-value < 0.001).

~~3.2.2.4. Abdomen~~

The abdominal diameter, simultaneously with the thorax diameter, was obtained for the first time on day 52 (± 48 h) of gestation.

A positive linear relationship between the diameter of the abdomen and the day of gestation (± 48 h) was demonstrated (p-value < 0.001).

~~3.2.2.5. Gonads and fetal sexing~~

The first measurement of the fetal gonads was obtained on day 96 (± 48 h) of gestation.

A positive linear relationship between the greatest diameters of the gonads and the day of gestation (± 48 h) was demonstrated.

According to our data, the time frame for fetal sexing runs from the 96th to the 210th day (± 48 h) of gestation. A statistically significant association (p-value < 0.001) between the month of gestation and the ability to perform sex determination of the fetus was demonstrated. It was possible to determine the fetal sex in the 4th month of gestation in 32% of cases, in the 5th month in 60% of cases, in the 6th month in 26% of cases, and in the 7th month in 10% of cases. The diagnostic accuracy was 88%.

~~3.2.2.6. Fetal heart rate~~

The first determination of fetal heart rate was obtained on day 22 (± 48 h) of gestation. A negative linear relationship between the fetal heart rate and the day of gestation (± 48 h) was detected (p-value < 0.001).

~~3.2.2.7. Umbilical artery~~

The first measurement of the frequency of the umbilical artery was recorded on day 125 (± 48 h) of gestation. A negative linear relationship between the umbilical artery frequency and the day of gestation (± 48 h) was detected, even if the p-value was not significant.

3.2.3. Combined utero-placental thickness (CUPT)

It was possible to obtain the first measurement of the CUPT on day 94 (± 48 h) of gestation.

A statistically significant correlation between the CUPT and the day of gestation (± 48 h) was detected (Spearman r= 0.14; p-value < 0.05). In some cases, some portions of the CUPT were edematous (Figures 7 and 8).

3.2.4. Echogenicity of the fetal fluids

Data regarding echogenicity of the allantoic fluids are presented in Figure 9. ~~During the first two months of gestation, 99% of the fetal allantoic fluids showed an echogenicity of grade 0, and 1% showed an echogenicity of grades 1-2.~~

~~From the 3rd to the 5th month of gestation, 97% showed an echogenicity of grade 0, and 3% showed an echogenicity of grades 1-2.~~

~~From the 6th to the 8th month of gestation, 79% showed an echogenicity of grade 0, and 21% showed an echogenicity of grades 1-2.~~

~~From the 9th to the 11th month of gestation, 87% showed an echogenicity of grade 0, and 13% showed an echogenicity of grades 1-2.~~

A statistically significant association between the trimester of pregnancy and the fetal fluid echogenicity was demonstrated (p-value < 0.001).

3.2.5. Fetal motility

According to our data, the lowest motility (1) was detected during the 2nd and 3rd months of gestation, while the highest motility was recorded during the 6th and 7th months of gestation.

A statistically significant association between the trimester of pregnancy and fetal motility was demonstrated (p-value < 0.05).

The highest motility was recorded from the 6th to the 8th month of gestation. We presumed that this was due to the presence of more abundant space available for the fetus.

In all the examinations, absolute inactivity of the fetus was never recorded.

3.2.6. Fetal presentation

During the 3rd trimester of gestation, 56 fetuses were observed in anterior presentation, 13 in posterior presentation and none in transverse presentation.

A statistically significant association (p-value < 0.01) between the trimester of pregnancy and fetal presentation was demonstrated. The fetuses showed the final presentation at the 9th month of gestation.

3.2.5. Mammary gland development

In 98.65% of cases, we did not find thickening of the mammary glands until the 11th month of gestation. In just one case (1.35%), thickening of the mammary glands associated with a serous secretion following the application of slight pressure was observed. This jenny

~~had a physiological 351-day pregnancy that ended with the birth of a male foal, so this observation did not represent a pathological condition.~~

3.2.6. Evaluation of fetal orbital diameter in relation to maternal size

The regression equations for the orbital diameter were calculated starting from the 100th day (± 48 h) of gestation by grouping the jennies on the basis of their body weight (groups A and B; ~~Graph 4~~ Figure 10). The differences between the slopes of the regression line were not significant (p-value = 0.9), so there was no statistically significant difference in the orbital diameter from the 100th day (± 48 h) of gestation between the two groups.

4. Discussion

The present study demonstrated through clinical monitoring that the mean pregnancy length in mixed-breed jennies bred in a continental climate was 370.82±16.6 days (± 48 h), in accordance with the results obtained by most authors who reported an average pregnancy length of 372-374 days [1,10,13-18]; however, some authors reported a shorter duration (353.4±13 days) [12].

The observed sex ratio showed a slightly higher number of females and was similar to the ratio reported by other authors [12], even though we examined of a smaller number of jennies. Even though we could not demonstrate a statistically significant association between the sex of the newborn and the pregnancy outcome, we recorded that the pregnancies were longer in jennies pregnant with male fetuses than in jennies pregnant with female fetuses, and this was in accordance with previously reported data for jennies [12,16,18] and mares [19-22] in the literature.

Even though reproductive seasonality appeared to be absent in these animals, the pregnancy length seemed to be longer when conception occurred in the autumn and winter and shorter when it occurred in the summer. This fact, which was also noticed by other authors in a study on indigenous jennies on a farm located in the south of Spain [16], was not statistically supported.

The issue regarding the frequent abortions observed in this farm (22.2% during the study period) remains unsolved because bacteriological and virological analyses did not identify an etiological agent. In 3 jennies that were negative for EHV-1-4, the herpesvirus glycoprotein (HVG) was isolated, presumably due to the presence of other herpesvirus strains, such as equine herpesvirus 8 which was recently isolated [23] and is common in donkeys. Surely the clinical signs, characterized by late-term abortion after 8 months of gestation without premonitory signs, could correspond to ~~H~~EHV symptomatology.

The most reliable hypothesis seemed to be linked to the season, as the abortions occurred at the end of winter to early spring, when the animals had been subjected to cold winter temperatures for few months, and then ended in late-spring. The donkey, despite being a rustic animal, is well adapted to life in arid and desert areas where it originates from, and it is possible that donkeys lack optimal adaptation to our latitudes, so they may be very stressed if subjected to low temperatures. This stress factor may trigger the onset of latent pathologies in the population. The need to have pregnancies spread throughout the entire solar year lies in the requirement to ensure constant milk production, but despite that, the breeder is now trying to avoid early spring parturitions in hopes that this could reduce the incidence of abortion.

The embryonic vesicle was detected for the first time 12 days after the last mating (day 12 ± 48 h of pregnancy, due to the non-identification of the exact moment of the ovulation), in accordance with reports by many authors (days 12-13 of gestation) who have evaluated jennies [1,2,10-12,17,24] and mares [25-27]; however, other authors [28] have reported the first detection of the embryonic vesicle in jennies on day 14. An early pregnancy diagnosis is very useful in production animals because it allows reduction in number of days needed to inseminate the jenny again in cases of negative outcomes.

Another important reason for the early pregnancy diagnosis is to assess the risk of twin pregnancy. Compared with mares, donkeys have been reported to have a higher frequency of multiple ovulations [8,29-31], and this was also observed in our study. The early diagnosis of pregnancy allowed us to choose the best option, conservative or not, since spontaneous regression of one of the two vesicles seemed to be the most likely scenario [32].

According to our experience, the breeder decided several times not to intervene in cases of twin pregnancies, and this always resulted in the resorption of one or both of the vesicles during the first 30 days of pregnancy.

The mean vesicle diameter, measured from 16 to 18 days after the last mating (23.4 ± 6.8 mm), was in accordance with the data obtained in jennies by some authors ~~11~~(26.4 ± 0.7 mm) [11], but it seems to be higher compared to the data observed by other authors in jennies ~~24~~(21.8 mm) [24], ~~10~~(22.3 mm) [10] and in mares ~~33~~(23 mm) [33]. We should highlight that these authors monitored the ovulation follicles daily to unambiguously identify the exact moment of ovulation. Since we could not do that, our day 0 corresponded to the last day on which the jenny had accepted mating.

Therefore, because we did not know exactly the ovulation time, because we had to evaluate the jennies 24-36 h after mating, and because the growth of the embryonic vesicle is exponential until approximately the 16th day of gestation, we obtained vesicle measurements with a higher standard deviation than those reported in the literature [10, 24]. In addition, the diameter of the embryonic vesicle has been proven to be positively correlated with the day of gestation by other authors [10,12,17,24]. The data regarding the loss of sphericity of the embryonic vesicle from the 21st day (± 48 h) of gestation and the significant association with the third week ~~of the first month~~ of pregnancy were in accordance with the results obtained in jennies and mares by many authors [10-12,17,24,28,33-36].

The day of first detection of the embryo (23 days after the last mating) and its antimesometrial position were in accordance with reports by many authors regarding mares and jennies [10-12,17,24,33,34].

The embryo depolarization within the vesicle was observed from day 32 (± 48 h) of gestation, which is an earlier date than that reported by other authors, who detected embryo depolarization from day 35 to 53 [24], from day 41 to 47 [10], or even on day 50 [11].

Because of the positive linear relationship between the vesicle diameter, the longitudinal length of the embryo and the day of gestation, we can state that during the first stage of embryo development, it is possible to date the pregnancy and evaluate the physiological growth of the fetus.

The first day of gestation when identification and measurement of the orbit was performed was earlier than that reported by other studies on jennies. Crisci A. et al. (2014) [12] reported first detection of the orbit on day 71-96 of gestation, while we reported detection on day 70, however variability (up to 48 hours) linked to assisted natural mating and to the non-identification of the exact moment of the ovulation, must be taken in account. Early detection of the orbit, similar to all the other examined structures, depends on how the conceptus is positioned in relation to the ultrasound probe and on the quality of the transducer, which may or may not allow the correct identification of very small structures. The statistically significant correlation between the orbital dimensions and the day of gestation that we found has been proven in jennies [12] and in mares [37-40].

Moreover, ultrasonographic measurement of the orbit is simple (because the orbit stays visible until the end of the pregnancy) and provides the best indication for fetal growth, as emphasized by other authors who evaluated mares [38-40]. As a parameter that is closely related to the gestational period, it also allows dating of the pregnancy physiologically in cases in which the mating date is not known and therefore allows estimation of the time at which parturition may occur, which is very useful in clinical practice.

Even though we grouped the jennies on the basis of their weight, we did not observe a statistically significant difference in the growth of the orbit from the 100th day (± 48 h) of gestation in the two groups. This finding is in agreement with what other authors have found for donkeys [17].

Since the orbit reflects fetal growth during gestation, it can be assumed that maternal size does not affect the extent of fetal development. Indeed, in the equine species, this category of dystocia, which is common in other species, is rare [41].

The thorax and abdomen were measured for the first time 52 days after the last mating. No references were found in the literature regarding identification of the abdomen of the donkey fetus; however, the date of thorax identification was in accordance with the literature [12]. In mares, the measurement of these two structures is reported on day 100 of gestation [39].

The gastric bubble was identified for the first time earlier in our study (day 51 ± 48 h of gestation) than in other works on jennies (day 60-71 of gestation [12]).

As reported by other authors who evaluated mares [39] and applied the same statistical analysis, we found a correlation between all the studied fetal structures and the gestational age from the 100th day (± 48 h) onwards. The orbital measurements were confirmed to be the best parameter to calculate the gestational age of the fetus. Other structures may also be useful but are not as reliable. For example, the size of the gastric bubble can be considered a parameter to calculate the gestational age of the fetus; however, it can be detected for a shorter period than the orbit, as previously reported for donkeys [12] and mares [40].

Thorax and abdominal measurements may also provide an indication of the gestational age, and this finding was in agreement with other authors who described a positive linear relationship between thorax measurements and day of gestation in jennies [12] and in mares [39], as well as between the abdominal size and the day of gestation in mares [39].

The fetal gonads were observed for the first time on day 96 after the last mating and up to day 210. To perform the determination of fetal sex, the best period was found to be the 5th month of gestation (60% of the performed examinations). This range is wider than that reported by several authors (between day 100 and day 150 of gestation) for donkeys [42] and for mares [39, 43]. On the other hand, other authors reported the possibility of sexing the horse fetus from day 90 to day 180 of gestation [44] or from day 120 to day 210 [45]. The factors involved in fetal sex determination are many. Above all, the most important factors include the following: the use of an ultrasound machine equipped with a high-quality probe, possibly with the help of the Color Doppler; good environmental brightness conditions; adequate restraint of the animal; mother and fetus remaining still during the examination; relatively small size of the fetus; and posterior presentation. Most likely, more than one of these conditions occurred simultaneously in our study, and this explains why it was possible to determine the fetal sex in a wider temporal range than what has been reported in the previous literature. We often had the opportunity to observe the fetuses in posterior presentation at the time of the examination, which is a condition that is more likely to occur in jennies than in mares, as the donkey fetus is typically smaller in size than a horse fetus. In addition, with the progression of gestational age, other structures, such as the external genitalia and the mammary glands, became visible, which allowed more accurate sex determination than mere observation of the gonads.

Gonad identification, besides being essential for sexing the fetus, also allowed estimation of the gestational age, which was in agreement with other studies on mares [39,40].

The first observation of the fetal heartbeat was obtained 22 days after the last mating. The negative linear relationship between the fetal heart rate and the day of gestation was in accordance with other studies in jennies [12] and in mares [40]. Indeed, in all species, the fetal heart rate is very high in the early embryonic and fetal stages and tends to decrease, almost reaching the maternal value, close to parturition.

Additionally, a negative linear relationship ($R^2 = 0.02$) was found between the umbilical artery frequency (measured for the first time 125 days after the last mating) and the gestational age. However, as other authors have emphasized [46], it is very difficult to obtain realistic and reliable data on umbilical artery blood flow because this artery has a winding course, and it is not always possible to place the ultrasound probe in an optimal position for the angle of incidence between the Doppler waves and the direction of the blood flow. The same authors [46] also observed a reduction in blood flow resistance from mid-pregnancy onwards. Human clinicians believe that proper determination of the umbilical arterial blood flow may provide extremely useful data regarding the growth and health of the fetus and placenta [47]. Therefore, it is worth checking this parameter despite the technical difficulties.

The CUPT measurement was performed exclusively transrectal ~~(TRU)~~ because of the impossibility, in field conditions, to adequately prepare the jennies (clipping of the abdomen, extended restraint time) for performing transabdominal ultrasound ~~(TAU)~~ imaging. Our first detection of CUPT was earlier than what has been reported by other authors (day 94 ± 48 h of gestation vs. day 154) [12], but it was in accordance with the data reported on mares [6].

The authors' choice to start measuring the CUPT from day 154 [12] is presumably due to the fact that in the equine species, the placenta is considered fully functional beginning on the 150th day of pregnancy. However, we chose to start measuring the CUPT when it became visible, even if it was still in development. The average CUPT found in our study from the 4th to the 9th month of pregnancy (M4: 4.97±1.73 cm; M5: 4.33±1.31 cm; M6: 4.41±0.94 cm; M7: 4.74±1.13 cm; M8: 4.48±1.89 cm; M9: 5.03±1.48 cm; M10: 4.87±1.98 cm; M11: 4.89±1.26 cm) were in accordance with that reported in mares by Renaudin C.D. et al. [7] and Bucca S. et al. [40]; however, it was lower than that described in jennies by Crisci A. [12]. In the mare, from the 10th to the 12th month, the average CUPT increases by 1.5-2 mm every month [7,40]. This was not found in our study, where CUPT remained virtually unchanged during gestation, even in animals that aborted. Conversely, some authors [12] reported an increase in CUPT during pathology in pregnancies. Instead, in some cases we observed a CUPT that was partially edematous. This sign in the mare is normally associated with placentitis but seems to be irrelevant in the jenny. All this data seems to indicate that these placental morphological characteristics are peculiar of donkeys and probably attributable to the longer gestation length in donkeys compared with horses [48].

Because of the current legislation on drugs in Italy, which explicitly prohibits the use of any product on equines bred for the production of milk for human consumption, the animals were not treated despite the ultrasound evidence of pathology. However, none of the jennies aborted with clinical signs of placentitis, and no placental abnormalities were found after parturition, suggesting that this aspect of CUPT may be physiologic in the donkey, may be related to the length of donkey pregnancies and may be associated with lower placental function, as described by Carluccio A. et al. [2].

In agreement with other authors [12], no abnormal echogenicity of the fetal fluids was found in all the examined pregnancies, but we detected a progressive increase in turbidity during pregnancy due to the deposition of particles within the fetal fluids, as described in mares [7,40]. This turbidity may also depend on the degree of fetal motility during the examination since the particles are settled during fetal rest and become suspended during fetal movement. The echoes emitted by the transducer through the fetal fluids stimulate the fetus, that responds by increasing its motility, causing an apparent increase in the echogenicity of the fetal fluids in advanced pregnancies. With respect to fetal motility, as reported by other authors using the same evaluation scale [12], we never observed total fetal immobility, and on the contrary, we often recorded high grade 3 motility, particularly from the 6th to the 8th month of gestation, presumably due to the greater amount of space available to the fetus. Similar results are also reported in mares [40].

In agreement with data reported for mares [40], in the last trimester of gestation, we never observed fetal presentations other than anterior presentation; however, some authors [12] have reported the possibility that the fetus may reach its final presentation even later than the 9th month of gestation in the jenny because of its relatively small size.

~~According to our field experience, jennies began to have mammary gland development approximately 10-15 days before parturition. In one case, thickening of the mammary glands associated with serous secretions following the application of slight pressure was observed, but this jenny had a physiological pregnancy of 351 days; thus, this sporadic~~

~~observation suggests that, as in the mare, some jennies can physiologically begin to have mammary gland development as early as 1 month before parturition.~~

5. Conclusions

~~Although donkeys have been used as working animals for thousands of years, interest in donkeys have arisen in various branches of the veterinary medicine very recently. For a long time, donkeys have been considered “little horses” and have played second fiddle to horses. Only now have we begun to understand how different these two species are.~~

Our work has suggested that the jenny has peculiarities that characterize its gestational features and distinguish them from those of the mare.

~~In equine gynecology, the use of ultrasound imaging for the monitoring of pregnancy dates back to 1980 and, to date, this technique is so developed that it is comparable to human medicine. The monitoring of the pregnancy in jennies was previously unnecessary because donkeys were mainly used for work (especially in developing countries), and in industrialized countries, their presence and use did not justify the application of such powerful and expensive technology.~~

~~Today, the donkey is used as a pet, for onotherapy, for hiking in the mountains and, above all, for milk production. The use of the donkey for milk production in particular, gave this species value as livestock and shifted the attitudes of breeders and veterinarians. Nevertheless, to date the literature is still very scarce, and the lack of knowledge appears to be much wider than expected.~~

~~We hope that this study can add pieces to the mosaic of knowledge on this topic~~. In this regard, the obtained results can contribute to the definition of reference values for this species and can be used by clinicians to evaluate embryonic and fetal development, to calculate the gestational age when unknown, and to identify any anomalies that occur during the pregnancy. Nevertheless, it has to be considered that this is a field study, with all the inherent limitations of this type of work (the interval between the examinations could not be reduced, losing accuracy; the light conditions were variable; transabdominal ultrasonography could not be performed; etc.). In the future, experimental studies following all the animals from the beginning of the pregnancy until parturition, with more standardized environmental conditions, shortened interval between the ultrasound examinations and eventually the possibility to perform transabdominal ultrasonography could be very useful to widen the knowledge regarding the ultrasound evaluation of gestational parameters in this species.

6. Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

7. Bibliography

1. Tosi U, Bernabò N, Verni F, Valbonetti L, Muttini A, Mattioli M, Barboni B. Postpartum reproductive activities and gestation length in Martina Franca jennies, an endangered Italian donkey breed. Theriogenology 2013;80:120-124.
2. Carluccio A, Panzani S, Tosi U, Riccaboni P, Contri A, Veronesi MC. Morphological features of the placenta at term in the Martina Franca donkey. Theriogenology 2008;69:918-924.

3. Ginther OJ & Griffin PG. Natural outcome and ultrasonic identification of equine fetal twins. Theriogenology 1994;41:1193-1199.
4. Ginther OJ & Bergfelt DR. Embryo reduction before day 11 in mares with twin conceptuses. J Anim Sci 1988;66:1727-1731.
5. Pearson RA, Ouassat M. A guide to live weight estimation and body condition scoring of donkeys. Centre for Tropical Veterinary Medicine, University of Edinburgh. Colours Printers Ltd, 14 Carnoustic Place, Glasgow; 2000.
6. Renaudin CD, Troedsson MHT, Gillis CL, King VL, Bodena A. Ultrasonographic evaluation of the equine placenta by transrectal and transabdominal approach in the normal pregnant mare. Theriogenology 1997;47:559-573.
7. Renaudin CD, Troedsson MHT, Gillis CL. Transrectal ultrasonographic evaluation of the normal equine placenta. Equine Veterinary Education 2003;6:16-17.
8. Hagstrom DJ. Donkeys are Different: An Overview of Reproductive Variations from Horses. University of Illinois, USA, Department of Agriculture, Local Extension Councils Cooperating, 2004;January: p. 1-5.
9. Matthews N. Donkeys – Not just small horse. The North American Veterinary Conference, Gainesville, USA, 2010;January: p. 214-216.
10. Meira C, Ferreira JCP, Papa FO, Henry M. Ultrasonographic evaluation of the conceptus from days 10 to 60 of pregnancy in jennies. Theriogenology 1998;49:1475-1482.
11. Carluccio A, Villani M, Contri A, Tosi U, Veronesi MC. Rilievi ecografici della gravidanza precoce nell'asina di Martina Franca. Ippologia 2005;4:31-35.
12. Crisci A, Rota A, Panzani D, Sgorbini M, Ousey JC, Camillo F. Clinical, ultrasonographic, and endocrinological studies on donkey pregnancy. Theriogenology 2014;81:275-283.

13. Fielding D. Reproductive Characteristics of the jenny donkey-Equus asinus: a review. Tropical Animal Health and Production 1988;20:161-166.
14. Pugh DG. Donkey Reproduction. Proceedings of the Annual Convention of the American Association of Equine Practitioners, 2002; p. 113-114.
15. Villani M, Wilsher S, Carluccio A, Contri A, Veronesi MC. Stereology in the term placenta of the donkey, Thoroughbred and pony: A comparative study. Animal Reproduction Science 2006;94:411-412.
16. Galisteo J, Perez-Marin CC. Factors affecting gestation length and estrus cycle characteristics in Spanish donkey breeds reared in southern Spain. Theriogenology 2010;74:443-450.
17. Brown WH. Embryonic and fetal development in the donkey. A Capstone Experience Manuscript 2012.
18. Carluccio A, Gloria A, Veronesi MC, De Amicis I, Noto F, Contri A. Factors affecting pregnancy lenght and phases of parturition in Martina Franca jennies. Theriogenology 2015;84:650-655.
19. Heidler B, Aurich JE, Pohl W, Aurich C. Body weight of mares and foals, estrous cycles and plasma glucose concentration in lactating and non-lactating Lipizzaner mares. Theriogenology 2004;61:883-893.
20. Valera M, Blesa F, Dos Santos R, Molina A. Genetic study of gestation length in Andalusian and Arabian mares. Animal Reproduction Science 2006;95:75-96.
21. McCue PM, Ferris RA. Parturition, dystocia and foal survival: a retrospective study of 1047 births. Equine Vet Journal Suppl 2012;(41):22-25.
22. Kuhl J, Stock KF, Wulf M, Aurich C. Maternal Lineage of Warmblood Mares Contributes to Variation of Gestation Length and Bias of Foal Sex Ratio. PLOS ONE 2015;10(10):e0139358.

23. Liu C, Guo W, Lu G, Xiang W, Wang X. Complete Genomic Sequence of an Equine Herpesvirus Type 8 Wh Strain Isolated from China. Journal of Virology 2012;86(9):5407. doi:10.1128/JVI.00445-12.
24. Gastal EL, Santos GF, Henry M, Piedade HM. Embryonic and foetal development in donkeys. Equine Veterinary Journal Suppl 1993;15:10-13.
25. Bessent C, Ginther OJ. Comparison of early conceptus mobility between mares and jennies. Theriogenology 1988;29:913-920.
26. Ley WB. Broodmare reproduction for the equine practitioner. 1st ed. New York: Teton New Media; 2004.
27. Derar DR, Ali A. Conceptual Orientation: A Unique Phenomenon of the Equine Pregnancy. Anatomy and Physiology 2016;2:1-4.
28. Lemma A, Schwartz HJ, Bekana M. Application of ultrasonography in the study of the reproductive system of tropical jennies (Equus asinus). Tropical Animal Health and Production 2006;38:267-274.
29. Blanchard TL, Taylor TS, Love CL. Estrous cycle characteristics and response to estrus synchronization in mammoth asses (Equus asinus americanus). Theriogenology 1999;52:827-834.
30. Taberner E, Medrano A, Penã A, Rigau T, Mirò J. Oestrus cycle characteristics and prediction of ovulation in Catalonian jennies. Theriogenology 2008;70:1489-1497.
31. Quaresma M, Payan-Carreira R. Characterization of the estrous cycle of Asinina de Miranda jennies (Equus asinus). Theriogenology 2015;83:616-624.
32. Ginther OJ. Reproductive biology of the mare (Basic and applied aspects). 2nd ed. Cross Plains, Wisconsin, Equiservices Publishing; 1992, p. 75-232.
33. Ginther OJ. Ultrasonic imaging and reproductive events in the mare. Cross Plains, Wisconsin, Equiservices Publishing; 1986, p. 378.

34. Ginther OJ. Fixation and orientation of the early equine conceptus. Theriogenology 1983;19:613-623.

35. Ginther OJ. Ultrasonic anatomy and pathology of the reproductive tract. Proceedings of the Equine Ultrasound Short Course, Fort Collins, Colorado State University; 1984a; p. 16-25.

36. Ginther OJ. Ultrasonic evaluation of the reproductive tract of the mare: the single embryo. Journal of Equine Veterinary Science 1984b;4:75-81.

37. Kahn W & Leidl W. Ultrasonic biometry of horse foetuses in utero and sonographic representation of their organs. Dtsch Tierarztl Wochenschr 1987;94:509-515.

38. McKinnon AO, Squires EL, Pickett BW. Equine Reproductive Ultrasonography, Bull. No. 4. Fort Collins, Colorado State University Animal Reproduction Laboratory, 1988; p. 31-40.

39. Renaudin CD, Gillis CL, Tarantal AF, Coleman DA. Evaluation of equine fetal growth from day 100 of gestation to parturition by ultrasonography. Journal of Reproduction and Fertility Suppl 2000;56:651-660.

40. Bucca S, Fogarty U, Collins A, Small V. Assessment of feto-placental well-being in the mare from mid-gestation to term: Transrectal and transabdominal ultrasonographic features. Theriogenology 2005;64:542-557.

41. Frazer G. Dystocia Management. In: McKinnon AO, Squires EL, Vaala WE, Varner DD, editors. Equine Reproduction - Vol.2. 2nd ed. Wiley-Blackwell Publishing; 2011, p. 2479-2496.

42. Mancuso R, Scollo C, Torrisi C, Catone G La determinazione del sesso fetale nell'asina [Fetal sex determination in donkey mare]. In: Proc. Annual Meeting of the Italian Association of Equine Veterinarians, Bologna, Italy, 2007; p. 173-174.

43. Livini M. Determination of Fetal Gender by Transrectal Ultrasound Examination: Field's Experience. Proceedings of the Annual Convention of the American Association of Equine Practitioners, Baltimore, MD, USA, 2010; p. 323-327.
44. Resende HL, Carmo MT, Ramires Neto C, Alvarenga A. Determination of equine fetal sex by Doppler ultrasonography of the gonads. Equine Veterinary Journal 2014;46:756-758.
45. Bucca S. Equine fetal gender determination from mid- to advanced-gestation by ultrasound. Theriogenology 2005;64:568-571.
46. Bollwein H, Weber F, Woschée I, Stolla R. Transrectal Doppler sonography of uterine and umbilical blood flow during pregnancy in mares. Theriogenology 2004;61:499-509.
47. Gudmundsson S, Flo K, Ghosh G., Wilsgaard T, Acharya G. Placental pulsatility index: a new more sensitive parameter for predicting adverse outcome in pregnancies suspected of fetal growth restriction. Acta Obstet Gynecol Scand 2016;November.
48. Carluccio A, Noto F, Parrillo S, Contri A, De Amicis I, Gloria A, Robbe D, Veronesi MC. Transrectal ultrasonographic evaluation of combined utero-placental thickness during the last half of pregnancy in Martina Franca donkeys. Theriogenology 2016;86(9):2296-2301.

8. Figures

Figure 1 CUPT measurement during the 8th month of gestation. D1: CUPT (5.20 mm); CS: cervical star; UA: uterine artery.

~~**Graph 1**~~ **Figure 2** Linear regression between the vesicle diameter and the gestational age during the first three months of gestation.

~~**Graph 2**~~ **Figure 3** Linear regression between the embryo length and the gestational age during the first three months of gestation.

Figure 4 (A) orbital diameter at 85-days (D1=0.89 cm), (B) at 152-days (D2=1.65), (C) at 211-days (D3=2.1 cm) and (D) at 261-days (D4=2.33 cm) of gestation.

~~**Graph 3**~~ **Figure 5** Linear regression between the fetal orbital diameter and the gestational age from the 100th day of gestation onwards.

Figure 6 (A) gastric bubble diameter at 52-days (D1=0.31 cm), (B) at 100-days (D2=0.72), (C) at 103-days (D3=1.03 cm) and (D) at 135-days (D4=1.25 cm) of gestation; TR: transversal diameter.

Figure ~~2~~ 7 CUPT measurement during the 10th month of gestation. Some portions of the CUPT are edematous. D1: CUPT (5.56 mm); CS: cervical star; UA: uterine artery.

Figure ~~3~~ 8 CUPT measurement during the 4th month of gestation. The placenta is diffusely edematous (yellow arrow).

Figure 9 Grade of echogenicity of the allantoic fluids. The percentages of fetuses showing grade 0 or 1-2 throughout pregnancy are indicated.

~~**Graph 4**~~ **Figure 10** Comparison between the fetal orbital diameter and maternal size from day 100 to day 351 of gestation.

Table 1 Gestational months (and corresponding days) in which maternal-fetal parameters were recorded.

Gestational Month	**Days**
First month - M1	0-31
Second month - M2	32-62
Third month - M3	63-93
Fourth month - M4	94-124
Fifth month - M5	125-155
Sixth month - M6	156-186
Seventh month - M7	187-217
Eighth month - M8	218-248
Ninth month - M9	249-279
Tenth month - M10	280-310
Eleventh month - M11	311-341

Table ~~1~~ 2 Measurements of maternal-fetal parameters taken in different months of gestation in jennies ~~(1 M-11 M)~~.

Gestationa~~l~~ (Month)	**Parameter (cm)**	**Mean ± DS (Range)**	**Median**
1st ~~M~~	Vesicle diameter ~~(cm)~~	2.50±0.86 (0.46-3.96)	2.40
	Embryo ~~(cm)~~	1.13±0.42 (0.58-2.29)	1.06
2nd ~~M~~	Vesicle diameter ~~(cm)~~	4.84±1.17 (2.83-7.3)	4.78
	Embryo ~~(cm)~~	2.19±0.92 (0.93-3.96)	1.90
	Thorax ~~(cm)~~	1.49±0.18 (1.2-1.68)	1.53
	Abdomen ~~(cm)~~	1.60±0.21 (1.22-1.86)	1.64
	Gastric bubble ~~(cm)~~	0.22±0.1 (0.1-0.37)	0.20
	BPM*	219.67±36.11 (158-270)	230
3rd ~~M~~	Vesicle diameter ~~(cm)~~	7.47±1.80 (3.56-10.70)	7.16
	Embryo ~~(cm)~~	4.98±1.03 (3.30-6.70)	5.50
	Thorax ~~(cm)~~	1.80±0.50 (1.13-2.30)	1.88

	Abdomen ~~(cm)~~	2.45±0.52 (1.24-3.09)	2.62
	Gastric bubble ~~(cm)~~	0.56±0.24 (0.15-0.95)	0.57
	Orbit ~~(cm)~~	0.66±0.14 (0.42-0.90)	0.70
4th ~~M~~	Thorax ~~(cm)~~	4.77±0.46 (4.27-5.20)	4.70
	Abdomen ~~(cm)~~	5.15±0.7 (3.93-6.20)	5.22
	Gastric bubble ~~(cm)~~	1.52±0.75 (0.22-3.01)	1.41
	BPM*	245.40±36.26 (183-290)	254.50
	Orbit ~~(cm)~~	1.13±0.19 (0.84-1.51)	1.16
	Gonad ~~(cm)~~	1.57±0.20 (1.32-1.90)	1.51
	CUPT ~~(CUPT - cm)~~	4.97±1.73 (3.15-9.90)	4.30
5th ~~M~~	Thorax ~~(cm)~~	5.31±0.65 (4.72-6.86)	5.14
	Abdomen ~~(cm)~~	5.75±0.66 (4.72-7.03)	5.61
	Gastric bubble ~~(cm)~~	2.26±0.82 (1.22-3.56)	2.18
	BPM*	198.18±35.17 (154-252)	193
	Orbit ~~(cm)~~	1.50±0.14 (1.28-1.78)	1.49
	Gonad ~~(cm)~~	3.44±1.01 (2.2-5.31)	3.10
	CUPT ~~(CUPT - cm)~~	4.33±1.31 (2.01-7.77)	4.32
	Umbilical artery (beats/min)	258.25±32.87 (204-295)	268.5
6th ~~M~~	Thorax ~~(cm)~~	5.72±0.41 (5.04-6.12)	5.80
	Abdomen ~~(cm)~~	6.23±0.32 (5.83-6.83)	6.21
	Gastric bubble ~~(cm)~~	2.86±0.89 (1.26-4.03)	3.08
	BPM*	193.22±47.79 (156-299)	171
	Orbit ~~(cm)~~	1.91±0.20 (1.55-2.3)	1.91
	Gonad ~~(cm)~~	3.34±0.45 (2.47-4.05)	3.47
	CUPT ~~(CUPT - cm)~~	4.41±0.94 (2.68-6.39)	4.40
	Umbilical artery (beats/min)	235.33±40.69 (164-280)	235

7th ~~M~~	Gastric bubble ~~(cm)~~	2.82±2.93 (2.57-2.96)	2.93
	BPM*	152.33±26.50 (122-171)	164
	Orbit ~~(cm)~~	2.11±0.25 (1.55-2.51)	2.16
	CUPT ~~(CUPT - cm)~~	4.74±1.13 (2.68-6.81)	4.75
	Umbilical artery (beats/min)	267.50±4.93 (262-295)	267.5
8th ~~M~~	Orbit ~~(cm)~~	2.44±0.22 (2.18-2.95)	2.45
	CUPT ~~(CUPT - cm)~~	4.48±1.89 (0.8-8.08)	4.36
9th ~~M~~	Orbit ~~(cm)~~	2.51±0.26 (2.00-2.82)	2.53
	CUPT ~~(CUPT - cm)~~	5.03±1.48 (1.20-7.30)	5.51
10th ~~M~~	Orbit ~~(cm)~~	2.56±0.28 (2.00-3.13)	2.58
	CUPT ~~(CUPT - cm)~~	4.87±1.98 (0.70-8.36)	4.69
11th ~~M~~	Orbit ~~(cm)~~	2.80±0.31 (2.39-3.20)	2.84
	CUPT ~~(CUPT - cm)~~	4.89±1.26 (3.20-7.75)	4.63

CUPT (combined utero-placental thickness); * (heart beat/min).

Table ~~2~~ 3 Linear regression of the embryonic vesicle and embryo length in the first three months of gestation. ~~r^2: correlation coefficient. b_0 and b_1: coefficients of the linear regression equation ($y = b_0 + b_1x$); y: calculated value for the variable; x: gestational age.~~

Embryo growth parameter	N	Range	Days	r^2	b_0	b_1	p-value
Embryo vesicle (mm)	99	4.6-1.07	12-91	0.83	7.13	0.88	< 0.001
Embryo length (cm)	49	0.58-6.70	23-90	0.75	-0.84	0.07	< 0.001

r^2: correlation coefficient. b_0 and b_1: coefficients of the linear regression equation ($y = b_0 + b_1x$); y: calculated value for the variable; x: gestational age.

Table ~~3~~ 4 Linear regression of the fetal parameters from the 100th day of gestation onwards. ~~r^2: correlation coefficient. b_0 and b_1: coefficients of the linear regression equation ($y = b_0 + b_1x$); y: calculated value for the variable; x: gestational age.~~

Fetal growth parameters (cm)	N	Range	Days	r^2	b_0	b_1	p-value
Orbit ~~(cm)~~	128	0.92-3.2	100-351	0.79	0.60	0.01	< 0.001
Gastric bubble ~~(cm)~~	48	0.72-4.12	100-210	0.23	0.10	0.02	< 0.001
Thorax ~~(cm)~~	21	4.27-6.86	100-185	0.70	2.43	0.02	< 0.001
Abdomen ~~(cm)~~	24	4.72-7.03	100-185	0.46	3.74	0.02	< 0.001
Gonad ~~(cm)~~	27	1.32-5.31	100-185	0.59	-2.02	0.03	< 0.001
Heart rate* ~~(beats/min)~~	31	122-299	101-212	0.37	340.70	-0.93	< 0.001
Umbilical artery* ~~(beats/min)~~	27	164-295	125-212	0.02	286.74	-0.20	0.44

r^2: correlation coefficient. b_0 and b_1: coefficients of the linear regression equation ($y = b_0 + b_1x$); y: calculated value for the variable; x: gestational age. * (heart beat/min).

Dear Editorial Team,
We would like to thank you and the Reviewers for the helpful suggestions and comments that offered us the opportunity to improve our manuscript. We complain because, before submitting the manuscript to your journal, it has been sent to an author services company (American Journal Experts, Certificate Verification Key: D789-53BA-B8C3-63A3-7093) that has revised it for English. Moreover we did our best to improve language and grammar also with native English speakers. We hope to satisfy your request.
The manuscript has been completely revised according the Reviewers' suggestions.
Our responses to the Reviewers' comments are below.

Reviewer #1
Initially the title gives us the impression that some correlation or relationship exists between the production of milk for human consumption (title), but this have being forgotten in the course of the paper.
There is no well defined objective, it is not known if the production of milk for human consumption is part, or if the goal is to compare jennies with different weights or the fetal biometry analyses without groups comparation.
Answer: Thank you for your suggestion, we agree and, for that reason, we decided to modify the title because milk production was not influencing the results.

One of the proposed objectives is to determine the gestational age by the measurements of the embryonic vesicle and fetal biometry, however the date of ovulation was not verified since the system used was natural mating. Therefore all accuracy of measurements can not be considered.
Answer: We thank you for your comment, it is true that we did not verify the ovulation, but we are considering a period up to 24 h because we recorded the exact date of the last assisted mating (in fact probably less than 24 h considering the time window in which jennies accept the stallion and the limited viability of the oocyte after ovulation in terms of time). We performed the pregnancy diagnosis on day 12 after the last mating in order to include the animals in the study as soon as possible, thus, maybe we detected the embryonic vesicle on day 13 of pregnancy instead of 12, as we mentioned in the text, obtaining a higher standard deviation of the vesicle diameter at the beginning of the pregnancy, but then it decreased rapidly. The same can be affirmed about the embryo length. However we inserted in the manuscript sentences that state clearly the presence of this variability linked to the assisted natural mating and to the non-identification of the exact moment of the ovulation. Moreover, most of the parameters considered in the study are focused on later stages of pregnancy (>3 weeks).
Furthermore it should be considered that this is a field study, performed in a family-managed farm located in a rural area, with a difficult logistic, so the experimental design was planned considering these complications and the fact that even if we could have performed a more frequent ultrasound examination of the jennies (for example every 12 h after mating), the standard deviation of the above mentioned parameters would not have decreased significantly.

The animals were divided into two groups of weights, but these groups were not compared in most of the analyzes.
Answer: Thank you for your observation. We evaluated all the parameters but we reported just the orbital diameter in relation to maternal size because this structure is the easiest to measure and also because this parameter appear to be the most related to the gestational age.

In some moments, the authors reported two reproductive seasons in another one season only ... the animals presented different gestational phases in different periods of the year, with some the evaluations starting at the end of gestation and others at the beginning (not the same animal), which generated confusion.
Answer: Thank you for your observation. At first, we performed a field study, so examination of the animals started at the reproductive stage at which they were in at the moment of the first clinical examination. In the third season we will monitor all the animals starting from the beginning of the pregnancy and following them until parturition.

The method used for transrectal ultrasonography is not the most suitable for evaluation of fetal biometry from the middle third of gestation ... therefore the measurements performed may have less acuracy.
Answer: Thank you for your observation. Yes, we know that, but in our field condition the transrectal ultrasonography was the best choice because of its suitability and rapidity also in relation to the impossibility to adequately prepare the jennies (clipping of the abdomen, extended restraint time, light condition, etc.) to perform transabdominal ultrasonography. Moreover, in our climatic condition, winter season is very cold and being the jennies so sensitive to low temperatures, the breeder did not give us permission to clip them.

lines 116 to 120 can be removed
Answer: Thank you for your suggestion. We removed the lines as you indicated, we just let information regarding the restraints in stocks, because the thought that this is important since allowed as to perform a more detailed and precise measuring. We also let the details regarding the person who performed the examination thus, in our experience, measurements can vary if taken by unskilled operators.

lines 156 to 159 is a result and not methodology
Answer: We moved these lines in the result section, thank you for your suggestion.

line 162 first trimester of gestation goes from day 0 to day 90, the authors report later in the text that at the beginning of gestation it is not possible to evaluate orbit diameter and this evaluation depends a lot on the accuracy of the ultrasound device used.
Answer: Thank you for your suggestion, we add in the text that the orbit is visible starting from day 70.

line 231, it is not clear whether mammary gland evaluation was also a parameter evaluated.
Answer: We did not evaluate this parameter. Thank you for your observation, we removed this part.

line 318, sentence does not make sense
Answer: This sentence has been modified, thank you for your suggestion.

line 383, does not describe that gestational fluid is being evaluated, allantoid or amniotic, which causes problems since the echogenicity proposed in the methodology is different between them, especially in the final third of gestation. So this evaluation needs to be discarged.
Answer: Thank you for your observation, we evaluated the allantoic fluids. A graphical representation of these data was added to the paper.

line 417 to 418, the foaling does not mean that it does not have some gestational or placental problem/disease.
Answer: Thank you for your observation. Yes, we are aware of it, but we analysed the morphological aspect of all the placentas and they were normal, moreover the Apgar scores of the foals were in the physiological range.

line 451, HEV = EHV
Answer: We corrected it, thank you for your observation.

line 470 to 473, the authors describe a high frequency of multiple ovulations for the species, which was also observed by them, but in the methodology natural mating was performed and no follicular growing and ovulation was evaluated.
Answer: Thank you for your observation. We performed transrectal ultrasonography after mating and in case of multiple ovulations, multiple active (as confirmed by doppler) corpora lutea and eventually multiple (two in our experience) embryonic vesicles were detected.

line 506 to 507, one day of difference in the results can not be considered when natural mating was used.**Answer**: Thank you for your observation. Yes, we agree. We inserted in the text a sentence regarding the need to consider the variability (as described before, up to 24 hours) linked to assisted natural mating and to the non-identification of the exact moment of the ovulation.

The results of CUPT are controversial and the conclusions do not coincide with the results. There is an affirmation by the authors that it is impossible to diagnose placentitis by means of the evaluation of CUPT in jennies, unfortunately the work was not designed for this, nor does it present a compatible methodology for such an incisive affirmation.
Answer: Thank you for your observation probably there is a misunderstanding. We mentioned placentitis and placental alterations only in the discussion. In our work we observed thickening or edema of placenta probably due only to the prolonged pregnancy of jennies compared with the mare.

The linear regression tables become unnecessary with the presentation of the equations and graphs.
Answer: Thank you for your observation, they can be eliminated.

Most of the results lack p value (probability)
Answer: Thank you for your observation, we inserted p-values also in the text.

The authors conclude that the measurements and parameters are different from equines, but this comparison was not performed.
Answer: Thank you for your observation. We wrote our conclusions on the basis of the literature, so we discussed about consideration made by different authors on jennies and mares.

First, third and fourth paragraphs of the conclusion are not conclusions and the fifth paragraph does not correspond to the objectives.
Answer: Thank you for your observation. We have modified the text following your indications.

I strongly recommend that the authors review all article, simplify evaluations and results with a focus on fetal biometrics, showing the limitations of the methodology.
Answer: Thank you for your suggestion. We have modified the text following your indications.

Reviewer #2:
The authors studied the "Evaluation of some gestational parameters in jennies bred for the production of milk for human consumption". The research topic is within the scope of the journal. However, there is no data supporting the milk production and analyses and the title should be changed to: Evaluation of some gestational parameters in Jennies bred. This manuscript contains some innovative information compared to published articles related to gestational parameters from this specie. The manuscript is interesting and with new insight on fetal-maternal evaluation during the 2-years period studied. The general idea is organized and provides useful findings. Furthermore, the editing needs minor corrections as indicated into the manuscript upload into the system. I can recommend this paper for publication in this journal.
The uploaded document is included into the system. Some points are describe below:

-The uses of Doppler ultrasoun to evaluate fetal growth may improve the manuscript importance. Authors should include the data on fetal hemodynamics to provide some additional information regard this specie.
Answer: We used the Doppler only for the evaluation of the CL and for the sexing of the fetus.
The instrument was new and we were not trained to make other measurements; moreover, the operating conditions were not optimal. Thus, data regarding the other determinations that we tried to do did not seem to be reliable, and we decided to not report them in the paper.

- Authors should take into consideration a more appropriated design figures to address the manuscript. They should also add more pictures to show and to compare ultrasound findings during the gestational period. Such as the orbital, gut, and other parameters measurements on ultrasound figures. Perhaps, they can use in the same figure (E.g.: Figure 4 (a) orbital growth at 6-months of age, (b) orbital growth at 11-months of age, and so on…). Graphs and figures need a highly resolution definition. Tables are not in the journal format.
Answer: Thank you for your observation. We have added pictures in the manuscript, as you suggested. The table format has been modified and we tried our best to improve the resolution of graphs and figures.

- There are too many subsections in the material and methods section. Avoid using them. Readers tend to become lost when too many subsections are described with few sentences in each. Try to combine some of the subsections. We tried to change this using the review track in the uploaded manuscript.
Answer: Thank you for your observation. We followed your suggestion and now most of the subsections are combined as you indicated in the manuscript.

- Provide a graph, simple bars, to show the results that describe the % of echogenicity and the evaluation thought out the gestational period. (see comment in the uploaded document).
Answer: We inserted the graph as you indicated, thank you for your suggestion.

Reviewer #4:
The study about EVALUATION OF SOME GESTATIONAL PARAMETERS IN JENNIES BRED FOR THE PRODUCTION OF MILK FOR HUMAN CONSUMPTION. The aim was to create the ideal conditions
96 for the animals to show the typical behavioral patterns of the species for animal welfare reasons. By following this procedure, it was possible to know the exact day of mating and avoid excessive stress to the jenny caused by the aggressiveness of the stallions.
Considerations
1- The abstract is too long and needs to be shortened
Answer: We shortened it, thank you for your suggestion.

2- Line 100: "After parturition, the jennies were left with the foals for approximately one month without being milked. After this period, the jennies were considered in lactation for the subsequent six months". This sentence should be in the material and methods.
Answer: Yes, it is in material and methods.

3- Line 84: "chest circumference". Change for Thoracic circumference
Answer: We changed it. Thank you for your suggestion.

4- Lines 131 to 140: for this information it is more appropriate to use a table
Answer: Thank you for your suggestion, we added a table.

5- 2.5.2.5. Orbit The orbit of the fetus was measured along its major axis from the first trimester to the end of gestation. What are the measures used? Measures for donkeys already exist?
Answer: The operator measured the orbit along the major axis, repeated the measurement twice and we reported the average of the two values. In the discussion we referred to data derived from bibliography.

6-The study is relevant. However, the need for the study
The article needs to be rewritten from Material and Methods, Discussion and Conclusion. For it is very difficult to read and understand.
Answer: The manuscript has been completely revised according to the Reviewers' suggestions, thank you for your observations.

The charts and tables need to be republished. I suggest using a program to produce the graphics.
Answer: We re-edited tables and charts, thank you for your observation.

Finally, the article needs a complete reformulation for submission to this paper.
Answer: Thank you for your suggestion. The manuscript has been fully reviewed and reformulated, we hope that it flows better now.

FIELD ULTRASOUND EVALUATION OF SOME GESTATIONAL PARAMETERS IN JENNIES

Tiziana Nervo[1], Alessia Bertero[2], Mariagrazia Poletto[1], Paola Pregel[1], Roberta Leone[3], Valentina Toffoli[4], Leila Vincenti[1]

1 Department of Veterinary Science, University of Torino, Largo Braccini 2/4, 10095 Grugliasco (TO) - Italy

2 Department of Veterinary Science, University of Milan, Via Celoria 10, 20133 Milano - Italy

3 Private practice, roby.leon.rl@gmail.com

4 Private practice, valentaine.d@hotmail.it

Corresponding author: alessia.bertero@unimi.it

Abstract

The aim of this study was to collect and analyze ultrasound measurements of fetal-maternal structures during normal and pathological pregnancies in jennies, a livestock species of growing interest. For two breeding seasons, 38 jennies of different breeds and crossbreeds aged between 3 and 18 years were monitored weekly by transrectal examination using a portable Esaote ultrasound (MyLab™ 30 GOLD VET) with a 5-7.5 MHz probe. The jennies were divided into two groups, < 250 kg and > 250 kg body weight, and the dates of conception and parturition/abortion were recorded to calculate pregnancy length. Descriptive statistics were performed for the following variables: pregnancy length and maternal-fetal parameters (measurements of the orbit, gastric bubble, thorax, abdomen, gonads, heart rate, umbilical artery velocimetry, and combined utero-placental thickness). A total of 68 pregnancies were studied, 36 of which ended during the study period. The average pregnancy length was 370.82 ± 16.6 days for full-term pregnancies (N = 28, 77.8%) and 316.13 ± 36.6 days for abortions (N = 8, 22.2%). The season of conception and fetal gender did not affect the pregnancy length. Pregnancy examination can reasonably be performed by two weeks after last service if ovulation date is not known. The orbital diameter was the most reliable parameter for monitoring the physiological development of the embryo and fetus, and it was strongly related to the gestational age. No differences in fetal development were observed in relation to the mother’s body weight. The combined utero-placental thickness was not associated with the gestational age and thickening and edema, frequently observed, were not associated with fetal pathologies.

Keywords: Jenny, Donkey, Pregnancy, Ultrasonography, Fetus, Embryo

1. Introduction

Although donkeys have been used as working animals for thousands of years, interest in this animal have arisen in various branches of the veterinary medicine very recently. For a long time, donkeys have been considered “little horses” and have played second fiddle to horses, but now the increase in knowledge is revealing significant differences between these two species. In recent years, due to the rising economic interest in donkeys, the reproductive physiology of donkeys has drawn the attention of researchers and veterinary clinicians [1]. Today, the donkey is used as a pet, for onotherapy, for hiking in the mountains and, above all, for milk production. The use of the donkey for milk production in particular, gave this species value as livestock and shifted the attitudes of breeders and veterinarians. Nevertheless, to date the literature is still very scarce, and the lack of knowledge appears to be much wider than expected. Detailed knowledge of the typical reproductive patterns of the species [2] allows to improve the reproductive performance of these animals and increases the economic returns for breeders. Transrectal ultrasonography is a technique that has been used since 1980, and it has revolutionized the study of early pregnancy in mares [3,4]. The monitoring of the pregnancy in jennies was previously unnecessary because donkeys were mainly used for work (especially in developing countries), and in industrialized countries, their presence and use did not justify the application of such powerful and expensive technology. So far, very few studies have been performed on donkeys, and most of them only address the early stages of pregnancy. The aim of this work was to collect and process data and measurements, obtained by transrectal ultrasonography, regarding the maternal-fetal structures throughout donkey gestation. Moreover it has been evaluated the presence of a possible relationship between the body weight of the mother and fetal dimensions during growth.

2. Materials and methods

2.1. Animals

This field study was carried out over a period of 14 months, from February 2014 to March 2015, on a semi-extensive family-managed farm located near Turin, Italy, that breeds donkeys to produce milk for human consumption and for the production of cosmetics. The jennies were Provencal, Martina Franca or Ragusa breeds, but most of them were crossbreeds. The age range was 3-18 years.

All the pregnant jennies were examined and subjected to transrectal ultrasonography on a weekly basis. Monitoring of the animals started at the reproductive stage at which they were in at that moment. Data were recorded and statistically analyzed. The animals were fed hay and grass in stables or pastures, depending on weather conditions. No feed supplements or concentrates were used. Thirty-eight jennies in good health were enrolled in the trial, during which each animal was subjected to clinical and ultrasound examinations of the reproductive system on a weekly basis for one or more pregnancies, for a total of 68 monitored pregnancies. Thus, some of the animals were first examined from the beginning of a pregnancy to the end of that pregnancy and again for the length of the following pregnancy, while other animals were first examined at a more advanced stage of the first pregnancy, but the subsequent pregnancy was fully monitored. During the study 5 animals were eliminated from the trial due to concurrent diseases (33 subjects remaining). For each jenny, the thorax circumference and the length from the olecranon to the ischial tuberosity were measured to estimate weight according to the guidelines previously published by Pearson R.A. and Ouassat M. [5]: Live weight (kg) = (circumference thoracic $^{2.12}$) x (olecranon-ischial tuberosity length (cm) $^{0.688}$) / 3801. The jennies were then divided into two groups: animals weighing less than 250 kg (11 subjects) and those weighing more than 250 kg (22 subjects).

2.2. Reproductive Management

The two stallions of proven fertility used for natural service at the farm were of the Ragusana breed. The mating occurred under farmer supervision, and the female was left in contact with the male just for the time required for copulation. Mating was repeated every other day until the female did not show any further signs of estrus. The aim was to create the ideal conditions for the animals to show the typical behavioral patterns of the species for animal welfare reasons.

After parturition, the jennies were left with the foals for approximately one month without being milked. After this period, the jennies were considered in lactation for the subsequent six months.

When the foals reached 6 months of age and the mothers were carrying new pregnancies, it was possible to wean the foals and to dry off the mothers. If the jennies became pregnant at the foal heat, the dry period lasted for approximately four months, and if they did not become pregnant, then it lasted longer. During lactation, approximately one liter of milk per jenny per day was produced. The milk production at the farm was constant because the herd included both females in early lactation that produced approximately 1.4 liters per animal and females at the end of lactation that produced no more than 0.7-0.8 liters per animal.

2.3. Animals and ultrasound examination procedure.

The jennies were restrained in stocks. No sedation was used to examine the animals. All the examinations were carried out by the same skilled practitioner. Ultrasound examination was performed using a portable ultrasound machine (Esaote MyLab ™ 30 GOLD VET). To perform serial scans of the reproductive tract and of the fetus, a linear endorectal probe with a frequency of 5-7.5 MHz was used. Doppler ultrasonography has been used for fetal sexing, for the detection of the heartbeat and for the evaluation of the corpora lutea (data not reported).

2.4. Embryo and fetal evaluation parameters

During the study, mating dates, birth/abortion dates and fetal or newborn sex were recorded to evaluate pregnancy length.

Maternal-fetal parameter data were organized into different groups based on the gestational month in which they were recorded, as indicated in Table 1.

The maximum diameter of the embryonic vesicle was measured from the time of pregnancy diagnosis until the third month. During the first month of pregnancy, the loss of sphericity of the embryonic vesicle was also recorded.

The day of pregnancy at which the embryo proper became visible within the embryonic vesicle was recorded, and from that time until the 3rd month of gestation, the embryo length was measured along the major axis.

The gestational age when it was possible to observe migration of the embryo within the vesicle was recorded.

The orbit of the fetus was measured along its major axis from day 70 to the end of gestation.

The fetal stomach, that ultrasonographically looks like a mobile, anechoic, bean-shaped structure due to its gaseous contents, was measured along its long axis from the 2nd until the 7th month of gestation.

The thorax was identified by visualization of the hyperechoic ribs and by the presence of cardiac motion. The measurement was performed at the point of maximal amplitude of the thorax from the 2nd to the 6th month of gestation.

The landmarks used to identify the abdomen were the gastric bubble and the intestines. The abdominal diameter was taken at the point of maximum amplitude, just caudal to the gastric bubble, from the 2nd to the 6th month of gestation.

The fetal gonads appeared as oval structures in the ventral and caudal portions of the abdomen near the kidneys. Once identified, the fetal gonads were measured along their long axis, and then, color Doppler was used to visualize the blood supply to the gonads to facilitate sexing of the fetus. The measurements of the gonads and sex determination were performed from the 4th to the 7th month of gestation.

After the identification of the heart in the thorax, Doppler echocardiography was used to precisely determine the heart rate in beats per minute (BPM). It was possible to measure the BPM in the second month of gestation and then from the 4th to the 7th month of gestation.

To locate the vascular triad at the level of the umbilical cord, we tried to follow the course of the umbilical cord in a short-axis view, in which it was distinguishable by four defined circular structures: the two veins, the umbilical artery and the urachus. Then, continuous-wave Doppler velocimetry was performed to obtain the numerical value for the umbilical artery velocimetry. These data were recorded between the 5th and 7th months of gestation.

2.4.1. Combined utero-placental thickness (CUPT) evaluation

The ultrasound measurement of the CUPT was recorded from the 4th month of gestation onwards. It was performed in the caudal portion of the uterine body, close to the cervical star, as described by Renaudin C.D. et al. [6,7], using the uterine artery as a landmark position (Figure 1).

2.4.2. Echogenicity of the fetal fluids

For each ultrasound examination of each jenny, the echogenicity of the amniotic and allantoic fluids was evaluated.

The different echogenicity levels were classified as reported below:

- 0: presence of widespread anechogenicity
- 1: presence of some particles that made the fetal fluids more turbid and then slightly more echogenic
- 2: echogenicity much greater than in condition 0

2.4.3. Fetal motility

For each ultrasound examination of each jenny, fetal motility was evaluated. The evaluation was subjective and was based on the ultrasound images and on the physical/tactile perception of the operator.

The fetal motility was classified into 3 groups:

- 1: the fetus was sleeping; it did not move or made extremely limited movements.
- 2: the fetus showed good motility but was still enough to allow the operator to perform a thorough ultrasound examination and measure the fetal structures.
- 3: the fetus moved excessively to the extent that measurement of the fetal structures became extremely difficult.

From the 6th month of gestation onwards, on the basis of the observed structures, the fetal presentation (anterior or posterior) was evaluated.

2.5. Statistical analysis

Pregnancies were divided into 4 groups depending on the season in which mating occurred (spring, summer, autumn, winter). The jennies were divided into 2 categories (A and B) based on their weight, with a cut-off value of 250 kg, because if the standard cut-off described in the literature had been used [8,9], almost all the jennies would have been included in the same category. Descriptive statistics (mean, median, standard deviation, range) were performed for the following:

- pregnancy length (parturition and abortion);

- pregnancy length for jennies at term, in relation to the season in which conception occurred;
- maternal-fetal parameters: measurements of the orbit, gastric bubble, thorax, abdomen, gonads, fetal heart rate, umbilical artery velocimetry, and CUPT.

The normality of the distributions was assessed by means of the Kolmogorov and Smirnov test. The differences in pregnancy length in relation to the season when conception occurred were analyzed by means of analysis of variance (ANOVA).

Fisher's test was used to verify the presence of a possible statistical association between the sex of the newborn and pregnancy outcome, the loss of sphericity of the embryonic vesicle and the day of gestation if changes were observed, and between the fetal presentation and the trimester of pregnancy.

The correlation between the orbital diameter and gestational age, and between CUPT and gestational age was evaluated with Spearman's test.

The chi-square test was used to detect possible associations between the echogenicity of the fetal fluids and the trimester of pregnancy, fetal motility and the trimester of pregnancy, and the ability to determine fetal sex and the month of gestation.

Differences in the duration of pregnancy in relation to the fetal sex were assessed by means of Student's t test for unpaired samples.

Linear regression analysis was performed including the days of pregnancy and the following parameters:

- dimensions of the embryonic vesicle, embryo length, measurements of orbit, gastric bubble, thorax, abdomen, gonads, fetal heart rate, and umbilical artery velocimetry;

- dimensions of the orbit from 100th day of pregnancy onwards, comparing jennies in groups A and B.

In all the analyses, differences were considered statistically significant when $P<0.05$.

A statistical analysis similar to that retrieved from the literature [10-12] was performed in order to compare data in the most appropriate way.

3. Results.

3.1 Pregnancy

The study was performed on 68 pregnancies, 36 of which came to term during the trial; of these, 28 ended with the birth of alive and vital foals (77.80%), while 8 ended with abortion (22.20%). The mean pregnancy length was 370.82 ± 16.60 days (range: 342-402 days) for pregnancies that came to term and 316.13 ± 36.60 days (range: 236-356 days) for abortions, but a 48 h-error should be considered, since the exact moment of ovulation is unknown.

Among the 28 pregnancies that came to term, 16 newborns were females (57.10%), and 12 were males (42.90%). Among the 8 abortions, 4 fetuses were male (50.00%), and 4 were female (50.00%).

A statistically significant association between the sex of the newborn and the outcome of pregnancy was not demonstrated, and no significant association between the sex of the newborn and the pregnancy length was found (p-value= 0.58). Nevertheless, we observed a longer duration of pregnancy for males (372.8 ± 16.8 days; range: 351-402 days) and a shorter duration for females (369.3 ± 16.8 days; range: 342-395 days).

A statistically significant association between the season when conception occurred and pregnancy length was not observed, even though pregnancy length seemed to be longer when conception occurred in autumn (374.43 ± 19.62 days; range: 354-395 days) or in winter (375.83 ± 22.95 days; range: 342-402 days) compared to conception that occurred in spring (369.50 ± 13.67 days; range: 349-389 days) or in summer (362.40 ± 7.20 days; range: 353-371 days), independent of the sex of the newborn foal.

Regarding the 8 abortions, in 7 cases, conception occurred in spring (87.00%), and in only one case, it occurred in winter (13.00%). A higher percentage of abortions was found in winter (3/8, 37.00%) and spring (4/8, 50.00%) compared to autumn (1/8, 13.99%) and summer (no cases). However, no statistically significant association was observed between the season of conception and abortions.

3.2 Maternal-fetal parameters

The collected parameters, grouped by gestational month, are reported in Table 2.

3.2.1. Embryo growth parameters

In addition, embryo growth parameters measured during the first three months of gestation are showed in Table 3.

The embryonic vesicle was detected for the first time on day 12 of gestation (with a variability up to 48 hours linked to assisted natural mating and to the non-identification of the exact moment of the ovulation).

The mean vesicle diameter between days 16 and 18 (days from the last mating) was 23.4 ± 6.8 mm.

Linear regression between the vesicle diameter and the day of gestation calculated until day 90 is depicted ($y = 0.884x + 7.128$; $R^2 = 0.8304$; p-value < 0.001; Figure 2).

Loss of sphericity of the embryonic vesicle was observed from the 21st day (± 48h) of gestation. An extremely significant association (p-value < 0.001) was demonstrated between the loss of sphericity of the embryonic vesicle and the third week of pregnancy.

The embryo proper was observed for the first time on day 23 of pregnancy (with a variability up to 48 hours linked to assisted natural mating and to the non-identification of the exact moment of the ovulation).

A positive linear regression between the longitudinal dimension of the embryo and day of gestation (± 48 h) was calculated until day 90 and is depicted in Figure 3 ($y = 0.0708x - 0.8413$; $R^2 = 0.7519$; p-value < 0.001).

Initially, the embryo was located in an antimesometrial position, quite central within the vesicle, but then, it became more and more eccentric, reaching the ventral wall of the vesicle. This embryo depolarization within the vesicle was observed from day 32 (± 48 h) of gestation.

3.2.2. Fetal growth parameters

The fetal growth parameters, measured from the 100th day (± 48 h) of gestation onwards, are summarized in Table 4.

The orbit of the fetus (Figure 4) was detected and measured for the first time on day 70 (± 48 h) of pregnancy.

A statistically significant correlation between orbital diameter and day of gestation (± 48 h) was demonstrated (y = 0.0071x - 0.5951; R^2 = 0.7861; p-value <0.001).

A positive linear relationship between the orbital diameter and the day of gestation (± 48 h) was demonstrated (Figure 5).

The gastric bubble (Figure 6) was identified for the first time in our study on day 51 (± 48 h) of gestation.

A positive linear relationship between the diameter of the gastric bubble and the day of gestation (± 48 h) was demonstrated (p-value < 0.001).

The thorax was detected for the first time on day 52 (± 48 h) of gestation.

A positive linear relationship between the diameter of the thorax and the day of gestation (± 48 h) was demonstrated (p-value < 0.001).

The abdominal diameter, simultaneously with the thorax diameter, was obtained for the first time on day 52 (± 48 h) of gestation.

A positive linear relationship between the diameter of the abdomen and the day of gestation (± 48 h) was demonstrated (p-value < 0.001).

The first measurement of the fetal gonads was obtained on day 96 (± 48 h) of gestation.

A positive linear relationship between the greatest diameters of the gonads and the day of gestation (± 48 h) was demonstrated.

According to our data, the time frame for fetal sexing runs from the 96th to the 210th day (± 48 h) of gestation. A statistically significant association (p-value < 0.001) between the month of gestation and the ability to perform sex determination of the fetus was demonstrated. It was possible to determine the fetal sex in the 4th month of gestation in

32% of cases, in the 5th month in 60% of cases, in the 6th month in 26% of cases, and in the 7th month in 10% of cases. The diagnostic accuracy was 88%.

The first determination of fetal heart rate was obtained on day 22 (± 48 h) of gestation. A negative linear relationship between the fetal heart rate and the day of gestation (± 48 h) was detected (p-value < 0.001).

The first measurement of the frequency of the umbilical artery was recorded on day 125 (± 48 h) of gestation. A negative linear relationship between the umbilical artery frequency and the day of gestation (± 48 h) was detected, even if the p-value was not significant.

3.2.3. Combined utero-placental thickness (CUPT)

It was possible to obtain the first measurement of the CUPT on day 94 (± 48 h) of gestation.

A statistically significant correlation between the CUPT and the day of gestation (± 48 h) was detected (Spearman r= 0.14; p-value < 0.05). In some cases, some portions of the CUPT were edematous (Figures 7 and 8).

3.2.4. Echogenicity of the fetal fluids

Data regarding echogenicity of the allantoic fluids are presented in Figure 9.

A statistically significant association between the trimester of pregnancy and the fetal fluid echogenicity was demonstrated (p-value < 0.001).

3.2.5. Fetal motility

According to our data, the lowest motility (1) was detected during the 2nd and 3rd months of gestation, while the highest motility was recorded during the 6th and 7th months of gestation.

A statistically significant association between the trimester of pregnancy and fetal motility was demonstrated (p-value < 0.05).

The highest motility was recorded from the 6th to the 8th month of gestation. We presumed that this was due to the presence of more abundant space available for the fetus.

In all the examinations, absolute inactivity of the fetus was never recorded.

During the 3rd trimester of gestation, 56 fetuses were observed in anterior presentation, 13 in posterior presentation and none in transverse presentation.

A statistically significant association (p-value < 0.01) between the trimester of pregnancy and fetal presentation was demonstrated. The fetuses showed the final presentation at the 9th month of gestation.

3.2.6. Evaluation of fetal orbital diameter in relation to maternal size

The regression equations for the orbital diameter were calculated starting from the 100th day (± 48 h) of gestation by grouping the jennies on the basis of their body weight (groups A and B; Figure 10). The differences between the slopes of the regression line were not significant (p-value = 0.9), so there was no statistically significant difference in the orbital diameter from the 100th day (± 48 h) of gestation between the two groups.

4. Discussion

The present study demonstrated through clinical monitoring that the mean pregnancy length in mixed-breed jennies bred in a continental climate was 370.82±16.6 days (± 48 h), in accordance with the results obtained by most authors who reported an average pregnancy length of 372-374 days [1,10,13-18]; however, some authors reported a shorter duration (353.4±13 days) [12].

The observed sex ratio showed a slightly higher number of females and was similar to the ratio reported by other authors [12], even though we examined of a smaller number of jennies. Even though we could not demonstrate a statistically significant association between the sex of the newborn and the pregnancy outcome, we recorded that the pregnancies were longer in jennies pregnant with male fetuses than in jennies pregnant with female fetuses, and this was in accordance with previously reported data for jennies [12,16,18] and mares [19-22] in the literature.

Even though reproductive seasonality appeared to be absent in these animals, the pregnancy length seemed to be longer when conception occurred in the autumn and winter and shorter when it occurred in the summer. This fact, which was also noticed by other authors in a study on indigenous jennies on a farm located in the south of Spain [16], was not statistically supported.

The issue regarding the frequent abortions observed in this farm (22.2% during the study period) remains unsolved because bacteriological and virological analyses did not identify an etiological agent. In 3 jennies that were negative for EHV-1-4, the herpesvirus glycoprotein (HVG) was isolated, presumably due to the presence of other herpesvirus strains, such as equine herpesvirus 8 which was recently isolated [23] and is common in donkeys. Surely the clinical signs, characterized by late-term abortion after 8 months of gestation without premonitory signs, could correspond to EHV symptomatology.

The most reliable hypothesis seemed to be linked to the season, as the abortions occurred at the end of winter to early spring, when the animals had been subjected to cold winter temperatures for few months, and then ended in late-spring. The donkey, despite being a rustic animal, is well adapted to life in arid and desert areas where it originates from, and it is possible that donkeys lack optimal adaptation to our latitudes, so they may be very stressed if subjected to low temperatures. This stress factor may trigger the onset of latent pathologies in the population. The need to have pregnancies spread throughout the entire solar year lies in the requirement to ensure constant milk production, but despite that, the breeder is now trying to avoid early spring parturitions in hopes that this could reduce the incidence of abortion.

The embryonic vesicle was detected for the first time 12 days after the last mating (day 12 ± 48 h of pregnancy, due to the non-identification of the exact moment of the ovulation), in accordance with reports by many authors (days 12-13 of gestation) who have evaluated jennies [1,2,10-12,17,24] and mares [25-27]; however, other authors [28] have reported the first detection of the embryonic vesicle in jennies on day 14. An early pregnancy diagnosis is very useful in production animals because it allows reduction in number of days needed to inseminate the jenny again in cases of negative outcomes.

Another important reason for the early pregnancy diagnosis is to assess the risk of twin pregnancy. Compared with mares, donkeys have been reported to have a higher frequency of multiple ovulations [8,29-31], and this was also observed in our study. The early diagnosis of pregnancy allowed us to choose the best option, conservative or not, since spontaneous regression of one of the two vesicles seemed to be the most likely scenario [32].

According to our experience, the breeder decided several times not to intervene in cases of twin pregnancies, and this always resulted in the resorption of one or both of the vesicles during the first 30 days of pregnancy.

The mean vesicle diameter, measured from 16 to 18 days after the last mating (23.4 ± 6.8 mm), was in accordance with the data obtained in jennies by some authors (26.4 ± 0.7 mm) [11], but it seems to be higher compared to the data observed by other authors in jennies (21.8 mm) [24], (22.3 mm) [10] and in mares (23 mm) [33]. We should highlight that these authors monitored the ovulation follicles daily to unambiguously identify the exact moment of ovulation. Since we could not do that, our day 0 corresponded to the last day on which the jenny had accepted mating.

Therefore, because we did not know exactly the ovulation time, because we had to evaluate the jennies 24-36 h after mating, and because the growth of the embryonic vesicle is exponential until approximately the 16th day of gestation, we obtained vesicle measurements with a higher standard deviation than those reported in the literature [10, 24]. In addition, the diameter of the embryonic vesicle has been proven to be positively correlated with the day of gestation by other authors [10,12,17,24]. The data regarding the loss of sphericity of the embryonic vesicle from the 21st day (± 48 h) of gestation and the significant association with the third week of pregnancy were in accordance with the results obtained in jennies and mares by many authors [10-12,17,24,28,33-36].

The day of first detection of the embryo (23 days after the last mating) and its antimesometrial position were in accordance with reports by many authors regarding mares and jennies [10-12,17,24,33,34].

The embryo depolarization within the vesicle was observed from day 32 (± 48 h) of gestation, which is an earlier date than that reported by other authors, who detected embryo depolarization from day 35 to 53 [24], from day 41 to 47 [10], or even on day 50 [11].

Because of the positive linear relationship between the vesicle diameter, the longitudinal length of the embryo and the day of gestation, we can state that during the first stage of embryo development, it is possible to date the pregnancy and evaluate the physiological growth of the fetus.

The first day of gestation when identification and measurement of the orbit was performed was earlier than that reported by other studies on jennies. Crisci A. et al. (2014) [12] reported first detection of the orbit on day 71-96 of gestation, while we reported detection on day 70, however variability (up to 48 hours) linked to assisted natural mating and to the non-identification of the exact moment of the ovulation, must be taken in account. Early detection of the orbit, similar to all the other examined structures, depends on how the conceptus is positioned in relation to the ultrasound probe and on the quality of the transducer, which may or may not allow the correct identification of very small structures. The statistically significant correlation between the orbital dimensions and the day of gestation that we found has been proven in jennies [12] and in mares [37-40].

Moreover, ultrasonographic measurement of the orbit is simple (because the orbit stays visible until the end of the pregnancy) and provides the best indication for fetal growth, as emphasized by other authors who evaluated mares [38-40]. As a parameter that is closely related to the gestational period, it also allows dating of the pregnancy physiologically in cases in which the mating date is not known and therefore allows estimation of the time at which parturition may occur, which is very useful in clinical practice.

Even though we grouped the jennies on the basis of their weight, we did not observe a statistically significant difference in the growth of the orbit from the 100th day (± 48 h) of gestation in the two groups. This finding is in agreement with what other authors have found for donkeys [17].

Since the orbit reflects fetal growth during gestation, it can be assumed that maternal size does not affect the extent of fetal development. Indeed, in the equine species, this category of dystocia, which is common in other species, is rare [41].

The thorax and abdomen were measured for the first time 52 days after the last mating. No references were found in the literature regarding identification of the abdomen of the donkey fetus; however, the date of thorax identification was in accordance with the literature [12]. In mares, the measurement of these two structures is reported on day 100 of gestation [39].

The gastric bubble was identified for the first time earlier in our study (day 51 ± 48 h of gestation) than in other works on jennies (day 60-71 of gestation [12]).

As reported by other authors who evaluated mares [39] and applied the same statistical analysis, we found a correlation between all the studied fetal structures and the gestational age from the 100th day (± 48 h) onwards. The orbital measurements were confirmed to be the best parameter to calculate the gestational age of the fetus. Other structures may also be useful but are not as reliable. For example, the size of the gastric bubble can be considered a parameter to calculate the gestational age of the fetus; however, it can be detected for a shorter period than the orbit, as previously reported for donkeys [12] and mares [40].

Thorax and abdominal measurements may also provide an indication of the gestational age, and this finding was in agreement with other authors who described a positive linear relationship between thorax measurements and day of gestation in jennies [12] and in mares [39], as well as between the abdominal size and the day of gestation in mares [39].

The fetal gonads were observed for the first time on day 96 after the last mating and up to day 210. To perform the determination of fetal sex, the best period was found to be the 5th month of gestation (60% of the performed examinations). This range is wider than that reported by several authors (between day 100 and day 150 of gestation) for donkeys [42] and for mares [39, 43]. On the other hand, other authors reported the possibility of sexing the horse fetus from day 90 to day 180 of gestation [44] or from day 120 to day 210 [45]. The factors involved in fetal sex determination are many. Above all, the most important factors include the following: the use of an ultrasound machine equipped with a high-quality probe, possibly with the help of the Color Doppler; good environmental brightness conditions; adequate restraint of the animal; mother and fetus remaining still during the examination; relatively small size of the fetus; and posterior presentation. Most likely, more than one of these conditions occurred simultaneously in our study, and this explains why it was possible to determine the fetal sex in a wider temporal range than what has been reported in the previous literature. We often had the opportunity to observe the fetuses in posterior presentation at the time of the examination, which is a condition that is more likely to occur in jennies than in mares, as the donkey fetus is typically smaller in size than a horse fetus. In addition, with the progression of gestational age, other structures, such as the external genitalia and the mammary glands, became visible, which allowed more accurate sex determination than mere observation of the gonads.

Gonad identification, besides being essential for sexing the fetus, also allowed estimation of the gestational age, which was in agreement with other studies on mares [39,40].

The first observation of the fetal heartbeat was obtained 22 days after the last mating. The negative linear relationship between the fetal heart rate and the day of gestation was in accordance with other studies in jennies [12] and in mares [40]. Indeed, in all species, the fetal heart rate is very high in the early embryonic and fetal stages and tends to decrease, almost reaching the maternal value, close to parturition.

Additionally, a negative linear relationship (R^2 = 0.02) was found between the umbilical artery frequency (measured for the first time 125 days after the last mating) and the gestational age. However, as other authors have emphasized [46], it is very difficult to obtain realistic and reliable data on umbilical artery blood flow because this artery has a winding course, and it is not always possible to place the ultrasound probe in an optimal position for the angle of incidence between the Doppler waves and the direction of the blood flow. The same authors [46] also observed a reduction in blood flow resistance from mid-pregnancy onwards. Human clinicians believe that proper determination of the umbilical arterial blood flow may provide extremely useful data regarding the growth and health of the fetus and placenta [47]. Therefore, it is worth checking this parameter despite the technical difficulties.

The CUPT measurement was performed exclusively transrectal because of the impossibility, in field conditions, to adequately prepare the jennies (clipping of the abdomen, extended restraint time) for performing transabdominal ultrasound imaging. Our first detection of CUPT was earlier than what has been reported by other authors (day 94 ± 48 h of gestation vs. day 154) [12], but it was in accordance with the data reported on mares [6].

The authors’ choice to start measuring the CUPT from day 154 [12] is presumably due to the fact that in the equine species, the placenta is considered fully functional beginning on the 150th day of pregnancy. However, we chose to start measuring the CUPT when it became visible, even if it was still in development. The average CUPT found in our study from the 4th to the 9th month of pregnancy (M4: 4.97±1.73 cm; M5: 4.33±1.31 cm; M6: 4.41±0.94 cm; M7: 4.74±1.13 cm; M8: 4.48±1.89 cm; M9: 5.03±1.48 cm; M10: 4.87±1.98

cm; M11: 4.89±1.26 cm) were in accordance with that reported in mares by Renaudin C.D. et al. [7] and Bucca S. et al. [40]; however, it was lower than that described in jennies by Crisci A. [12]. In the mare, from the 10th to the 12th month, the average CUPT increases by 1.5-2 mm every month [7,40]. This was not found in our study, where CUPT remained virtually unchanged during gestation, even in animals that aborted. Conversely, some authors [12] reported an increase in CUPT during pathology in pregnancies. Instead, in some cases we observed a CUPT that was partially edematous. This sign in the mare is normally associated with placentitis but seems to be irrelevant in the jenny. All this data seems to indicate that these placental morphological characteristics are peculiar of donkeys and probably attributable to the longer gestation length in donkeys compared with horses [48].

Because of the current legislation on drugs in Italy, which explicitly prohibits the use of any product on equines bred for the production of milk for human consumption, the animals were not treated despite the ultrasound evidence of pathology. However, none of the jennies aborted with clinical signs of placentitis, and no placental abnormalities were found after parturition, suggesting that this aspect of CUPT may be physiologic in the donkey, may be related to the length of donkey pregnancies and may be associated with lower placental function, as described by Carluccio A. et al. [2].

In agreement with other authors [12], no abnormal echogenicity of the fetal fluids was found in all the examined pregnancies, but we detected a progressive increase in turbidity during pregnancy due to the deposition of particles within the fetal fluids, as described in mares [7,40]. This turbidity may also depend on the degree of fetal motility during the examination since the particles are settled during fetal rest and become suspended during fetal movement. The echoes emitted by the transducer through the fetal fluids stimulate the fetus, that responds by increasing its motility, causing an apparent increase in the echogenicity of the fetal fluids in advanced pregnancies. With respect to fetal motility, as reported by other authors using the same evaluation scale [12], we never observed total fetal immobility, and on the contrary, we often recorded high grade 3 motility, particularly from the 6th to the 8th month of gestation, presumably due to the greater amount of space available to the fetus. Similar results are also reported in mares [40].

In agreement with data reported for mares [40], in the last trimester of gestation, we never observed fetal presentations other than anterior presentation; however, some authors [12] have reported the possibility that the fetus may reach its final presentation even later than the 9th month of gestation in the jenny because of its relatively small size.

5. Conclusions

Our work has suggested that the jenny has peculiarities that characterize its gestational features and distinguish them from those of the mare.

In this regard, the obtained results can contribute to the definition of reference values for this species and can be used by clinicians to evaluate embryonic and fetal development, to calculate the gestational age when unknown, and to identify any anomalies that occur during the pregnancy. Nevertheless, it has to be considered that this is a field study, with all the inherent limitations of this type of work (the interval between the examinations could not be reduced, losing accuracy; the light conditions were variable; transabdominal ultrasonography could not be performed; etc.). In the future, experimental studies following all the animals from the beginning of the pregnancy until parturition, with more standardized environmental conditions, shortened interval between the ultrasound examinations and eventually the possibility to perform transabdominal ultrasonography could be very useful to widen the knowledge regarding the ultrasound evaluation of gestational parameters in this species.

6. Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

7. Bibliography

1. Tosi U, Bernabò N, Verni F, Valbonetti L, Muttini A, Mattioli M, Barboni B. Postpartum reproductive activities and gestation length in Martina Franca jennies, an endangered Italian donkey breed. Theriogenology 2013;80:120-124.
2. Carluccio A, Panzani S, Tosi U, Riccaboni P, Contri A, Veronesi MC. Morphological features of the placenta at term in the Martina Franca donkey. Theriogenology 2008;69:918-924.
3. Ginther OJ & Griffin PG. Natural outcome and ultrasonic identification of equine fetal twins. Theriogenology 1994;41:1193-1199.
4. Ginther OJ & Bergfelt DR. Embryo reduction before day 11 in mares with twin conceptuses. J Anim Sci 1988;66:1727-1731.
5. Pearson RA, Ouassat M. A guide to live weight estimation and body condition scoring of donkeys. Centre for Tropical Veterinary Medicine, University of Edinburgh. Colours Printers Ltd, 14 Carnoustic Place, Glasgow; 2000.
6. Renaudin CD, Troedsson MHT, Gillis CL, King VL, Bodena A. Ultrasonographic evaluation of the equine placenta by transrectal and transabdominal approach in the normal pregnant mare. Theriogenology 1997;47:559-573.

7. Renaudin CD, Troedsson MHT, Gillis CL. Transrectal ultrasonographic evaluation of the normal equine placenta. Equine Veterinary Education 2003;6:16-17.
8. Hagstrom DJ. Donkeys are Different: An Overview of Reproductive Variations from Horses. University of Illinois, USA, Department of Agriculture, Local Extension Councils Cooperating, 2004;January: p. 1-5.
9. Matthews N. Donkeys – Not just small horse. The North American Veterinary Conference, Gainesville, USA, 2010;January: p. 214-216.
10. Meira C, Ferreira JCP, Papa FO, Henry M. Ultrasonographic evaluation of the conceptus from days 10 to 60 of pregnancy in jennies. Theriogenology 1998;49:1475-1482.
11. Carluccio A, Villani M, Contri A, Tosi U, Veronesi MC. Rilievi ecografici della gravidanza precoce nell'asina di Martina Franca. Ippologia 2005;4:31-35.
12. Crisci A, Rota A, Panzani D, Sgorbini M, Ousey JC, Camillo F. Clinical, ultrasonographic, and endocrinological studies on donkey pregnancy. Theriogenology 2014;81:275-283.
13. Fielding D. Reproductive Characteristics of the jenny donkey-Equus asinus: a review. Tropical Animal Health and Production 1988;20:161-166.
14. Pugh DG. Donkey Reproduction. Proceedings of the Annual Convention of the American Association of Equine Practitioners, 2002; p. 113-114.
15. Villani M, Wilsher S, Carluccio A, Contri A, Veronesi MC. Stereology in the term placenta of the donkey, Thoroughbred and pony: A comparative study. Animal Reproduction Science 2006;94:411-412.
16. Galisteo J, Perez-Marin CC. Factors affecting gestation length and estrus cycle characteristics in Spanish donkey breeds reared in southern Spain. Theriogenology 2010;74:443-450.

17. Brown WH. Embryonic and fetal development in the donkey. A Capstone Experience Manuscript 2012.
18. Carluccio A, Gloria A, Veronesi MC, De Amicis I, Noto F, Contri A. Factors affecting pregnancy lenght and phases of parturition in Martina Franca jennies. Theriogenology 2015;84:650-655.
19. Heidler B, Aurich JE, Pohl W, Aurich C. Body weight of mares and foals, estrous cycles and plasma glucose concentration in lactating and non-lactating Lipizzaner mares. Theriogenology 2004;61:883-893.
20. Valera M, Blesa F, Dos Santos R, Molina A. Genetic study of gestation length in Andalusian and Arabian mares. Animal Reproduction Science 2006;95:75-96.
21. McCue PM, Ferris RA. Parturition, dystocia and foal survival: a retrospective study of 1047 births. Equine Vet Journal Suppl 2012;(41):22-25.
22. Kuhl J, Stock KF, Wulf M, Aurich C. Maternal Lineage of Warmblood Mares Contributes to Variation of Gestation Length and Bias of Foal Sex Ratio. PLOS ONE 2015;10(10):e0139358.
23. Liu C, Guo W, Lu G, Xiang W, Wang X. Complete Genomic Sequence of an Equine Herpesvirus Type 8 Wh Strain Isolated from China. Journal of Virology 2012;86(9):5407. doi:10.1128/JVI.00445-12.
24. Gastal EL, Santos GF, Henry M, Piedade HM. Embryonic and foetal development in donkeys. Equine Veterinary Journal Suppl 1993;15:10-13.
25. Bessent C, Ginther OJ. Comparison of early conceptus mobility between mares and jennies. Theriogenology 1988;29:913-920.
26. Ley WB. Broodmare reproduction for the equine practitioner. 1st ed. New York: Teton New Media; 2004.
27. Derar DR, Ali A. Conceptual Orientation: A Unique Phenomenon of the Equine Pregnancy. Anatomy and Physiology 2016;2:1-4.

28. Lemma A, Schwartz HJ, Bekana M. Application of ultrasonography in the study of the reproductive system of tropical jennies (Equus asinus). Tropical Animal Health and Production 2006;38:267-274.
29. Blanchard TL, Taylor TS, Love CL. Estrous cycle characteristics and response to estrus synchronization in mammoth asses (Equus asinus americanus). Theriogenology 1999;52:827-834.
30. Taberner E, Medrano A, Penã A, Rigau T, Mirò J. Oestrus cycle characteristics and prediction of ovulation in Catalonian jennies. Theriogenology 2008;70:1489-1497.
31. Quaresma M, Payan-Carreira R. Characterization of the estrous cycle of Asinina de Miranda jennies (Equus asinus). Theriogenology 2015;83:616-624.
32. Ginther OJ. Reproductive biology of the mare (Basic and applied aspects). 2nd ed. Cross Plains, Wisconsin, Equiservices Publishing; 1992, p. 75-232.
33. Ginther OJ. Ultrasonic imaging and reproductive events in the mare. Cross Plains, Wisconsin, Equiservices Publishing; 1986, p. 378.
34. Ginther OJ. Fixation and orientation of the early equine conceptus. Theriogenology 1983;19:613-623.
35. Ginther OJ. Ultrasonic anatomy and pathology of the reproductive tract. Proceedings of the Equine Ultrasound Short Course, Fort Collins, Colorado State University; 1984a; p. 16-25.
36. Ginther OJ. Ultrasonic evaluation of the reproductive tract of the mare: the single embryo. Journal of Equine Veterinary Science 1984b;4:75-81.
37. Kahn W & Leidl W. Ultrasonic biometry of horse foetuses in utero and sonographic representation of their organs. Dtsch Tierarztl Wochenschr 1987;94:509-515.
38. McKinnon AO, Squires EL, Pickett BW. Equine Reproductive Ultrasonography, Bull. No. 4. Fort Collins, Colorado State University Animal Reproduction Laboratory, 1988; p. 31-40.

39. Renaudin CD, Gillis CL, Tarantal AF, Coleman DA. Evaluation of equine fetal growth from day 100 of gestation to parturition by ultrasonography. Journal of Reproduction and Fertility Suppl 2000;56:651-660.

40. Bucca S, Fogarty U, Collins A, Small V. Assessment of feto-placental well-being in the mare from mid-gestation to term: Transrectal and transabdominal ultrasonographic features. Theriogenology 2005;64:542-557.

41. Frazer G. Dystocia Management. In: McKinnon AO, Squires EL, Vaala WE, Varner DD, editors. Equine Reproduction - Vol.2. 2nd ed. Wiley-Blackwell Publishing; 2011, p. 2479-2496.

42. Mancuso R, Scollo C, Torrisi C, Catone G La determinazione del sesso fetale nell’asina [Fetal sex determination in donkey mare]. In: Proc. Annual Meeting of the Italian Association of Equine Veterinarians, Bologna, Italy, 2007; p. 173-174.

43. Livini M. Determination of Fetal Gender by Transrectal Ultrasound Examination: Field’s Experience. Proceedings of the Annual Convention of the American Association of Equine Practitioners, Baltimore, MD, USA, 2010; p. 323-327.

44. Resende HL, Carmo MT, Ramires Neto C, Alvarenga A. Determination of equine fetal sex by Doppler ultrasonography of the gonads. Equine Veterinary Journal 2014;46:756-758.

45. Bucca S. Equine fetal gender determination from mid- to advanced-gestation by ultrasound. Theriogenology 2005;64:568-571.

46. Bollwein H, Weber F, Woschée I, Stolla R. Transrectal Doppler sonography of uterine and umbilical blood flow during pregnancy in mares. Theriogenology 2004;61:499-509.

47. Gudmundsson S, Flo K, Ghosh G., Wilsgaard T, Acharya G. Placental pulsatility index: a new more sensitive parameter for predicting adverse outcome in

pregnancies suspected of fetal growth restriction. Acta Obstet Gynecol Scand 2016;November.

48. Carluccio A, Noto F, Parrillo S, Contri A, De Amicis I, Gloria A, Robbe D, Veronesi MC. Transrectal ultrasonographic evaluation of combined utero-placental thickness during the last half of pregnancy in Martina Franca donkeys. Theriogenology 2016;86(9):2296-2301.

8. Figures

Figure 1 CUPT measurement during the 8th month of gestation. D1: CUPT (5.20 mm); CS: cervical star; UA: uterine artery.

Figure 2 Linear regression between the vesicle diameter and the gestational age during the first three months of gestation.

Figure 3 Linear regression between the embryo length and the gestational age during the first three months of gestation.

Figure 4 (A) orbital diameter at 85-days (D1=0.89 cm), (B) at 152-days (D2=1.65), (C) at 211-days (D3=2.1 cm) and (D) at 261-days (D4=2.33 cm) of gestation.

Figure 5 Linear regression between the fetal orbital diameter and the gestational age from the 100th day of gestation onwards.

Figure 6 (A) gastric bubble diameter at 52-days (D1=0.31 cm), (B) at 100-days (D2=0.72), (C) at 103-days (D3=1.03 cm) and (D) at 135-days (D4=1.25 cm) of gestation; TR: transversal diameter.

Figure 7 CUPT measurement during the 10th month of gestation. Some portions of the CUPT are edematous. D1: CUPT (5.56 mm); CS: cervical star; UA: uterine artery.

Figure 8 CUPT measurement during the 4th month of gestation. The placenta is diffusely edematous (yellow arrow).

Figure 9 Grade of echogenicity of the allantoic fluids. The percentages of fetuses showing grade 0 or 1-2 throughout pregnancy are indicated.

Figure 10 Comparison between the fetal orbital diameter and maternal size from day 100 to day 351 of gestation.

Table 1 Gestational months (and corresponding days) in which maternal-fetal parameters were recorded.

Gestational Month	Days
First month - M1	0-31
Second month - M2	32-62
Third month - M3	63-93
Fourth month - M4	94-124
Fifth month - M5	125-155
Sixth month - M6	156-186
Seventh month - M7	187-217
Eighth month - M8	218-248
Ninth month - M9	249-279
Tenth month - M10	280-310
Eleventh month - M11	311-341

Table 2 Measurements of maternal-fetal parameters taken in different months of gestation in jennies.

Gestation (Month)	Parameter (cm)	Mean ± DS (Range)	Median
1st	Vesicle diameter	2.50±0.86 (0.46-3.96)	2.40
	Embryo	1.13±0.42 (0.58-2.29)	1.06
2nd	Vesicle diameter	4.84±1.17 (2.83-7.3)	4.78
	Embryo	2.19±0.92 (0.93-3.96)	1.90
	Thorax	1.49±0.18 (1.2-1.68)	1.53
	Abdomen	1.60±0.21 (1.22-1.86)	1.64
	Gastric bubble	0.22±0.1 (0.1-0.37)	0.20
	BPM*	219.67±36.11 (158-270)	230
3rd	Vesicle diameter	7.47±1.80 (3.56-10.70)	7.16
	Embryo	4.98±1.03 (3.30-6.70)	5.50
	Thorax	1.80±0.50 (1.13-2.30)	1.88
	Abdomen	2.45±0.52 (1.24-3.09)	2.62
	Gastric bubble	0.56±0.24 (0.15-0.95)	0.57
	Orbit	0.66±0.14 (0.42-0.90)	0.70
4th	Thorax	4.77±0.46 (4.27-5.20)	4.70
	Abdomen	5.15±0.7 (3.93-6.20)	5.22
	Gastric bubble	1.52±0.75 (0.22-3.01)	1.41
	BPM*	245.40±36.26 (183-290)	254.50
	Orbit	1.13±0.19 (0.84-1.51)	1.16
	Gonad	1.57±0.20 (1.32-1.90)	1.51
	CUPT	4.97±1.73 (3.15-9.90)	4.30
5th	Thorax	5.31±0.65 (4.72-6.86)	5.14
	Abdomen	5.75±0.66 (4.72-7.03)	5.61
	Gastric bubble	2.26±0.82 (1.22-3.56)	2.18
	BPM*	198.18±35.17 (154-252)	193
	Orbit	1.50±0.14 (1.28-1.78)	1.49
	Gonad	3.44±1.01 (2.2-5.31)	3.10

	CUPT	4.33±1.31 (2.01-7.77)	4.32
	Umbilical artery (beats/min)	258.25±32.87 (204-295)	268.5
6th	Thorax	5.72±0.41 (5.04-6.12)	5.80
	Abdomen	6.23±0.32 (5.83-6.83)	6.21
	Gastric bubble	2.86±0.89 (1.26-4.03)	3.08
	BPM*	193.22±47.79 (156-299)	171
	Orbit	1.91±0.20 (1.55-2.3)	1.91
	Gonad	3.34±0.45 (2.47-4.05)	3.47
	CUPT	4.41±0.94 (2.68-6.39)	4.40
	Umbilical artery (beats/min)	235.33±40.69 (164-280)	235
7th	Gastric bubble	2.82±2.93 (2.57-2.96)	2.93
	BPM*	152.33±26.50 (122-171)	164
	Orbit	2.11±0.25 (1.55-2.51)	2.16
	CUPT	4.74±1.13 (2.68-6.81)	4.75
	Umbilical artery (beats/min)	267.50±4.93 (262-295)	267.5
8th	Orbit	2.44±0.22 (2.18-2.95)	2.45
	CUPT	4.48±1.89 (0.8-8.08)	4.36
9th	Orbit	2.51±0.26 (2.00-2.82)	2.53
	CUPT	5.03±1.48 (1.20-7.30)	5.51
10th	Orbit	2.56±0.28 (2.00-3.13)	2.58
	CUPT	4.87±1.98 (0.70-8.36)	4.69
11th	Orbit	2.80±0.31 (2.39-3.20)	2.84
	CUPT	4.89±1.26 (3.20-7.75)	4.63

CUPT (combined utero-placental thickness); * (heart beat/min).

Table 3 Linear regression of the embryonic vesicle and embryo length in the first three months of gestation.

Embryo growth parameter	**N**	**Range**	**Days**	**r^2**	**b_0**	**b_1**	**p-value**
Embryo vesicle (mm)	99	4.6-1.07	12-91	0.83	7.13	0.88	< 0.001
Embryo length (cm)	49	0.58-6.70	23-90	0.75	-0.84	0.07	< 0.001

r^2: correlation coefficient. b_0 and b_1: coefficients of the linear regression equation ($y = b_0 + b_1x$); y: calculated value for the variable; x: gestational age.

Table 4 Linear regression of the fetal parameters from the 100th day of gestation onwards.

Fetal growth parameters (cm)	**N**	**Range**	**Days**	**r^2**	**b_0**	**b_1**	**p-value**
Orbit	128	0.92-3.2	100-351	0.79	0.60	0.01	< 0.001
Gastric bubble	48	0.72-4.12	100-210	0.23	0.10	0.02	< 0.001
Thorax	21	4.27-6.86	100-185	0.70	2.43	0.02	< 0.001
Abdomen	24	4.72-7.03	100-185	0.46	3.74	0.02	< 0.001
Gonad	27	1.32-5.31	100-185	0.59	-2.02	0.03	< 0.001
Heart rate*	31	122-299	101-212	0.37	340.70	-0.93	< 0.001
Umbilical artery*	27	164-295	125-212	0.02	286.74	-0.20	0.44

r^2: correlation coefficient. b_0 and b_1: coefficients of the linear regression equation ($y = b_0 + b_1x$); y: calculated value for the variable; x: gestational age. * (heart beat/min).

Highlights.

1. To date the literature on donkey physiology is still scarce and the lack of knowledge appears to be wider than expected.
2. The aim of this study was to analyze ultrasound measurements of fetal-maternal structures during pregnancies in jennies.
3. Orbital diameter and CUPT were the most reliable parameters for monitoring the donkey pregnancy.
4. The CUPT evaluation may be useful for the diagnosis of placental diseases.
5. Our results can contribute to the definition of reference values for this species.

Dear Editorial Team,
We would like to thank you and the Reviewers for the helpful suggestions and comments that offered us the opportunity to improve our manuscript. We complain because, before submitting the manuscript to your journal, it has been sent to an author services company (American Journal Experts, Certificate Verification Key: D789-53BA-B8C3-63A3-7093) that has revised it for English. Moreover we did our best to improve language and grammar also with native English speakers. We hope to satisfy your request.
The manuscript has been completely revised according the Reviewers' suggestions.
Our responses to the Reviewers' comments are below.

Reviewer #1
Initially the title gives us the impression that some correlation or relationship exists between the production of milk for human consumption (title), but this have being forgotten in the course of the paper.
There is no well defined objective, it is not known if the production of milk for human consumption is part, or if the goal is to compare jennies with different weights or the fetal biometry analyses without groups comparation.
Answer: Thank you for your suggestion, we agree and, for that reason, we decided to modify the title because milk production was not influencing the results.

One of the proposed objectives is to determine the gestational age by the measurements of the embryonic vesicle and fetal biometry, however the date of ovulation was not verified since the system used was natural mating. Therefore all accuracy of measurements can not be considered.
Answer: We thank you for your comment, it is true that we did not verify the ovulation, but we are considering a period up to 24 h because we recorded the exact date of the last assisted mating (in fact probably less than 24 h considering the time window in which jennies accept the stallion and the limited viability of the oocyte after ovulation in terms of time). We performed the pregnancy diagnosis on day 12 after the last mating in order to include the animals in the study as soon as possible, thus, maybe we detected the embryonic vesicle on day 13 of pregnancy instead of 12, as we mentioned in the text, obtaining a higher standard deviation of the vesicle diameter at the beginning of the pregnancy, but then it decreased rapidly. The same can be affirmed about the embryo length. However we inserted in the manuscript sentences that state clearly the presence of this variability linked to the assisted natural mating and to the non-identification of the exact moment of the ovulation. Moreover, most of the parameters considered in the study are focused on later stages of pregnancy (>3 weeks).
Furthermore it should be considered that this is a field study, performed in a family-managed farm located in a rural area, with a difficult logistic, so the experimental design was planned considering these complications and the fact that even if we could have performed a more frequent ultrasound examination of the jennies (for example every 12 h after mating), the standard deviation of the above mentioned parameters would not have decreased significantly.

The animals were divided into two groups of weights, but these groups were not compared in most of the analyzes.
Answer: Thank you for your observation. We evaluated all the parameters but we reported just the orbital diameter in relation to maternal size because this structure is the easiest to measure and also because this parameter appear to be the most related to the gestational age.

In some moments, the authors reported two reproductive seasons in another one season only ... the animals presented different gestational phases in different periods of the year, with some the evaluations starting at the end of gestation and others at the beginning (not the same animal), which generated confusion.
Answer: Thank you for your observation. At first, we performed a field study, so examination of the animals started at the reproductive stage at which they were in at the moment of the first clinical examination. In the third season we will monitor all the animals starting from the beginning of the pregnancy and following them until parturition.

The method used for transrectal ultrasonography is not the most suitable for evaluation of fetal biometry from the middle third of gestation ... therefore the measurements performed may have less acuracy.
Answer: Thank you for your observation. Yes, we know that, but in our field condition the transrectal ultrasonography was the best choice because of its suitability and rapidity also in relation to the impossibility to adequately prepare the jennies (clipping of the abdomen, extended restraint time, light condition, etc.) to perform transabdominal ultrasonography. Moreover, in our climatic condition, winter season is very cold and being the jennies so sensitive to low temperatures, the breeder did not give us permission to clip them.

lines 116 to 120 can be removed
Answer: Thank you for your suggestion. We removed the lines as you indicated, we just let information regarding the restraints in stocks, because the thought that this is important since allowed as to perform a more detailed and precise measuring. We also let the details regarding the person who performed the examination thus, in our experience, measurements can vary if taken by unskilled operators.

lines 156 to 159 is a result and not methodology
Answer: We moved these lines in the result section, thank you for your suggestion.

line 162 first trimester of gestation goes from day 0 to day 90, the authors report later in the text that at the beginning of gestation it is not possible to evaluate orbit diameter and this evaluation depends a lot on the accuracy of the ultrasound device used.
Answer: Thank you for your suggestion, we add in the text that the orbit is visible starting from day 70.

line 231, it is not clear whether mammary gland evaluation was also a parameter evaluated.
Answer: We did not evaluate this parameter. Thank you for your observation, we removed this part.

line 318, sentence does not make sense
Answer: This sentence has been modified, thank you for your suggestion.

line 383, does not describe that gestational fluid is being evaluated, allantoid or amniotic, which causes problems since the echogenicity proposed in the methodology is different between them, especially in the final third of gestation. So this evaluation needs to be discarged.
Answer: Thank you for your observation, we evaluated the allantoic fluids. A graphical representation of these data was added to the paper.

line 417 to 418, the foaling does not mean that it does not have some gestational or placental problem/disease.
Answer: Thank you for your observation. Yes, we are aware of it, but we analysed the morphological aspect of all the placentas and they were normal, moreover the Apgar scores of the foals were in the physiological range.

line 451, HEV = EHV
Answer: We corrected it, thank you for your observation.

line 470 to 473, the authors describe a high frequency of multiple ovulations for the species, which was also observed by them, but in the methodology natural mating was performed and no follicular growing and ovulation was evaluated.
Answer: Thank you for your observation. We performed transrectal ultrasonography after mating and in case of multiple ovulations, multiple active (as confirmed by doppler) corpora lutea and eventually multiple (two in our experience) embryonic vesicles were detected.

line 506 to 507, one day of difference in the results can not be considered when natural mating was used. **Answer**: Thank you for your observation. Yes, we agree. We inserted in the text a sentence regarding the need to consider the variability (as described before, up to 24 hours) linked to assisted natural mating and to the non-identification of the exact moment of the ovulation.

The results of CUPT are controversial and the conclusions do not coincide with the results. There is an affirmation by the authors that it is impossible to diagnose placentitis by means of the evaluation of CUPT in jennies, unfortunately the work was not designed for this, nor does it present a compatible methodology for such an incisive affirmation.
Answer: Thank you for your observation probably there is a misunderstanding. We mentioned placentitis and placental alterations only in the discussion. In our work we observed thickening or edema of placenta probably due only to the prolonged pregnancy of jennies compared with the mare.

The linear regression tables become unnecessary with the presentation of the equations and graphs.
Answer: Thank you for your observation, they can be eliminated.

Most of the results lack p value (probability)
Answer: Thank you for your observation, we inserted p-values also in the text.

The authors conclude that the measurements and parameters are different from equines, but this comparison was not performed.
Answer: Thank you for your observation. We wrote our conclusions on the basis of the literature, so we discussed about consideration made by different authors on jennies and mares.

First, third and fourth paragraphs of the conclusion are not conclusions and the fifth paragraph does not correspond to the objectives.
Answer: Thank you for your observation. We have modified the text following your indications.

I strongly recommend that the authors review all article, simplify evaluations and results with a focus on fetal biometrics, showing the limitations of the methodology.
Answer: Thank you for your suggestion. We have modified the text following your indications.

Reviewer #2:
The authors studied the "Evaluation of some gestational parameters in jennies bred for the production of milk for human consumption". The research topic is within the scope of the journal. However, there is no data supporting the milk production and analyses and the title should be changed to: Evaluation of some gestational parameters in Jennies bred. This manuscript contains some innovative information compared to published articles related to gestational parameters from this specie. The manuscript is interesting and with new insight on fetal-maternal evaluation during the 2-years period studied. The general idea is organized and provides useful findings. Furthermore, the editing needs minor corrections as indicated into the manuscript upload into the system. I can recommend this paper for publication in this journal.
The uploaded document is included into the system. Some points are describe below:

-The uses of Doppler ultrasoun to evaluate fetal growth may improve the manuscript importance. Authors should include the data on fetal hemodynamics to provide some additional information regard this specie.
Answer: We used the Doppler only for the evaluation of the CL and for the sexing of the fetus.
The instrument was new and we were not trained to make other measurements; moreover, the operating conditions were not optimal. Thus, data regarding the other determinations that we tried to do did not seem to be reliable, and we decided to not report them in the paper.

- Authors should take into consideration a more appropriated design figures to address the manuscript. They should also add more pictures to show and to compare ultrasound findings during the gestational period. Such as the orbital, gut, and other parameters measurements on ultrasound figures. Perhaps, they can use in the same figure (E.g.: Figure 4 (a) orbital growth at 6-months of age, (b) orbital growth at 11-months of age, and so on…). Graphs and figures need a highly resolution definition. Tables are not in the journal format.
Answer: Thank you for your observation. We have added pictures in the manuscript, as you suggested. The table format has been modified and we tried our best to improve the resolution of graphs and figures.

- There are too many subsections in the material and methods section. Avoid using them. Readers tend to become lost when too many subsections are described with few sentences in each. Try to combine some of the subsections. We tried to change this using the review track in the uploaded manuscript.
Answer: Thank you for your observation. We followed your suggestion and now most of the subsections are combined as you indicated in the manuscript.

- Provide a graph, simple bars, to show the results that describe the % of echogenicity and the evaluation thought out the gestational period. (see comment in the uploaded document).
Answer: We inserted the graph as you indicated, thank you for your suggestion.

Reviewer #4:
The study about EVALUATION OF SOME GESTATIONAL PARAMETERS IN JENNIES BRED FOR THE PRODUCTION OF MILK FOR HUMAN CONSUMPTION. The aim was to create the ideal conditions
96 for the animals to show the typical behavioral patterns of the species for animal welfare reasons. By following this procedure, it was possible to know the exact day of mating and avoid excessive stress to the jenny caused by the aggressiveness of the stallions.
Considerations
1- The abstract is too long and needs to be shortened
Answer: We shortened it, thank you for your suggestion.

2- Line 100: "After parturition, the jennies were left with the foals for approximately one month without being milked. After this period, the jennies were considered in lactation for the subsequent six months". This sentence should be in the material and methods.
Answer: Yes, it is in material and methods.

3- Line 84: "chest circumference". Change for Thoracic circumference
Answer: We changed it. Thank you for your suggestion.

4- Lines 131 to 140: for this information it is more appropriate to use a table
Answer: Thank you for your suggestion, we added a table.

5- 2.5.2.5. Orbit The orbit of the fetus was measured along its major axis from the first trimester to the end of gestation. What are the measures used? Measures for donkeys already exist?
Answer: The operator measured the orbit along the major axis, repeated the measurement twice and we reported the average of the two values. In the discussion we referred to data derived from bibliography.

6-The study is relevant. However, the need for the study
The article needs to be rewritten from Material and Methods, Discussion and Conclusion. For it is very difficult to read and understand.
Answer: The manuscript has been completely revised according to the Reviewers' suggestions, thank you for your observations.

The charts and tables need to be republished. I suggest using a program to produce the graphics.
Answer: We re-edited tables and charts, thank you for your observation.

Finally, the article needs a complete reformulation for submission to this paper.
Answer: Thank you for your suggestion. The manuscript has been fully reviewed and reformulated, we hope that it flows better now.

Figure 1

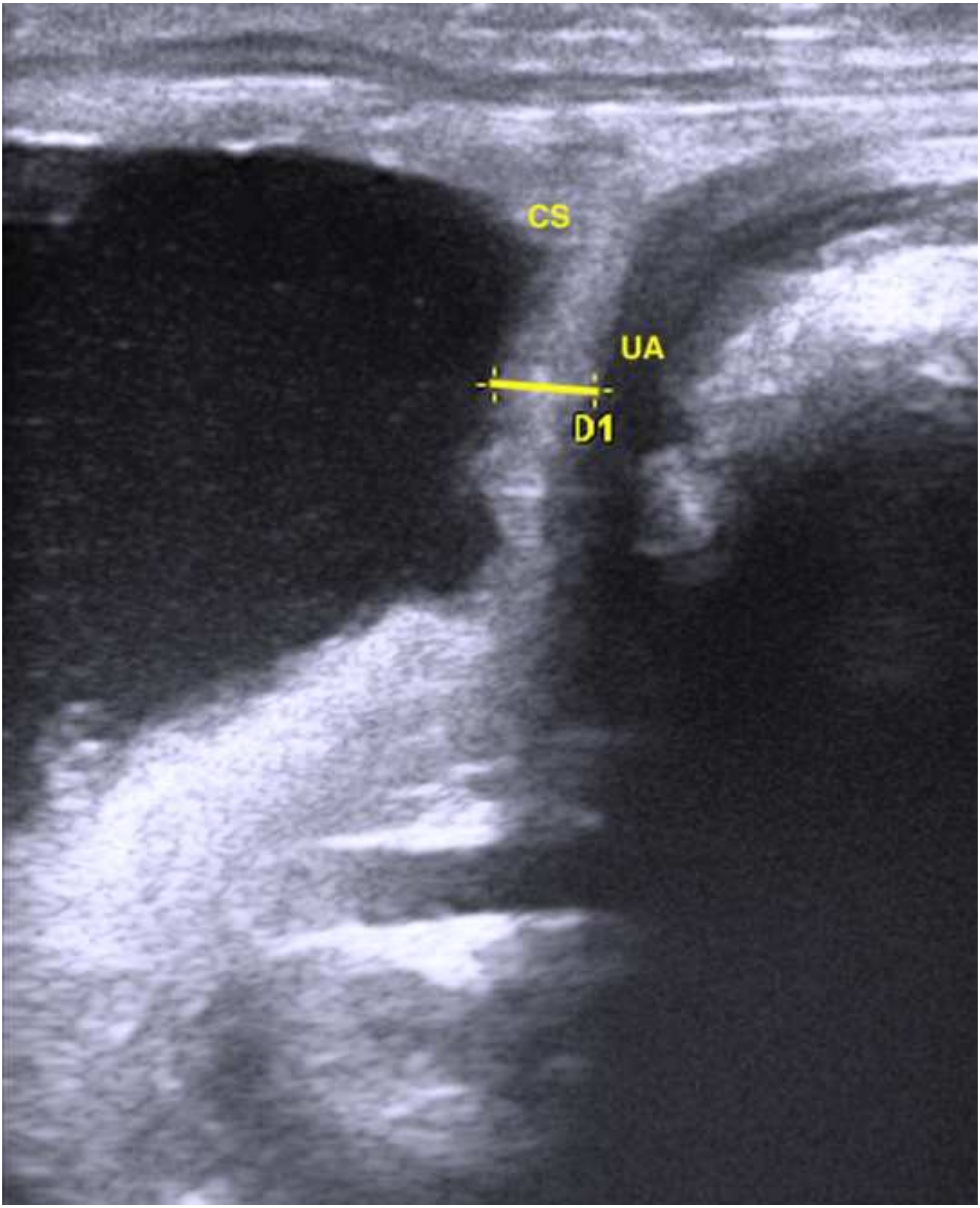
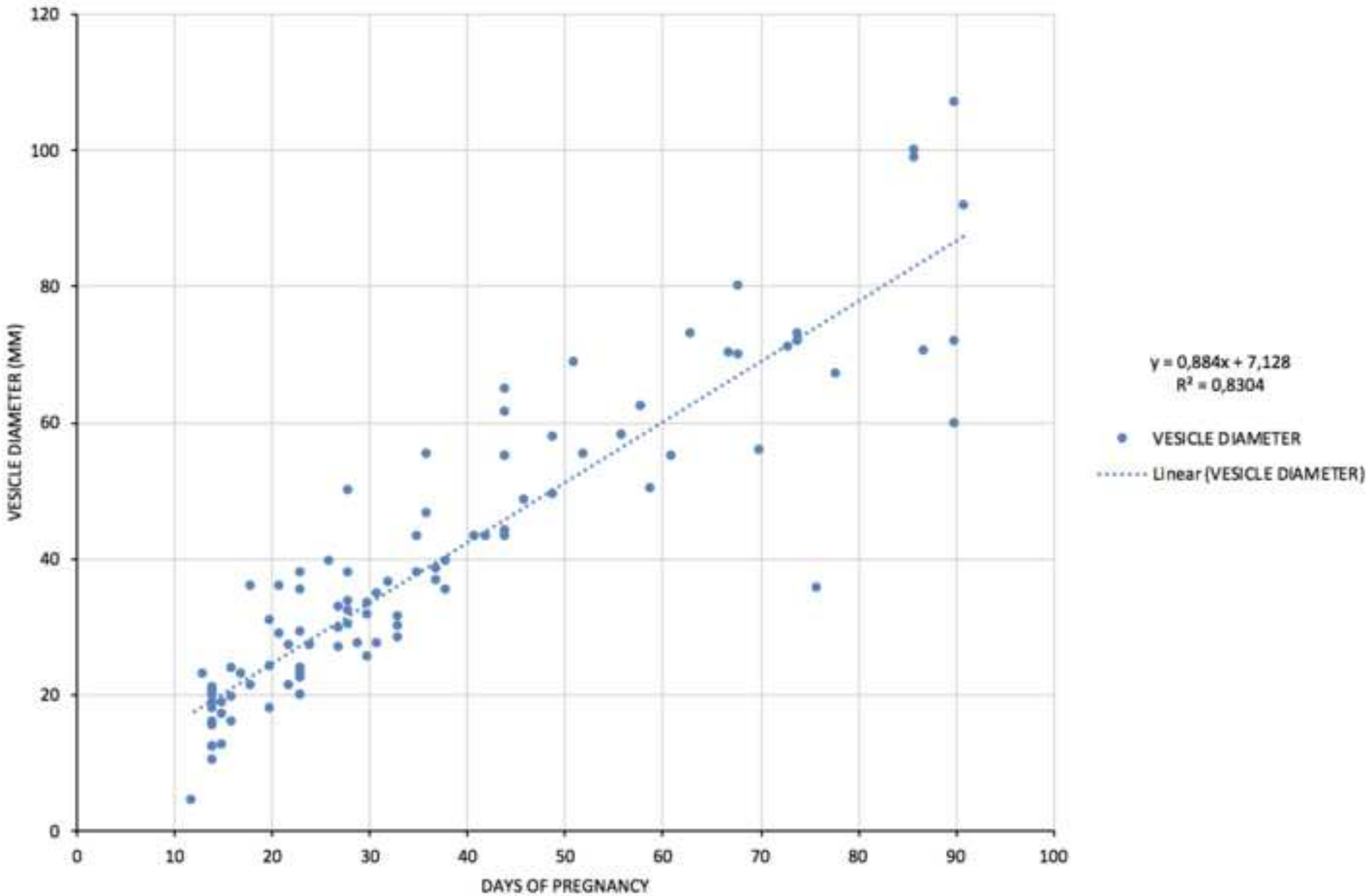
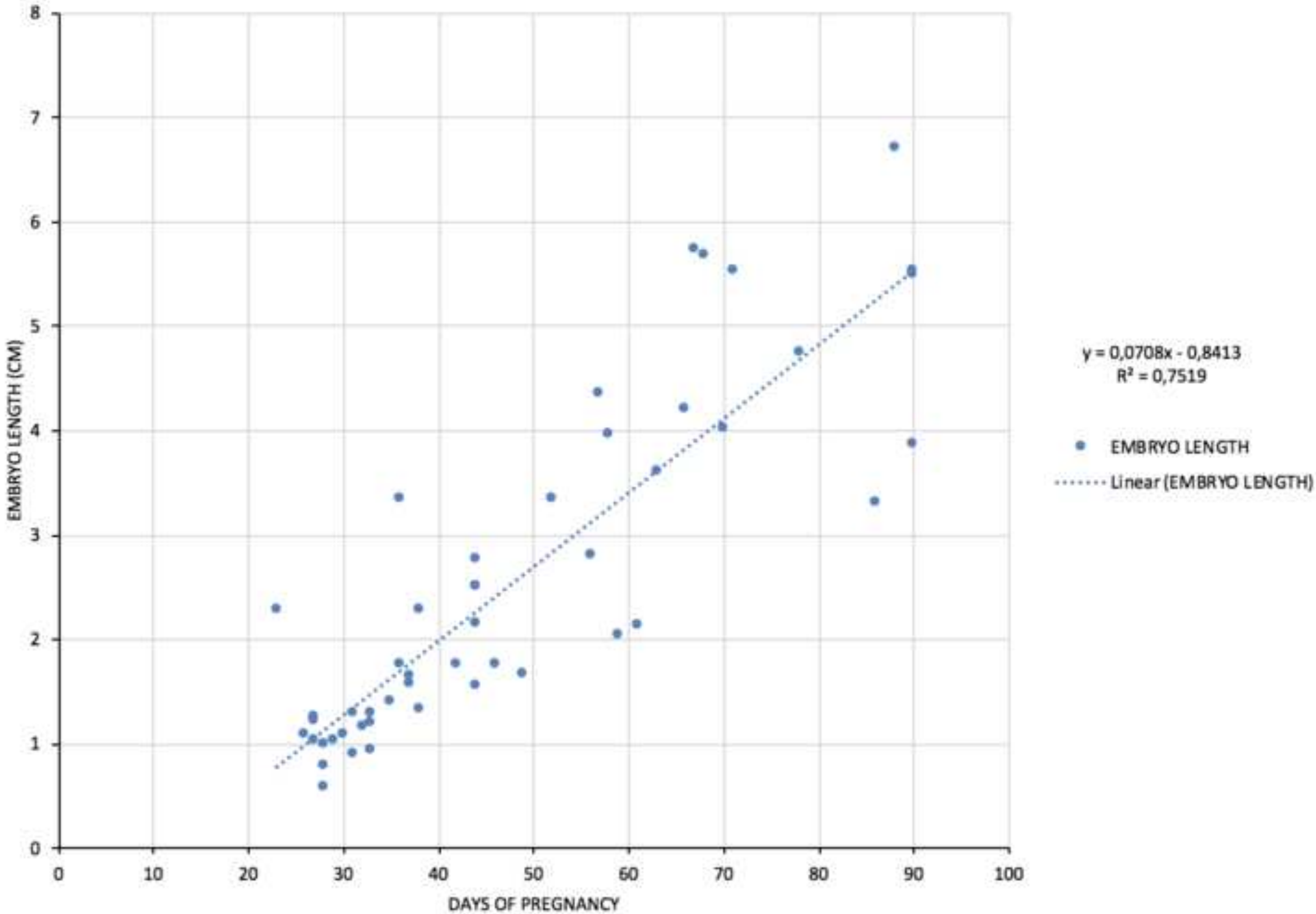


Figure 2



120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
VESICLE DIAMETER (MM)
DAYS OF PREGNANCY
y = 0,884x + 7,128
R² = 0,8304
VESICLE DIAMETER
Linear (VESICLE DIAMETER)

Figure 3



8
7
6
5
4
3
2
1
0
EMBRYO LENGTH (CM)
0
10
20
30
40
50
60
70
80
90
100
DAYS OF PREGNANCY
y = 0,0708x - 0,8413
R² = 0,7519
EMBRYO LENGTH
Linear (EMBRYO LENGTH)

Figure 4

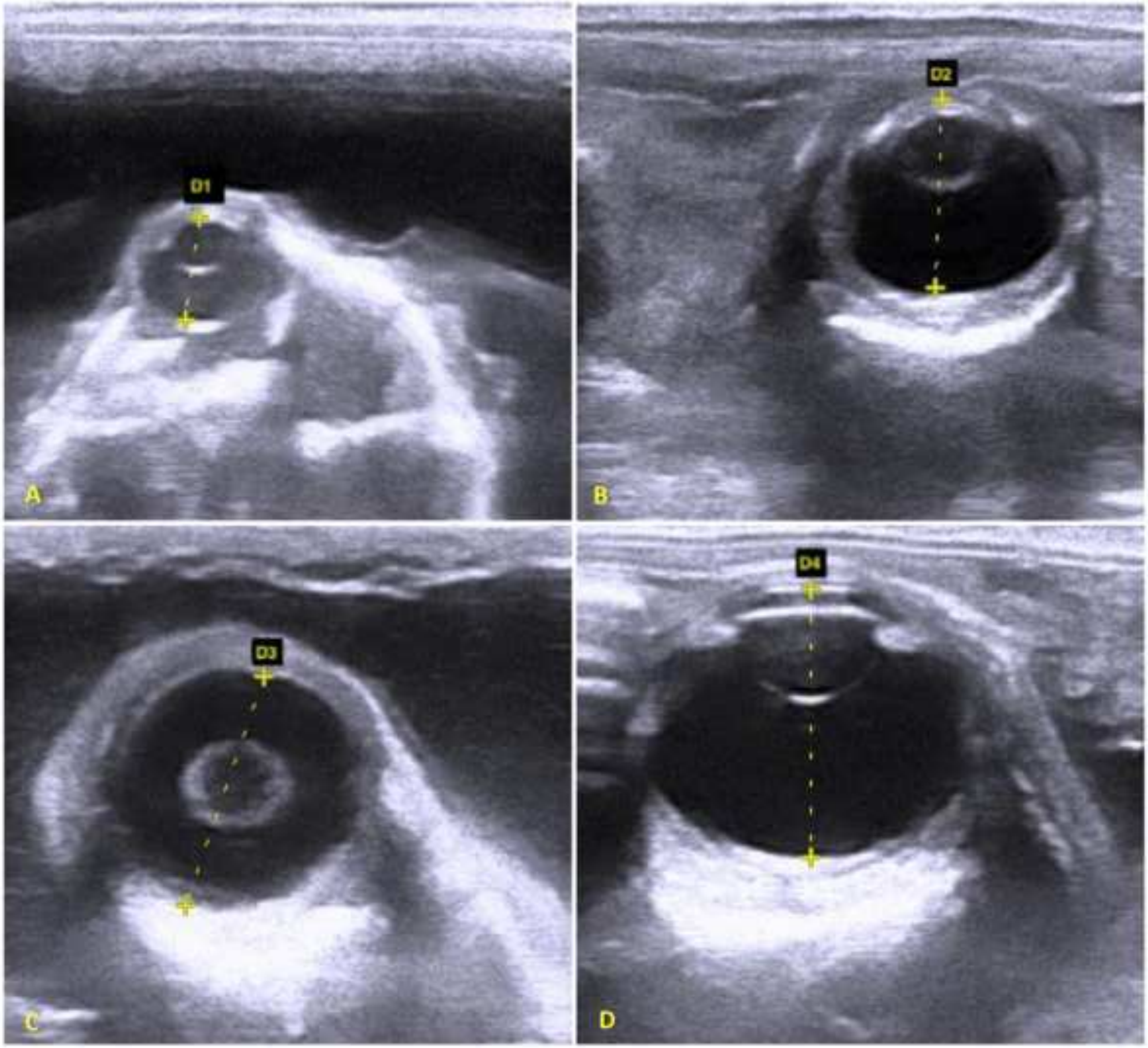
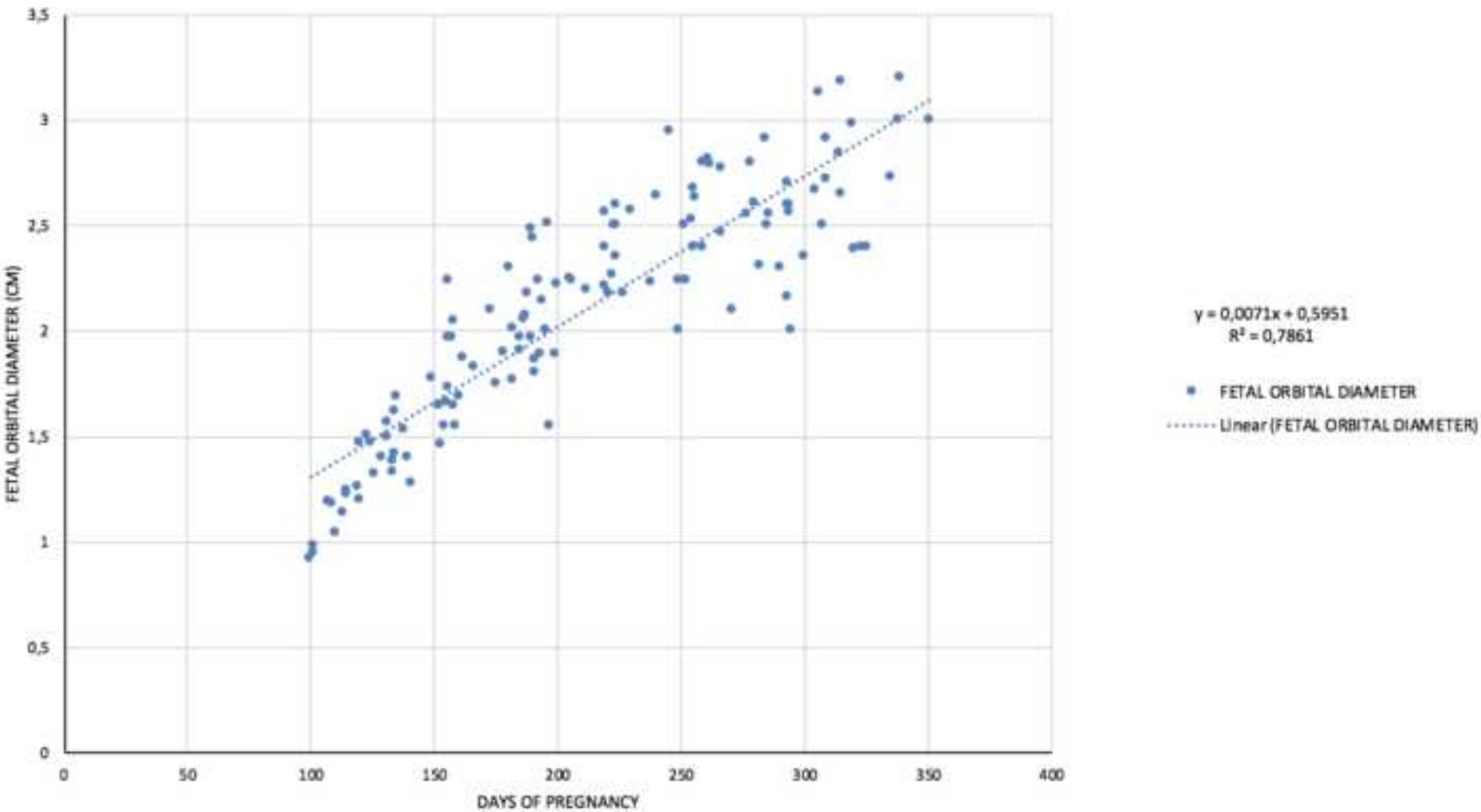


Figure 5



3,5
3
2,5
2
1,5
1
0,5
0
0
50
100
150
200
250
300
350
400
FETAL ORBITAL DIAMETER (CM)
DAYS OF PREGNANCY
y = 0,0071x + 0,5951
R² = 0,7861
FETAL ORBITAL DIAMETER
Linear (FETAL ORBITAL DIAMETER)

Figure 6

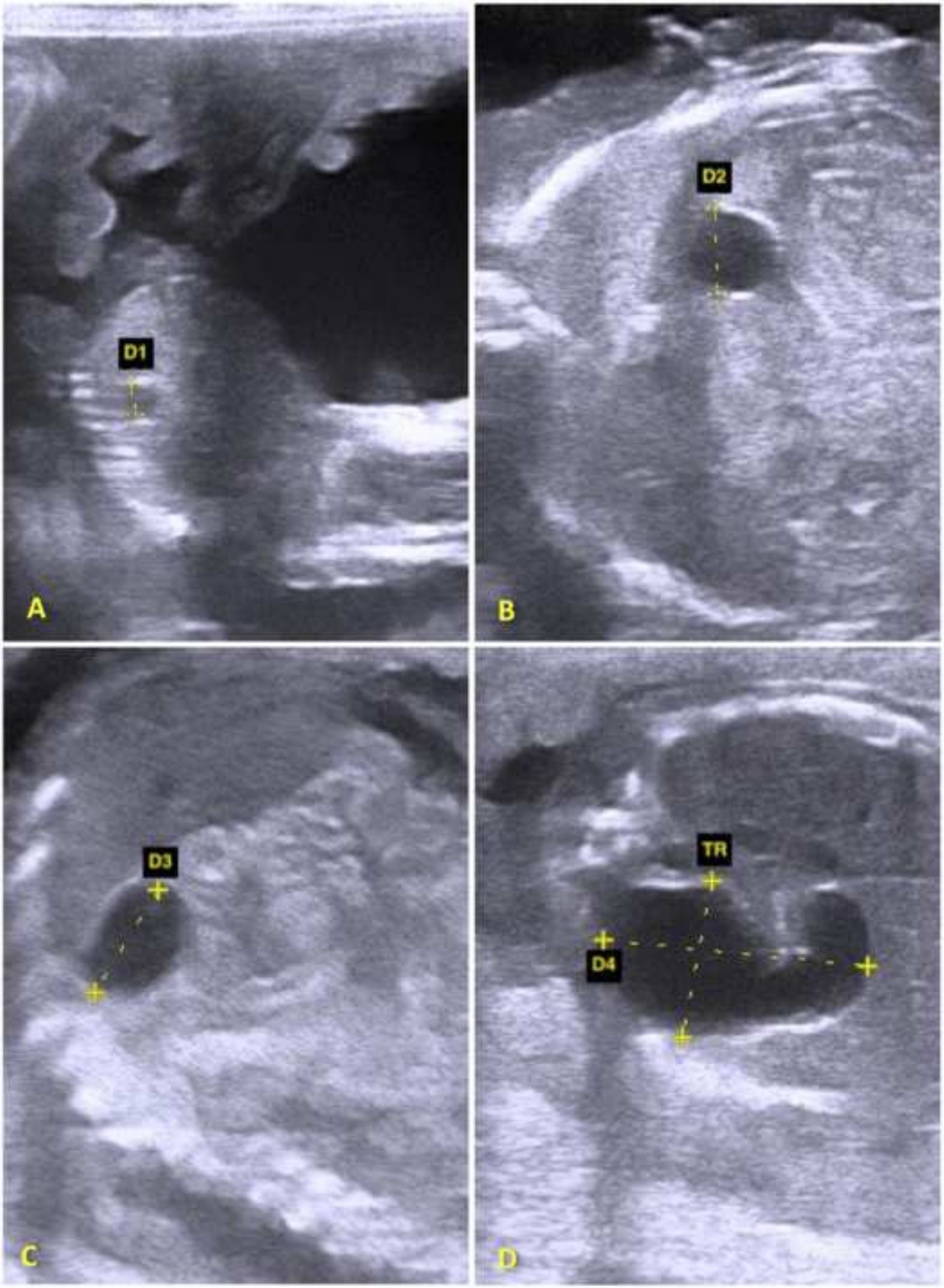


Figure 7

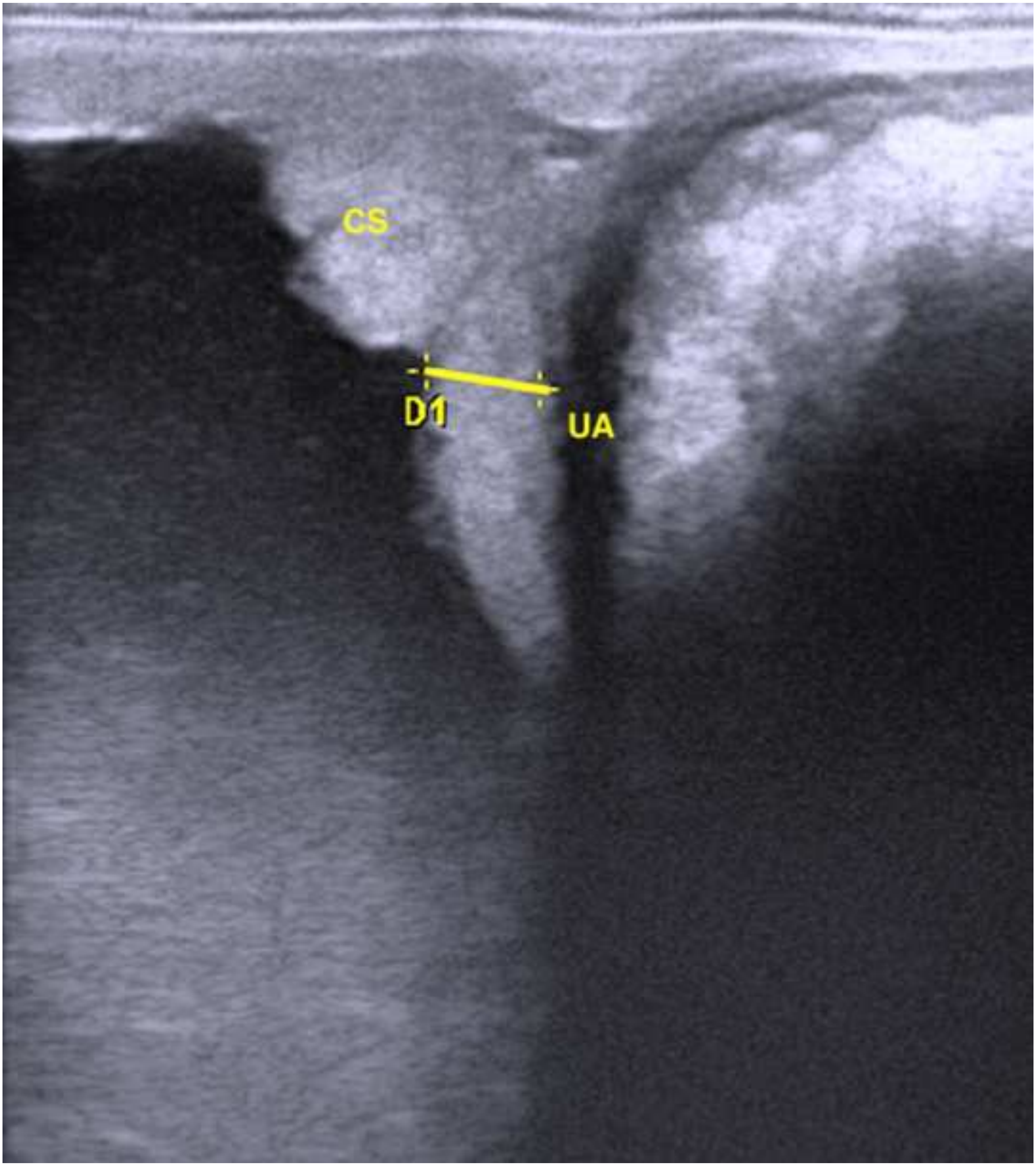


Figure 8

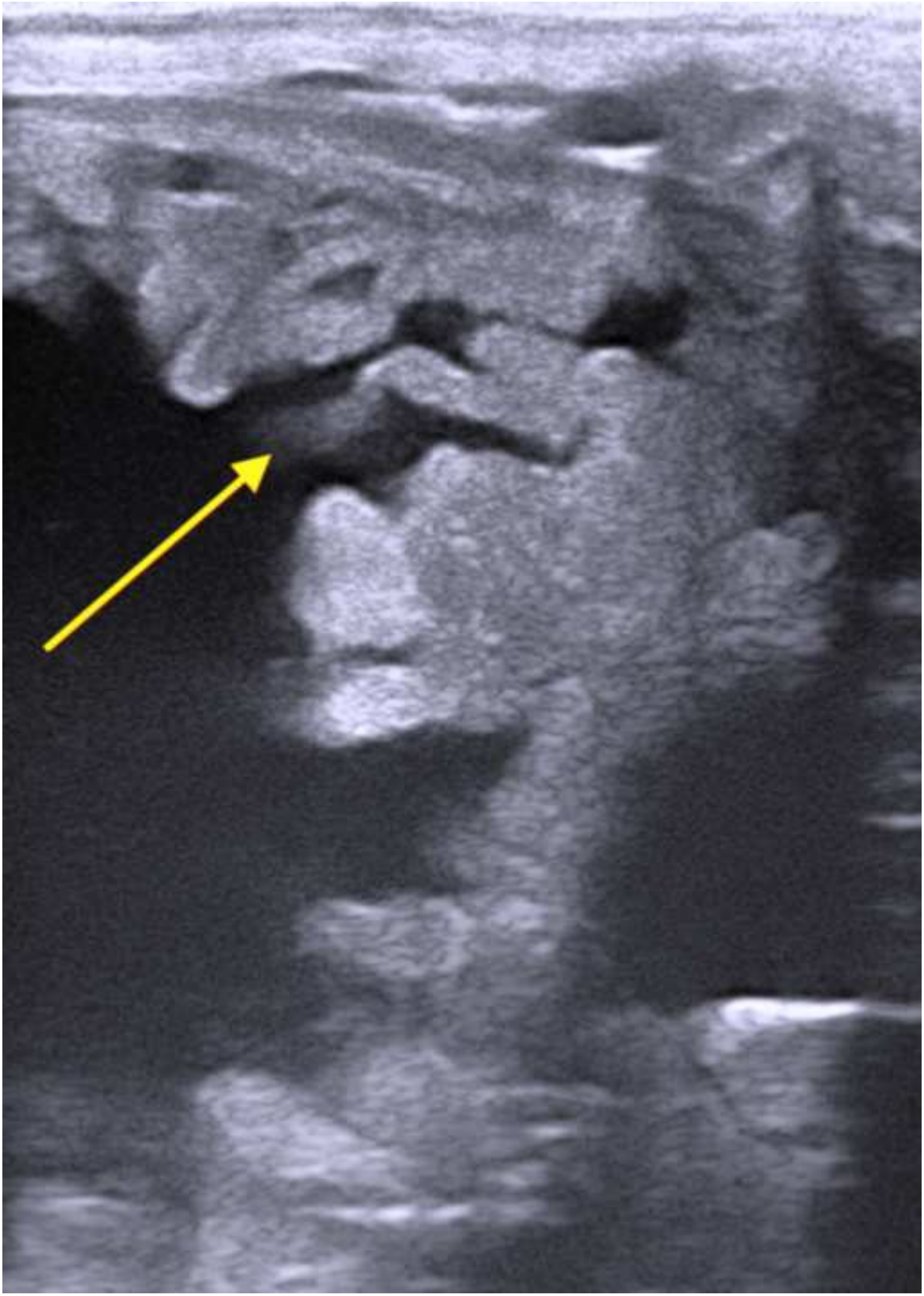


Figure 9

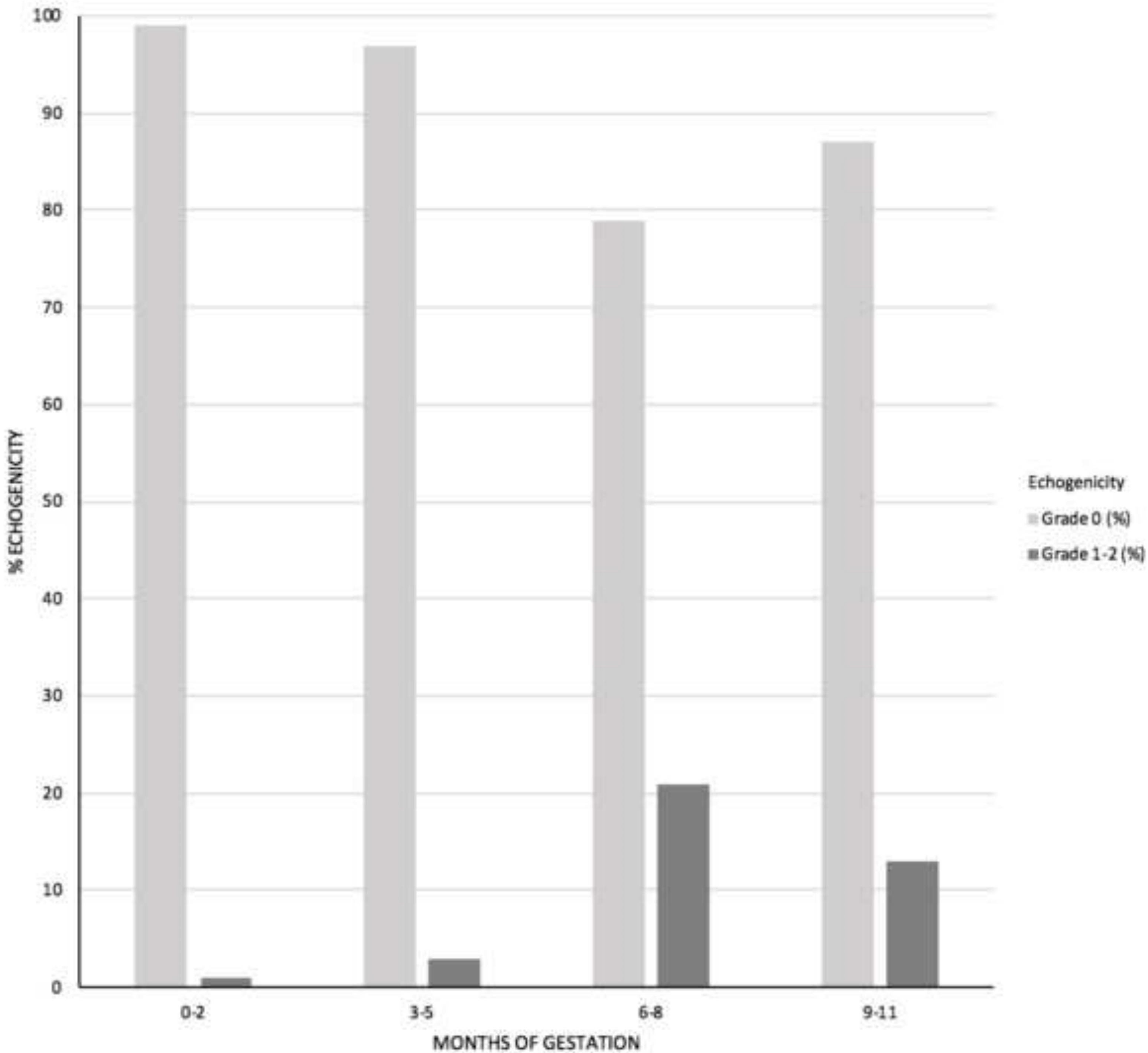
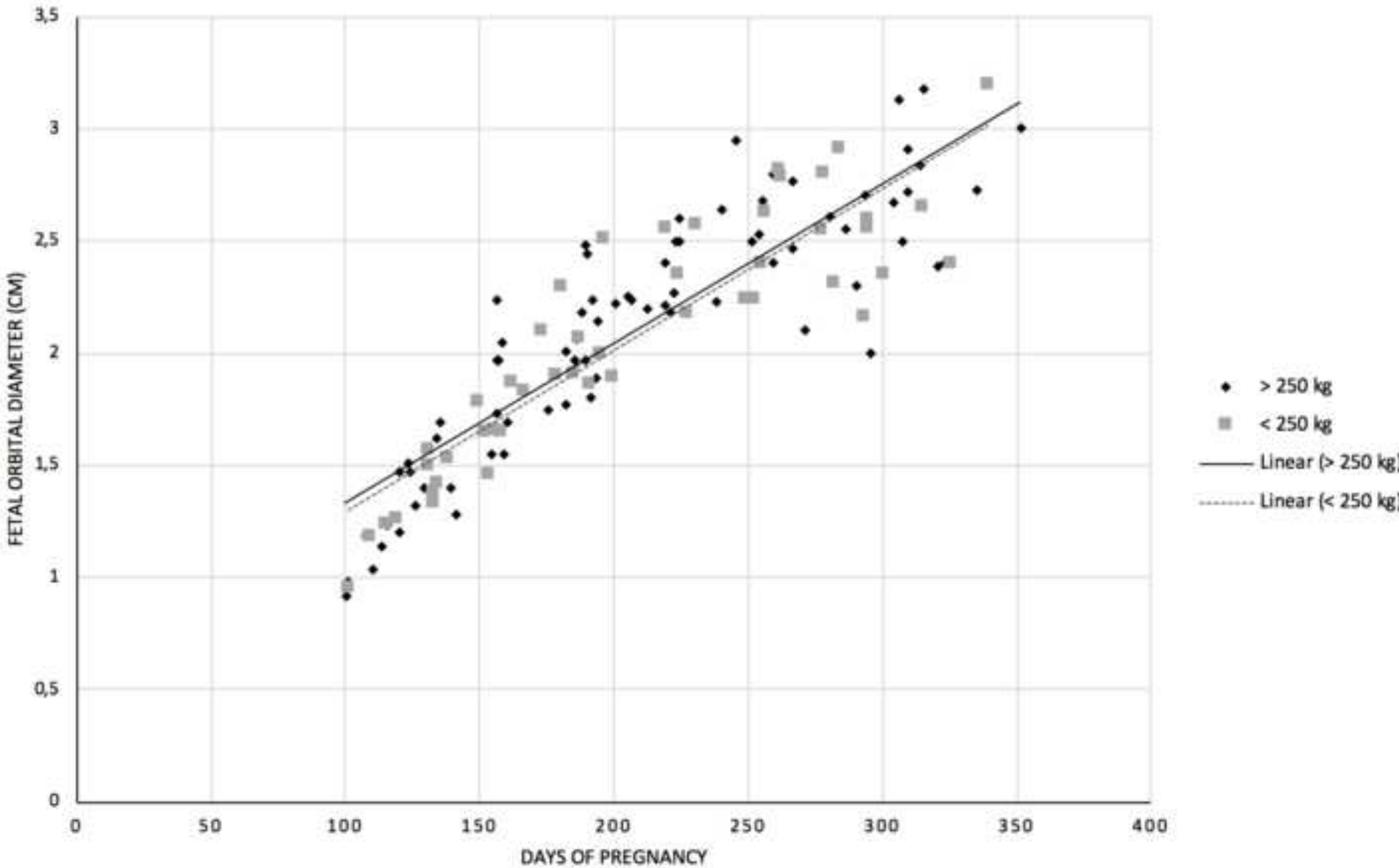

100
90
80
70
60
50
40
30
20
10
0
% ECHOGENICITY
0-2
3-5
6-8
9-11
MONTHS OF GESTATION
Echogenicity
Grade 0 (%)
Grade 1-2 (%)

Figure 10



3,5
3
2,5
2
1,5
1
0,5
0
FETAL ORBITAL DIAMETER (CM)
0
50
100
150
200
250
300
350
400
DAYS OF PREGNANCY
> 250 kg
< 250 kg
Linear (> 250 kg)
Linear (< 250 kg)

EDITORIAL CERTIFICATE

This document certifies that the manuscript listed below was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at American Journal Experts.

Manuscript title:

EVALUATION OF SOME GESTATIONAL PARAMETERS IN JENNIES BRED FOR THE PRODUCTION OF MILK FOR HUMAN CONSUMPTION

Authors:

Tiziana Nervo, Alessia Bertero, Mariagrazia Poletto, Paola Pregel, Roberta Leone, Valentina Toffoli, Leila Vincenti

Date Issued:

February 8, 2018

Certificate Verification Key:

D789-53BA-B8C3-63A3-7093

This certificate may be verified at www.aje.com/certificate. This document certifies that the manuscript listed above was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at American Journal Experts. Neither the research content nor the authors' intentions were altered in any way during the editing process. Documents receiving this certification should be English-ready for publication; however, the author has the ability to accept or reject our suggestions and changes. To verify the final AJE edited version, please visit our verification page. If you have any questions or concerns about this edited document, please contact American Journal Experts at support@aje.com.

Author Agreement

This piece of the submission is being sent via mail.