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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.

1

2 **FIELD ULTRASOUND EVALUATION OF SOME GESTATIONAL PARAMETERS IN**
3 **JENNIES**

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13

14

15 **Abstract**

16 The aim of this study was to collect and analyze ultrasound measurements of fetal-
17 maternal structures during normal and pathological pregnancies in jennies, a livestock
18 species of growing interest. For two breeding seasons, 38 jennies of different breeds and
19 crossbreeds aged between 3 and 18 years were monitored weekly by transrectal
20 examination using a portable Esaote ultrasound (MyLab™ 30 GOLD VET) with a 5-7.5
21 MHz probe. The jennies were divided into two groups, ~~those jennies weighing less than~~
22 ~~250 kg and those weighing more than 250 kg,~~ < 250 kg and > 250 kg body weight, and the
23 dates of conception and parturition/abortion were recorded to calculate pregnancy length.
24 Descriptive statistics were performed, ~~and the mean, median, standard deviation and~~
25 ~~range were calculated~~ for the following variables: pregnancy length (in relation to the
26 ~~season in which conception occurred) and maternal-fetal parameters, including~~
27 (measurements of the orbit, gastric bubble, thorax, abdomen, gonads, heart rate, umbilical
28 artery velocimetry, and combined utero-placental thickness) ~~(CUPT)~~. A total of 68
29 pregnancies were studied, 36 of which ended during the study period. ~~In agreement with~~

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

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

47

48 **1. Introduction**

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

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

80 possible to establish physiological growth parameters for one or more fetal structures
81 during pregnancy. Moreover it has been evaluated the presence of a possible relationship
82 between the body weight of the mother and fetal dimensions during growth.

83

84 **2. Materials and methods**

85 *2.1. Animals*

86 This **field** study was carried out over a period of 14 months, from February 2014 to March
87 2015, on a semi-extensive family-managed farm located near Turin, Italy, that breeds
88 donkeys to produce milk for human consumption and for the production of cosmetics. The
89 jennies were Provencal, Martina Franca or Ragusa breeds, but most of them were
90 crossbreeds. The age range was 3-18 years.

91 All the pregnant jennies were examined and subjected to transrectal ultrasonography on a
92 weekly basis. Monitoring of the animals started at the reproductive stage at which they
93 were in at that moment. Data were recorded and statistically analyzed. The animals were
94 fed hay and grass in stables or pastures, depending on weather conditions. No feed
95 supplements or concentrates were used. Thirty-eight jennies in good health were enrolled
96 in the trial, during which each animal was subjected to clinical and ultrasound
97 examinations of the reproductive system on a weekly basis for one or more pregnancies,
98 for a total of 68 monitored pregnancies. Thus, some of the animals were first examined
99 from the beginning of a pregnancy to the end of that pregnancy and again for the length of
100 the following pregnancy, while other animals were first examined at a more advanced
101 stage of the first pregnancy, but the subsequent pregnancy was fully monitored. During the
102 study 5 animals were eliminated from the trial due to concurrent diseases (33 subjects
103 remaining). For each jenny, the thorax circumference and the length from the olecranon to

104 the ischial tuberosity were measured to estimate weight according to the guidelines
105 previously published by Pearson R.A. and Ouassat M. [5]: Live weight (kg) =
106 $(\text{circumference thoracic}^{2.12}) \times (\text{olecranon-ischial tuberosity length (cm)}^{0.688}) / 3801$. The
107 jennies were then divided into two groups: animals weighing less than 250 kg (11 subjects)
108 and those weighing more than 250 kg (22 subjects).

109

110 *2.2. Reproductive Management*

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

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

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

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

130

131 *2.3. Equipment and technique*

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

135

136 *2.3. Animals and Preparation of animals and ultrasound examination procedure.*

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

145

146 *2.4. Examined parameters Embryo and fetal evaluation parameters*

147 2.5.1. Pregnancy length

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

150

151 2.5.2. Maternal-fetal parameters

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

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

165

166 2.5.2.1. Embryonic vesicle

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

170

171 2.5.2.2. Loss of sphericity of the embryonic vesicle

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

174

175 2.5.2.3. Embryo

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

179

180 2.5.2.4. Embryo Depolarization

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

185

186 2.5.2.5. Orbit

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

189

190 2.5.2.6. Gastric bubble

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

194

195 2.5.2.7. Chest

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

199

200 2.5.2.8. Abdomen

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

204

205 2.5.2.9. Gonads and fetal sexing

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

211

212 2.5.2.10. Fetal heart rate

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

217

218 2.5.2.11. Umbilical artery

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

225

226 2.4.1. Combined utero-placental thickness (CUPT) evaluation

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

231

232 2.4.2. Echogenicity of the fetal fluids

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

235 The different echogenicity levels were classified as reported below:

- 236 - 0: presence of widespread anechogenicity
- 237 - 1: presence of some particles that made the fetal fluids more turbid and then slightly
238 more echogenic
- 239 - 2: echogenicity much greater than in condition 0

240

241 2.4.3. Fetal motility

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

245 The fetal motility was classified into 3 groups:

- 246 - 1: the fetus was sleeping; it did not move or made extremely limited movements.
- 247 - 2: the fetus showed good motility but was still enough to allow the operator to
248 perform a thorough ultrasound examination and measure the fetal structures.
- 249 - 3: the fetus moved excessively to the extent that measurement of the fetal
250 structures became extremely difficult.

251

252 2.5. 2.15. Fetal presentation

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

255

256 2.4.6. Mammary gland development

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

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

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

263

264 **2.5.** *Statistical analysis*

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

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

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

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

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

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

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

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

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

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

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

299

300 **3. Results.**

301 *3.1 Pregnancy*

302 The study was performed on 68 pregnancies, 36 of which came to term during the trial; of
303 these, 28 ended with the birth of alive and vital foals (77.80%), while 8 ended with abortion
304 (22.20%). The mean pregnancy length was 370.82 ± 16.60 days (range: 342-402 days) for

305 pregnancies that came to term and 316.13 ± 36.60 days (range: 236-356 days) for
306 abortions, but a 48 h-error should be considered, since the exact moment of ovulation is
307 unknown.

308 Among the 28 pregnancies that came to term, 16 newborns were females (57.10%), and
309 12 were males (42.90%). Among the 8 abortions, 4 fetuses were male (50.00%), and 4
310 were female (50.00%).

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

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

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

327

328 *3.2 Maternal-fetal parameters*

329 The collected parameters, grouped by gestational month, are reported in Table 4 2.

330

331 3.2.1. Embryo growth parameters

332 In addition, embryo growth parameters measured during the first three months of gestation

333 are showed in Table 2 3. The embryo growth parameters, measured during the first 3

334 months of gestation, are summarized in Table 2.

335

336 3.2.1.1 Embryonic vesicle

337 The embryonic vesicle was detected for the first time on day 12 of gestation (with a

338 variability up to 48 hours linked to assisted natural mating and to the non-identification of

339 the exact moment of the ovulation).

340 The mean vesicle diameter between days 16 and 18 (days from the last mating) was 23.4

341 \pm 6.8 mm.

342 In graph 1, the linear regression between the vesicle diameter and the day of gestation

343 calculated until day 90 is depicted ($y = 0.884x + 7.128$; $R^2 = 0.8304$; $p\text{-value} < 0.001$;

344 Figure 2).

345

346 3.2.1.2 Loss of sphericity of the embryonic vesicle

347 Loss of sphericity of the embryonic vesicle was observed from the 21st day (\pm 48 h) of

348 gestation. An extremely significant association ($p\text{-value} < 0.001$) was demonstrated

349 between the loss of sphericity of the embryonic vesicle and the third week of the first

350 month of pregnancy.

351

352 3.2.1.3. Embryo

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

356 A positive linear regression between the longitudinal dimension of the embryo and day of
357 gestation (± 48 h) was calculated until day 90 and is depicted in graph 2 Figure 3 ($y =$
358 $0.0708x - 0.8413$; $R^2 = 0.7519$; $p\text{-value} < 0.001$).

359

360 3.2.1.4. Embryo Depolarization

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

365

366 3.2.2. Fetal growth parameters

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

369

370 3.2.2.1. Orbit

371 The orbit of the fetus (Figure 4) was detected and measured for the first time on day 70 (\pm
372 48 h) of pregnancy.

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

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

377

378 3.2.2.2. Gastric bubble

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

381 A positive linear relationship between the diameter of the gastric bubble and the day of
382 gestation (± 48 h) was demonstrated ($p\text{-value} < 0.001$).

383

384 3.2.2.3. Chest

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

386 A positive linear relationship between the diameter of the thorax and the day of gestation
387 (± 48 h) was demonstrated ($p\text{-value} < 0.001$).

388

389 3.2.2.4. Abdomen

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

392 A positive linear relationship between the diameter of the abdomen and the day of
393 gestation (± 48 h) was demonstrated ($p\text{-value} < 0.001$).

394

395 3.2.2.5. Gonads and fetal sexing

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

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

399 According to our data, the time frame for fetal sexing runs from the 96th to the 210th day
400 (± 48 h) of gestation. A statistically significant association (p -value < 0.001) between the
401 month of gestation and the ability to perform sex determination of the fetus was
402 demonstrated. It was possible to determine the fetal sex in the 4th month of gestation in
403 32% of cases, in the 5th month in 60% of cases, in the 6th month in 26% of cases, and in
404 the 7th month in 10% of cases. The diagnostic accuracy was 88%.

405

406 3.2.2.6. Fetal heart rate

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

410

411 3.2.2.7. Umbilical artery

412 The first measurement of the frequency of the umbilical artery was recorded on day 125 (\pm
413 48 h) of gestation. A negative linear relationship between the umbilical artery frequency
414 and the day of gestation (± 48 h) was detected, even if the p -value was not significant.

415

416 3.2.3. Combined utero-placental thickness (CUPT)

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

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

422

423 3.2.4. Echogenicity of the fetal fluids

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

427 From the 3rd to the 5th month of gestation, 97% showed an echogenicity of grade 0, and
428 3% showed an echogenicity of grades 1-2.

429 From the 6th to the 8th month of gestation, 79% showed an echogenicity of grade 0, and
430 21% showed an echogenicity of grades 1-2.

431 From the 9th to the 11th month of gestation, 87% showed an echogenicity of grade 0, and
432 13% showed an echogenicity of grades 1-2.

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

435

436 3.2.5. Fetal motility

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

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

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

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

446

447 3.2.6. Fetal presentation

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

450 A statistically significant association (p-value < 0.01) between the trimester of pregnancy
451 and fetal presentation was demonstrated. The fetuses showed the final presentation at the
452 9th month of gestation.

453

454 3.2.5. Mammary gland development

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

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

460

461 3.2.6. Evaluation of fetal orbital diameter in relation to maternal size

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

467

468 **4. Discussion**

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

474 The observed sex ratio showed a slightly higher number of females and was similar to the
475 ratio reported by other authors [12], even though we examined of a smaller number of
476 jennies. Even though we could not demonstrate a statistically significant association
477 between the sex of the newborn and the pregnancy outcome, we recorded that the
478 pregnancies were longer in jennies pregnant with male fetuses than in jennies pregnant
479 with female fetuses, and this was in accordance with previously reported data for jennies
480 [12,16,18] and mares [19-22] in the literature.

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

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

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

503 The embryonic vesicle was detected for the first time 12 days after the last mating (day 12
504 \pm 48 h of pregnancy, due to the non-identification of the exact moment of the ovulation), in
505 accordance with reports by many authors (days 12-13 of gestation) who have evaluated

506 jennies [1,2,10-12,17,24] and mares [25-27]; however, other authors [28] have reported
507 the first detection of the embryonic vesicle in jennies on day 14. An early pregnancy
508 diagnosis is very useful in production animals because it allows reduction in number of
509 days needed to inseminate the jenny again in cases of negative outcomes.

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

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

519 The mean vesicle diameter, measured from 16 to 18 days **after the last mating** (23.4 ± 6.8
520 mm), was in accordance with the data obtained in jennies by some authors $44(26.4 \pm 0.7$
521 mm) [11], but it seems to be higher compared to the data observed by other authors in
522 jennies $24(21.8 \text{ mm})$ [24], $40(22.3 \text{ mm})$ [10] and in mares $33(23 \text{ mm})$ [33]. We should
523 highlight that these authors monitored the ovulation follicles daily to unambiguously identify
524 the exact moment of ovulation. Since we could not do that, our day 0 corresponded to the
525 last day on which the jenny had accepted mating.

526 Therefore, because we did not know exactly the ovulation time, because we had to
527 evaluate the jennies 24-36 h after mating, and because the growth of the embryonic
528 vesicle is exponential until approximately the 16th day of gestation, we obtained vesicle
529 measurements with a higher standard deviation than those reported in the literature [10,

530 24]. In addition, the diameter of the embryonic vesicle has been proven to be positively
531 correlated with the day of gestation by other authors [10,12,17,24]. The data regarding the
532 loss of sphericity of the embryonic vesicle from the 21st day (± 48 h) of gestation and the
533 significant association with the third week of the first month of pregnancy were in
534 accordance with the results obtained in jennies and mares by many authors [10-
535 12,17,24,28,33-36].

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

539 The embryo depolarization within the vesicle was observed from day 32 (± 48 h) of
540 gestation, which is an earlier date than that reported by other authors, who detected
541 embryo depolarization from day 35 to 53 [24], from day 41 to 47 [10], or even on day 50
542 [11].

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

547 The first day of gestation when identification and measurement of the orbit was performed
548 was earlier than that reported by other studies on jennies. Crisci A. et al. (2014) [12]
549 reported first detection of the orbit on day 71-96 of gestation, while we reported detection
550 on day 70, however variability (up to 48 hours) linked to assisted natural mating and to the
551 non-identification of the exact moment of the ovulation, must be taken in account. Early
552 detection of the orbit, similar to all the other examined structures, depends on how the
553 conceptus is positioned in relation to the ultrasound probe and on the quality of the

554 transducer, which may or may not allow the correct identification of very small structures.
555 The statistically significant correlation between the orbital dimensions and the day of
556 gestation that we found has been proven in jennies [12] and in mares [37-40].

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

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

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

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

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

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

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

589 The fetal gonads were observed for the first time on day 96 **after the last mating** and up to
590 day 210. To perform the determination of fetal sex, the best period was found to be the 5th
591 month of gestation (60% of the performed examinations). This range is wider than that
592 reported by several authors (between day 100 and day 150 of gestation) for donkeys [42]
593 and for mares [39, 43]. On the other hand, other authors reported the possibility of sexing
594 the horse fetus from day 90 to day 180 of gestation [44] or from day 120 to day 210 [45].

595 The factors involved in fetal sex determination are many. Above all, the most important
596 factors include the following: the use of an ultrasound machine equipped with a high-
597 quality probe, possibly with the help of the Color Doppler; good environmental brightness
598 conditions; adequate restraint of the animal; mother and fetus remaining still during the
599 examination; relatively small size of the fetus; and posterior presentation. Most likely, more
600 than one of these conditions occurred simultaneously in our study, and this explains why it
601 was possible to determine the fetal sex in a wider temporal range than what has been

602 reported in the previous literature. We often had the opportunity to observe the fetuses in
603 posterior presentation at the time of the examination, which is a condition that is more
604 likely to occur in jennies than in mares, as the donkey fetus is typically smaller in size than
605 a horse fetus. In addition, with the progression of gestational age, other structures, such as
606 the external genitalia and the mammary glands, became visible, which allowed more
607 accurate sex determination than mere observation of the gonads.

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

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

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

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

632 The authors' choice to start measuring the CUPT from day 154 [12] is presumably due to
633 the fact that in the equine species, the placenta is considered fully functional beginning on
634 the 150th day of pregnancy. However, we chose to start measuring the CUPT when it
635 became visible, even if it was still in development. The average CUPT found in our study
636 from the 4th to the 9th month of pregnancy (M4: 4.97 ± 1.73 cm; M5: 4.33 ± 1.31 cm; M6:
637 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
638 cm; M11: 4.89 ± 1.26 cm) were in accordance with that reported in mares by Renaudin C.D.
639 et al. [7] and Bucca S. et al. [40]; however, it was lower than that described in jennies by
640 Crisci A. [12]. In the mare, from the 10th to the 12th month, the average CUPT increases
641 by 1.5-2 mm every month [7,40]. This was not found in our study, where CUPT remained
642 virtually unchanged during gestation, even in animals that aborted. Conversely, some
643 authors [12] reported an increase in CUPT during pathology in pregnancies. Instead, in
644 some cases we observed a CUPT that was partially edematous. This sign in the mare is
645 normally associated with placentitis but seems to be irrelevant in the jenny. All this data
646 seems to indicate that these placental morphological characteristics are peculiar of
647 donkeys and probably attributable to the longer gestation length in donkeys compared with
648 horses [48].

649 Because of the current legislation on drugs in Italy, which explicitly prohibits the use of any
650 product on equines bred for the production of milk for human consumption, the animals

651 were not treated despite the ultrasound evidence of pathology. However, none of the
652 jennies aborted with clinical signs of placentitis, and no placental abnormalities were found
653 after parturition, suggesting that this aspect of CUPT may be physiologic in the donkey,
654 may be related to the length of donkey pregnancies and may be associated with lower
655 placental function, as described by Carluccio A. et al. [2].

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

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

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

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

678

679 **5. Conclusions**

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

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

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

692 Today, the donkey is used as a pet, for onotherapy, for hiking in the mountains and, above
693 all, for milk production. The use of the donkey for milk production in particular, gave this
694 species value as livestock and shifted the attitudes of breeders and veterinarians.

695 Nevertheless, to date the literature is still very scarce, and the lack of knowledge appears
696 to be much wider than expected.

697 We hope that this study can add pieces to the mosaic of knowledge on this topic. In this
698 regard, the obtained results can contribute to the definition of reference values for this

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

710

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714

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840

841 **8. Figures**

842 **Figure 1** CUPT measurement during the 8th month of gestation. **D1: CUPT (5.20 mm);**

843 **CS: cervical star; UA: uterine artery.**

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

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

848 **Figure 4** (A) orbital diameter at 85-days (D1=0.89 cm), (B) at 152-days (D2=1.65), (C) at
849 211-days (D3=2.1 cm) and (D) at 261-days (D4=2.33 cm) of gestation.

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

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

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

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

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

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

863

864 **Table 1** Gestational months (and corresponding days) in which maternal-fetal parameters
865 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

866

867 **Table 4 2** Measurements of maternal-fetal parameters taken in different months of
868 gestation in jennies (1 M-11 M).

Gestational (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

869

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

871

872 **Table 2 3** Linear regression of the embryonic vesicle and embryo length in the first three

873 months of gestation. r^2 : correlation coefficient. b_0 and b_1 : coefficients of the linear

874 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

875

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

878

879 **Table 3 4** Linear regression of the fetal parameters from the 100th day of gestation
 880 onwards. r^2 : correlation coefficient. b_0 and b_1 : coefficients of the linear regression equation
 881 ($y = b_0 + b_1x$); 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

882

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

885

886

887

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 to 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 comparison.

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 accuracy.

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 discarded.

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 ultrasound 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

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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

30 can reasonably be performed by two weeks after last service if ovulation date is not
31 known. The orbital diameter was the most reliable parameter for monitoring the
32 physiological development of the embryo and fetus, and it was strongly related to the
33 gestational age. No differences in fetal development were observed in relation to the
34 mother's body weight. The combined utero-placental thickness was not associated with
35 the gestational age and thickening and edema, frequently observed, were not associated
36 with fetal pathologies.

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

38

39 **1. Introduction**

40 Although donkeys have been used as working animals for thousands of years, interest in
41 this animal have arisen in various branches of the veterinary medicine very recently. For a
42 long time, donkeys have been considered "little horses" and have played second fiddle to
43 horses, but now the increase in knowledge is revealing significant differences between
44 these two species. In recent years, due to the rising economic interest in donkeys, the
45 reproductive physiology of donkeys has drawn the attention of researchers and veterinary
46 clinicians [1]. Today, the donkey is used as a pet, for onotherapy, for hiking in the
47 mountains and, above all, for milk production. The use of the donkey for milk production in
48 particular, gave this species value as livestock and shifted the attitudes of breeders and
49 veterinarians. Nevertheless, to date the literature is still very scarce, and the lack of
50 knowledge appears to be much wider than expected. Detailed knowledge of the typical
51 reproductive patterns of the species [2] allows to improve the reproductive performance of
52 these animals and increases the economic returns for breeders. Transrectal
53 ultrasonography is a technique that has been used since 1980, and it has revolutionized

54 the study of early pregnancy in mares [3,4]. The monitoring of the pregnancy in jennies
55 was previously unnecessary because donkeys were mainly used for work (especially in
56 developing countries), and in industrialized countries, their presence and use did not justify
57 the application of such powerful and expensive technology. So far, very few studies have
58 been performed on donkeys, and most of them only address the early stages of
59 pregnancy. The aim of this work was to collect and process data and measurements,
60 obtained by transrectal ultrasonography, regarding the maternal-fetal structures throughout
61 donkey gestation. Moreover it has been evaluated the presence of a possible relationship
62 between the body weight of the mother and fetal dimensions during growth.

63

64 **2. Materials and methods**

65 *2.1. Animals*

66 This field study was carried out over a period of 14 months, from February 2014 to March
67 2015, on a semi-extensive family-managed farm located near Turin, Italy, that breeds
68 donkeys to produce milk for human consumption and for the production of cosmetics. The
69 jennies were Provencal, Martina Franca or Ragusa breeds, but most of them were
70 crossbreeds. The age range was 3-18 years.

71 All the pregnant jennies were examined and subjected to transrectal ultrasonography on a
72 weekly basis. Monitoring of the animals started at the reproductive stage at which they
73 were in at that moment. Data were recorded and statistically analyzed. The animals were
74 fed hay and grass in stables or pastures, depending on weather conditions. No feed
75 supplements or concentrates were used. Thirty-eight jennies in good health were enrolled
76 in the trial, during which each animal was subjected to clinical and ultrasound
77 examinations of the reproductive system on a weekly basis for one or more pregnancies,

78 for a total of 68 monitored pregnancies. Thus, some of the animals were first examined
79 from the beginning of a pregnancy to the end of that pregnancy and again for the length of
80 the following pregnancy, while other animals were first examined at a more advanced
81 stage of the first pregnancy, but the subsequent pregnancy was fully monitored. During the
82 study 5 animals were eliminated from the trial due to concurrent diseases (33 subjects
83 remaining). For each jenny, the thorax circumference and the length from the olecranon to
84 the ischial tuberosity were measured to estimate weight according to the guidelines
85 previously published by Pearson R.A. and Ouassat M. [5]: Live weight (kg) =
86 $(\text{circumference thoracic}^{2.12}) \times (\text{olecranon-ischial tuberosity length (cm)}^{0.688}) / 3801$. The
87 jennies were then divided into two groups: animals weighing less than 250 kg (11 subjects)
88 and those weighing more than 250 kg (22 subjects).

89

90 *2.2. Reproductive Management*

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

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

100 When the foals reached 6 months of age and the mothers were carrying new pregnancies,
101 it was possible to wean the foals and to dry off the mothers. If the jennies became

102 pregnant at the foal heat, the dry period lasted for approximately four months, and if they
103 did not become pregnant, then it lasted longer. During lactation, approximately one liter of
104 milk per jenny per day was produced. The milk production at the farm was constant
105 because the herd included both females in early lactation that produced approximately 1.4
106 liters per animal and females at the end of lactation that produced no more than 0.7-0.8
107 liters per animal.

108

109 *2.3. Animals and ultrasound examination procedure.*

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

117

118 *2.4. Embryo and fetal evaluation parameters*

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

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

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

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

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

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

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

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

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

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

147 After the identification of the heart in the thorax, Doppler echocardiography was used to
148 precisely determine the heart rate in beats per minute (BPM). It was possible to measure

149 the BPM in the second month of gestation and then from the 4th to the 7th month of
150 gestation.

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

157

158 2.4.1. Combined utero-placental thickness (CUPT) evaluation

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

163

164 2.4.2. Echogenicity of the fetal fluids

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

167 The different echogenicity levels were classified as reported below:

- 168 - 0: presence of widespread anechogenicity
- 169 - 1: presence of some particles that made the fetal fluids more turbid and then slightly
170 more echogenic
- 171 - 2: echogenicity much greater than in condition 0

172

173 2.4.3. Fetal motility

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

177 The fetal motility was classified into 3 groups:

- 178 - 1: the fetus was sleeping; it did not move or made extremely limited movements.
- 179 - 2: the fetus showed good motility but was still enough to allow the operator to
180 perform a thorough ultrasound examination and measure the fetal structures.
- 181 - 3: the fetus moved excessively to the extent that measurement of the fetal
182 structures became extremely difficult.

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

185

186 *2.5. Statistical analysis*

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

- 193 - pregnancy length (parturition and abortion);

194 - pregnancy length for jennies at term, in relation to the season in which conception
195 occurred;

196 - maternal-fetal parameters: measurements of the orbit, gastric bubble, thorax,
197 abdomen, gonads, fetal heart rate, umbilical artery velocimetry, and CUPT.

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

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

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

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

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

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

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

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

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

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

221

222 **3. Results.**

223 *3.1 Pregnancy*

224 The study was performed on 68 pregnancies, 36 of which came to term during the trial; of
225 these, 28 ended with the birth of alive and vital foals (77.80%), while 8 ended with abortion
226 (22.20%). The mean pregnancy length was 370.82 ± 16.60 days (range: 342-402 days) for
227 pregnancies that came to term and 316.13 ± 36.60 days (range: 236-356 days) for
228 abortions, but a 48 h-error should be considered, since the exact moment of ovulation is
229 unknown.

230 Among the 28 pregnancies that came to term, 16 newborns were females (57.10%), and
231 12 were males (42.90%). Among the 8 abortions, 4 fetuses were male (50.00%), and 4
232 were female (50.00%).

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

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

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

249

250 *3.2 Maternal-fetal parameters*

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

252

253 3.2.1. Embryo growth parameters

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

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

259 The mean vesicle diameter between days 16 and 18 (days from the last mating) was 23.4
260 ± 6.8 mm.

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

263

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

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

270 A positive linear regression between the longitudinal dimension of the embryo and day of
271 gestation (± 48 h) was calculated until day 90 and is depicted in Figure 3 ($y = 0.0708x -$
272 0.8413 ; $R^2 = 0.7519$; p -value < 0.001).

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

277

278 3.2.2. Fetal growth parameters

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

281 The orbit of the fetus (Figure 4) was detected and measured for the first time on day 70 (\pm
282 48 h) of pregnancy.

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

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

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

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

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

292 A positive linear relationship between the diameter of the thorax and the day of gestation
293 (± 48 h) was demonstrated (p -value < 0.001).

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

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

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

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

301 According to our data, the time frame for fetal sexing runs from the 96th to the 210th day
302 (± 48 h) of gestation. A statistically significant association (p -value < 0.001) between the
303 month of gestation and the ability to perform sex determination of the fetus was
304 demonstrated. It was possible to determine the fetal sex in the 4th month of gestation in

305 32% of cases, in the 5th month in 60% of cases, in the 6th month in 26% of cases, and in
306 the 7th month in 10% of cases. The diagnostic accuracy was 88%.

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

310 The first measurement of the frequency of the umbilical artery was recorded on day 125 (\pm
311 48 h) of gestation. A negative linear relationship between the umbilical artery frequency
312 and the day of gestation (\pm 48 h) was detected, even if the p-value was not significant.

313

314 3.2.3. Combined utero-placental thickness (CUPT)

315 It was possible to obtain the first measurement of the CUPT on day 94 (\pm 48 h) of
316 gestation.

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

320

321 3.2.4. Echogenicity of the fetal fluids

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

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

325

326 3.2.5. Fetal motility

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

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

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

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

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

338 A statistically significant association (p-value < 0.01) between the trimester of pregnancy
339 and fetal presentation was demonstrated. The fetuses showed the final presentation at the
340 9th month of gestation.

341

342 3.2.6. Evaluation of fetal orbital diameter in relation to maternal size

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

348

349 **4. Discussion**

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

355 The observed sex ratio showed a slightly higher number of females and was similar to the
356 ratio reported by other authors [12], even though we examined of a smaller number of
357 jennies. Even though we could not demonstrate a statistically significant association
358 between the sex of the newborn and the pregnancy outcome, we recorded that the
359 pregnancies were longer in jennies pregnant with male fetuses than in jennies pregnant
360 with female fetuses, and this was in accordance with previously reported data for jennies
361 [12,16,18] and mares [19-22] in the literature.

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

367 The issue regarding the frequent abortions observed in this farm (22.2% during the study
368 period) remains unsolved because bacteriological and virological analyses did not identify
369 an etiological agent. In 3 jennies that were negative for EHV-1-4, the herpesvirus
370 glycoprotein (HVG) was isolated, presumably due to the presence of other herpesvirus
371 strains, such as equine herpesvirus 8 which was recently isolated [23] and is common in

372 donkeys. Surely the clinical signs, characterized by late-term abortion after 8 months of
373 gestation without premonitory signs, could correspond to EHV symptomatology.

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

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

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

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

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

407 Therefore, because we did not know exactly the ovulation time, because we had to
408 evaluate the jennies 24-36 h after mating, and because the growth of the embryonic
409 vesicle is exponential until approximately the 16th day of gestation, we obtained vesicle
410 measurements with a higher standard deviation than those reported in the literature [10,
411 24]. In addition, the diameter of the embryonic vesicle has been proven to be positively
412 correlated with the day of gestation by other authors [10,12,17,24]. The data regarding the
413 loss of sphericity of the embryonic vesicle from the 21st day (± 48 h) of gestation and the
414 significant association with the third week of pregnancy were in accordance with the
415 results obtained in jennies and mares by many authors [10-12,17,24,28,33-36].

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

419 The embryo depolarization within the vesicle was observed from day 32 (± 48 h) of
420 gestation, which is an earlier date than that reported by other authors, who detected

421 embryo depolarization from day 35 to 53 [24], from day 41 to 47 [10], or even on day 50
422 [11].

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

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

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

443 Even though we grouped the jennies on the basis of their weight, we did not observe a
444 statistically significant difference in the growth of the orbit from the 100th day (± 48 h) of

445 gestation in the two groups. This finding is in agreement with what other authors have
446 found for donkeys [17].

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

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

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

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

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

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

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

490 The first observation of the fetal heartbeat was obtained 22 days after the last mating. The
491 negative linear relationship between the fetal heart rate and the day of gestation was in
492 accordance with other studies in jennies [12] and in mares [40]. Indeed, in all species, the

493 fetal heart rate is very high in the early embryonic and fetal stages and tends to decrease,
494 almost reaching the maternal value, close to parturition.

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

506 The CUPT measurement was performed exclusively transrectal because of the
507 impossibility, in field conditions, to adequately prepare the jennies (clipping of the
508 abdomen, extended restraint time) for performing transabdominal ultrasound imaging. Our
509 first detection of CUPT was earlier than what has been reported by other authors (day $94 \pm$
510 48 h of gestation vs. day 154) [12], but it was in accordance with the data reported on
511 mares [6].

512 The authors' choice to start measuring the CUPT from day 154 [12] is presumably due to
513 the fact that in the equine species, the placenta is considered fully functional beginning on
514 the 150th day of pregnancy. However, we chose to start measuring the CUPT when it
515 became visible, even if it was still in development. The average CUPT found in our study
516 from the 4th to the 9th month of pregnancy (M4: 4.97 ± 1.73 cm; M5: 4.33 ± 1.31 cm; M6:
517 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

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

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

536 In agreement with other authors [12], no abnormal echogenicity of the fetal fluids was
537 found in all the examined pregnancies, but we detected a progressive increase in turbidity
538 during pregnancy due to the deposition of particles within the fetal fluids, as described in
539 mares [7,40]. This turbidity may also depend on the degree of fetal motility during the
540 examination since the particles are settled during fetal rest and become suspended during
541 fetal movement. The echoes emitted by the transducer through the fetal fluids stimulate
542 the fetus, that responds by increasing its motility, causing an apparent increase in the

543 echogenicity of the fetal fluids in advanced pregnancies. With respect to fetal motility, as
544 reported by other authors using the same evaluation scale [12], we never observed total
545 fetal immobility, and on the contrary, we often recorded high grade 3 motility, particularly
546 from the 6th to the 8th month of gestation, presumably due to the greater amount of space
547 available to the fetus. Similar results are also reported in mares [40].

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

552

553 **5. Conclusions**

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

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

566 could be very useful to widen the knowledge regarding the ultrasound evaluation of
567 gestational parameters in this species.

568

569 **6. Acknowledgements**

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571 commercial, or not-for-profit sectors.

572

573 **7. Bibliography**

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698

699 **8. Figures**

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

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

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

706 **Figure 4** (A) orbital diameter at 85-days (D1=0.89 cm), (B) at 152-days (D2=1.65), (C) at
707 211-days (D3=2.1 cm) and (D) at 261-days (D4=2.33 cm) of gestation.

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

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

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

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

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

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

721

722 **Table 1** Gestational months (and corresponding days) in which maternal-fetal parameters
723 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

724

725 **Table 2** Measurements of maternal-fetal parameters taken in different months of gestation
726 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
3rd	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
	4th	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
5th	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

727

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

729

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

Embryo growth parameter	N	Range	Days	r²	b₀	b₁	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

732

733 r²: correlation coefficient. b₀ and b₁: coefficients of the linear regression equation (y = b₀ +
734 b₁x); y: calculated value for the variable; x: gestational age.

735

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

Fetal growth parameters (cm)	N	Range	Days	r²	b₀	b₁	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

737

738 r²: correlation coefficient. b₀ and b₁: coefficients of the linear regression equation (y = b₀ +
739 b₁x); y: calculated value for the variable; x: gestational age. * (heart beat/min).

1 **Highlights.**

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

10

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 to 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 comparison.

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 accuracy.

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, allantoic 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 discarded.

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 ultrasound 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

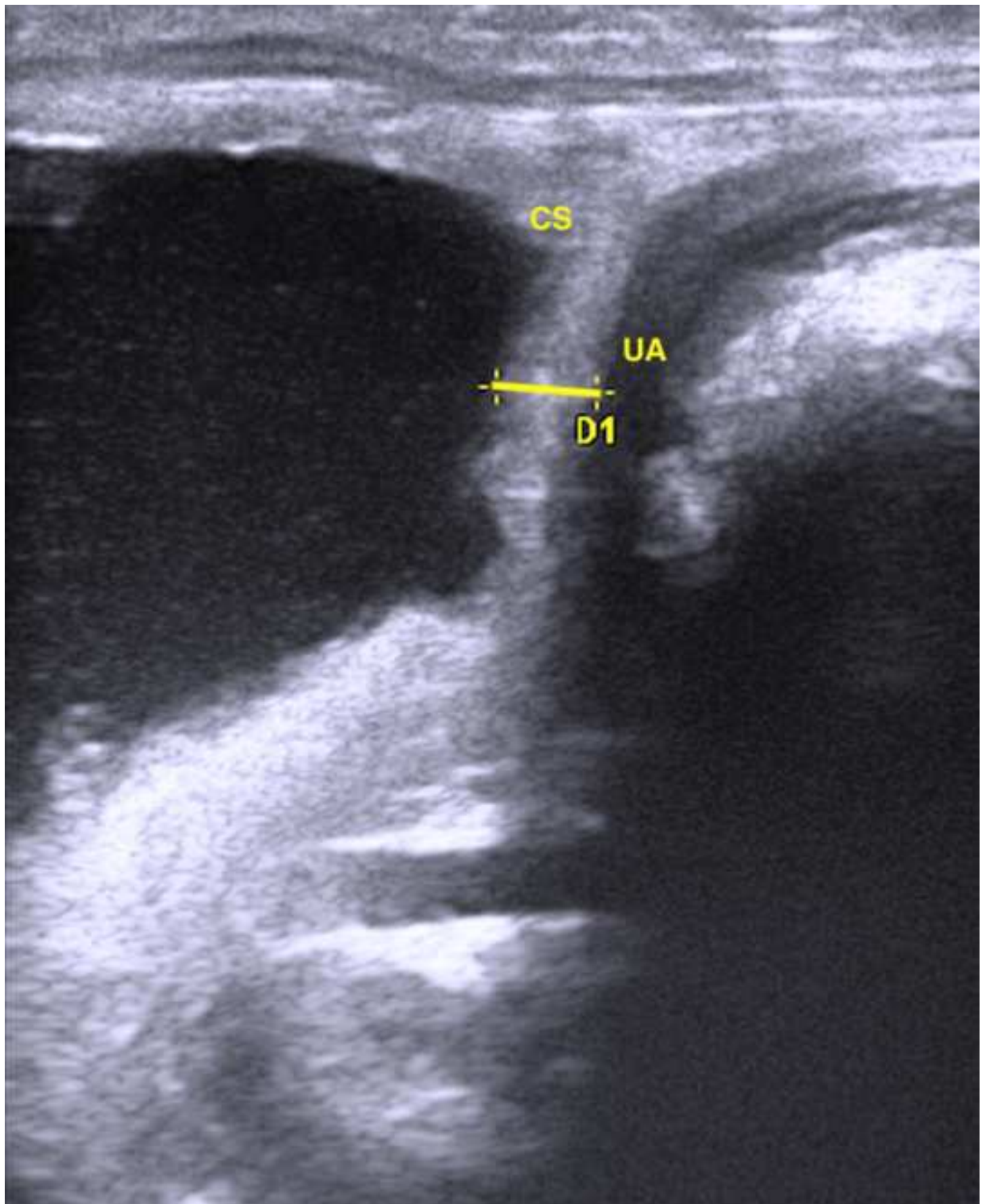


Figure 2

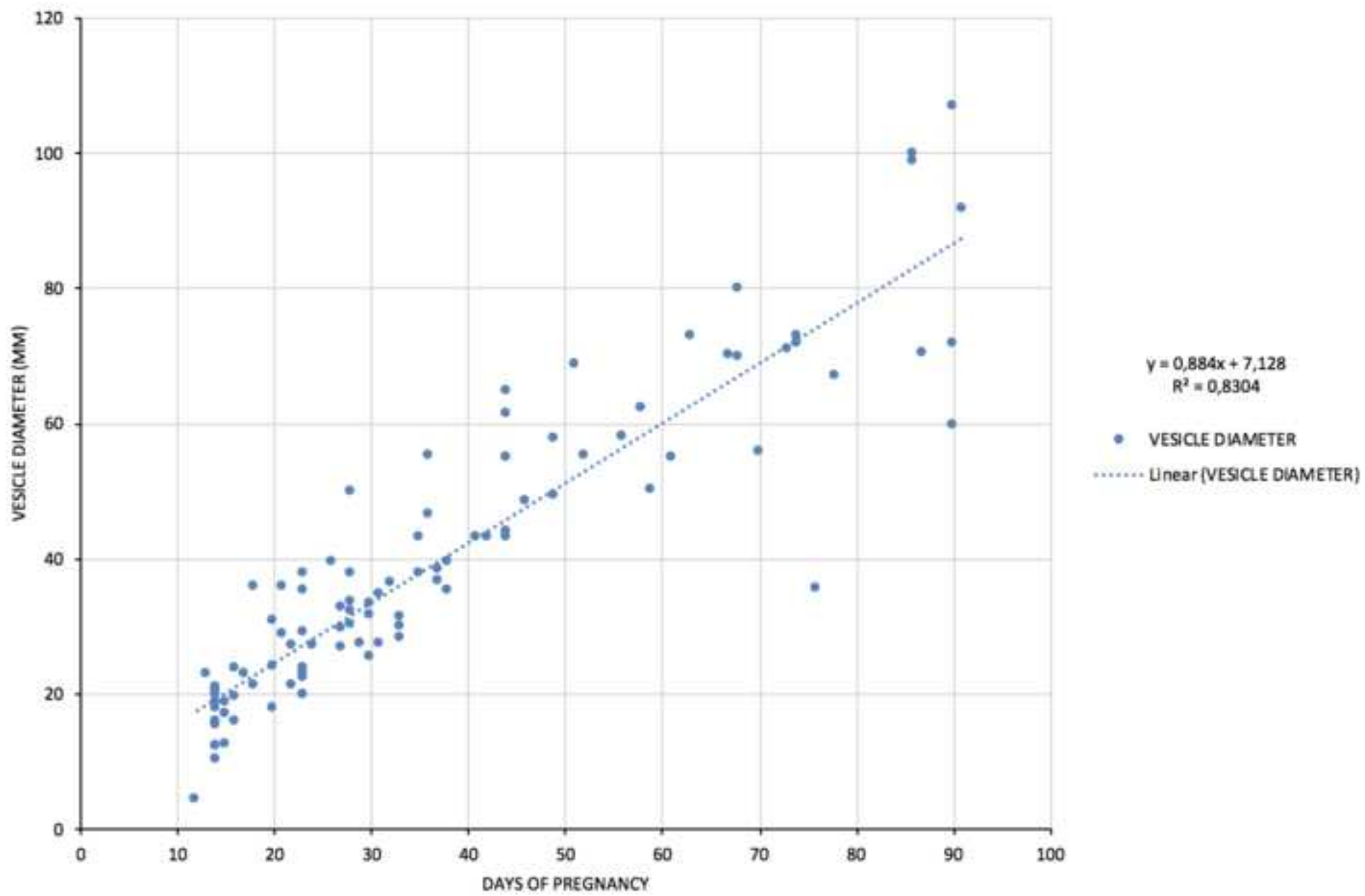


Figure 3

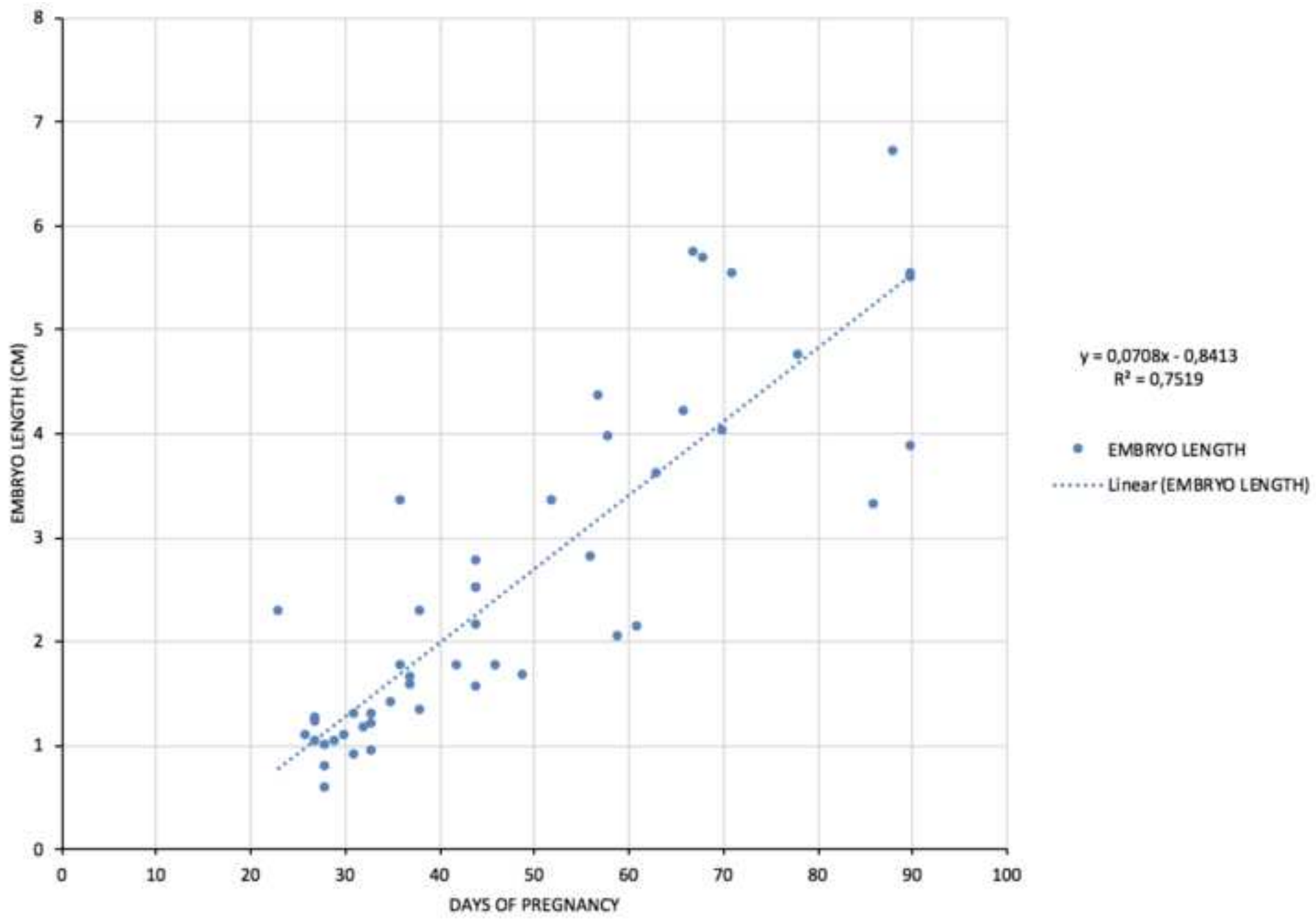


Figure 4

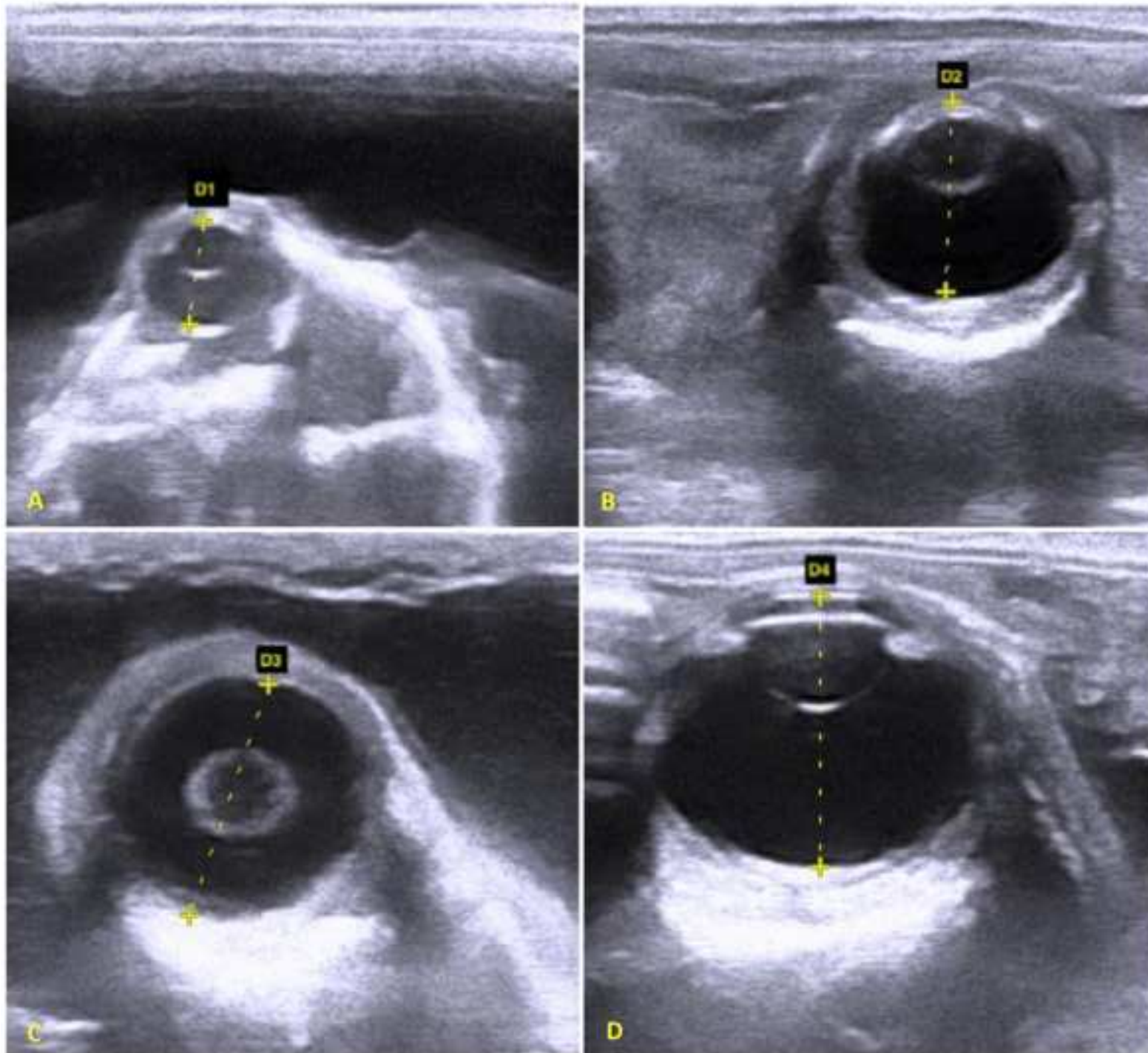


Figure 5

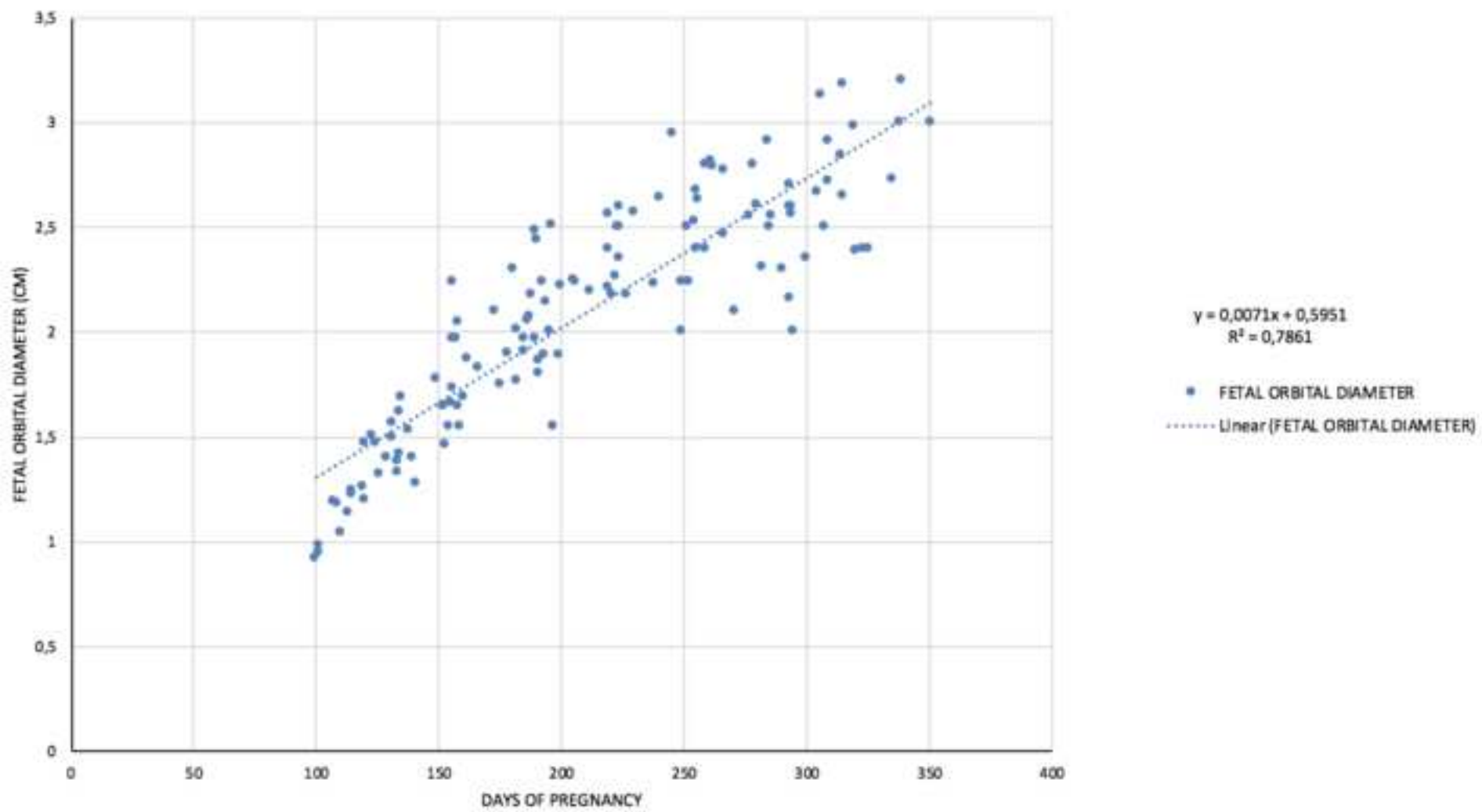


Figure 6

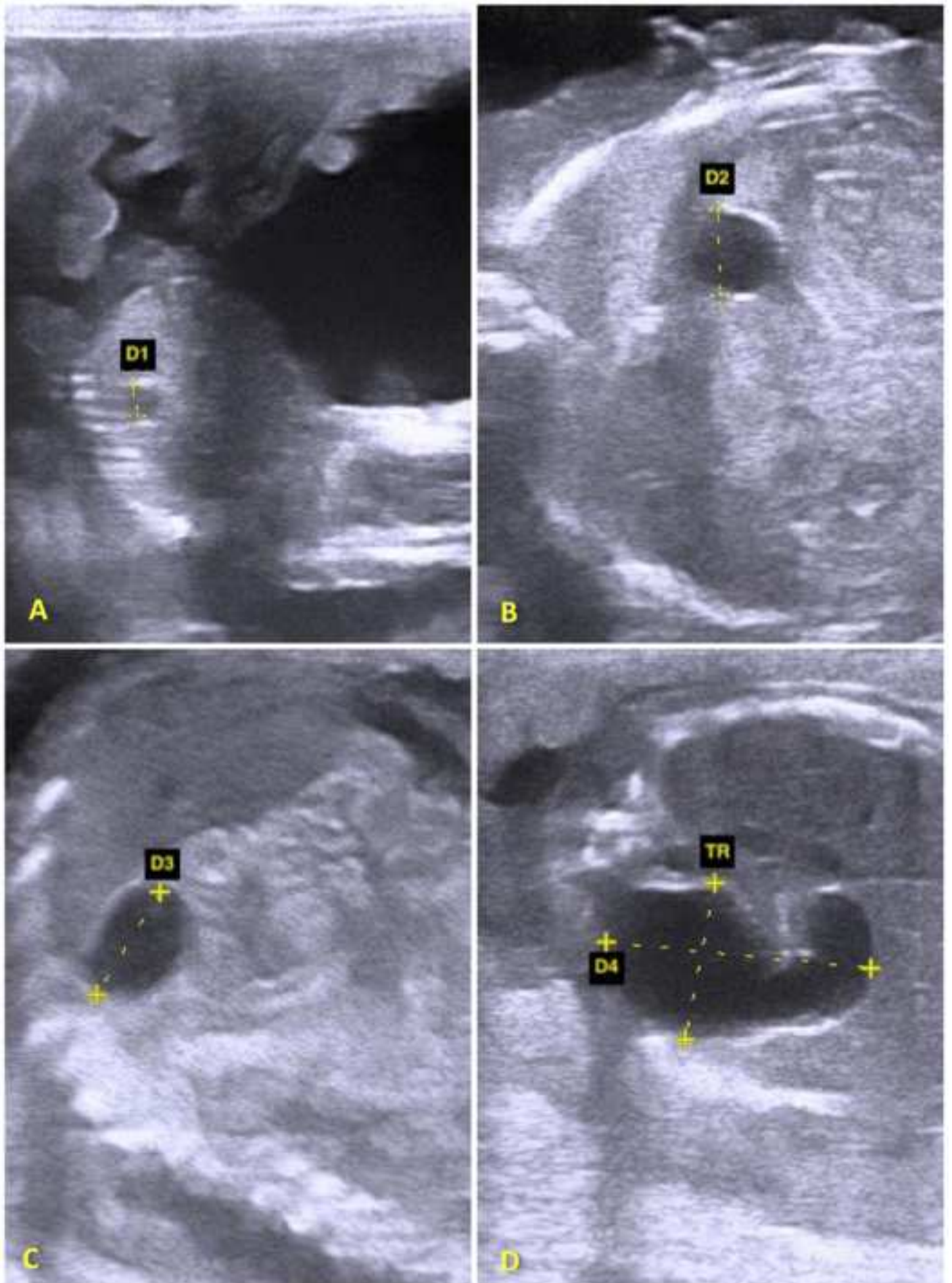


Figure 7

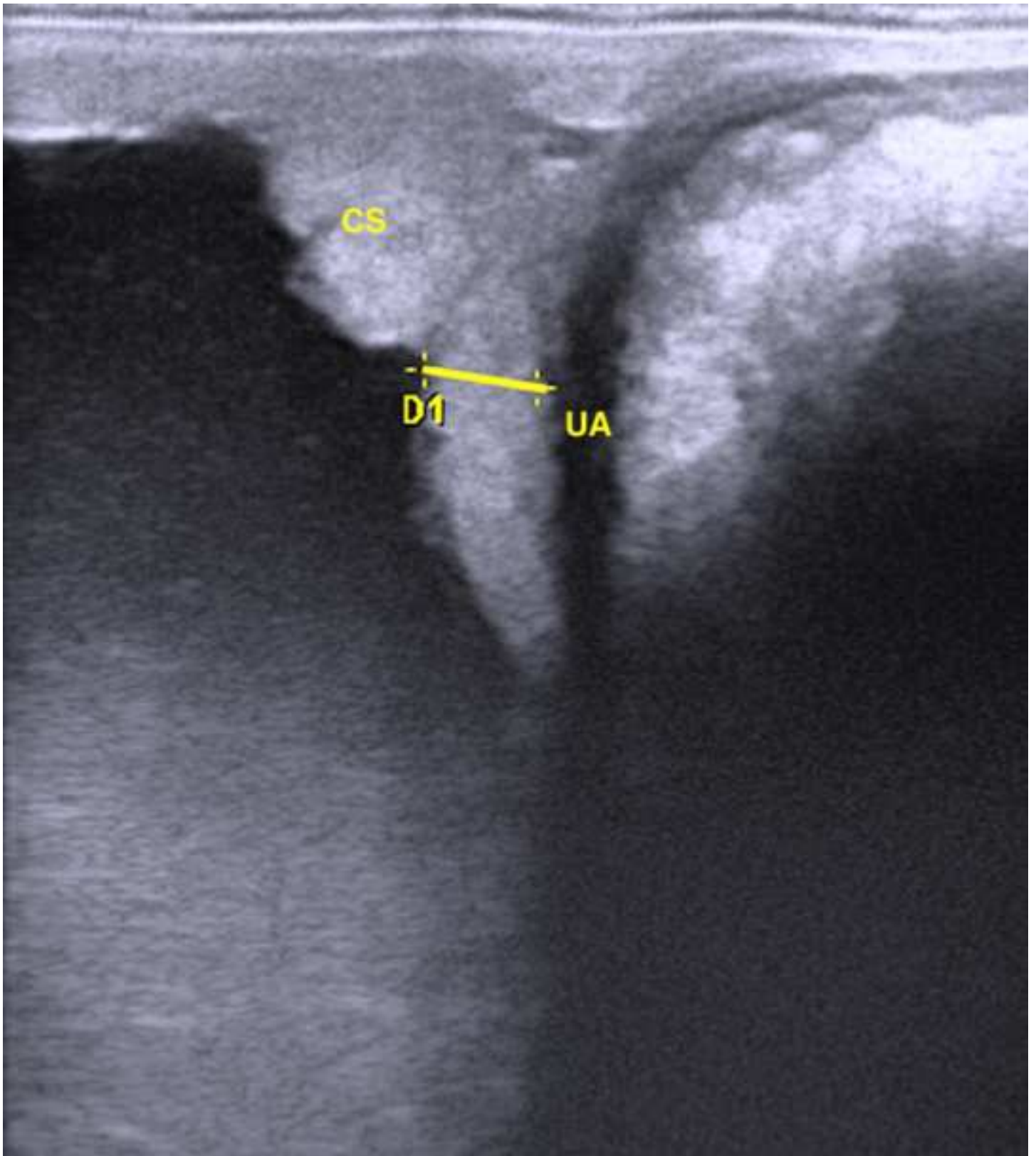


Figure 8



Figure 9

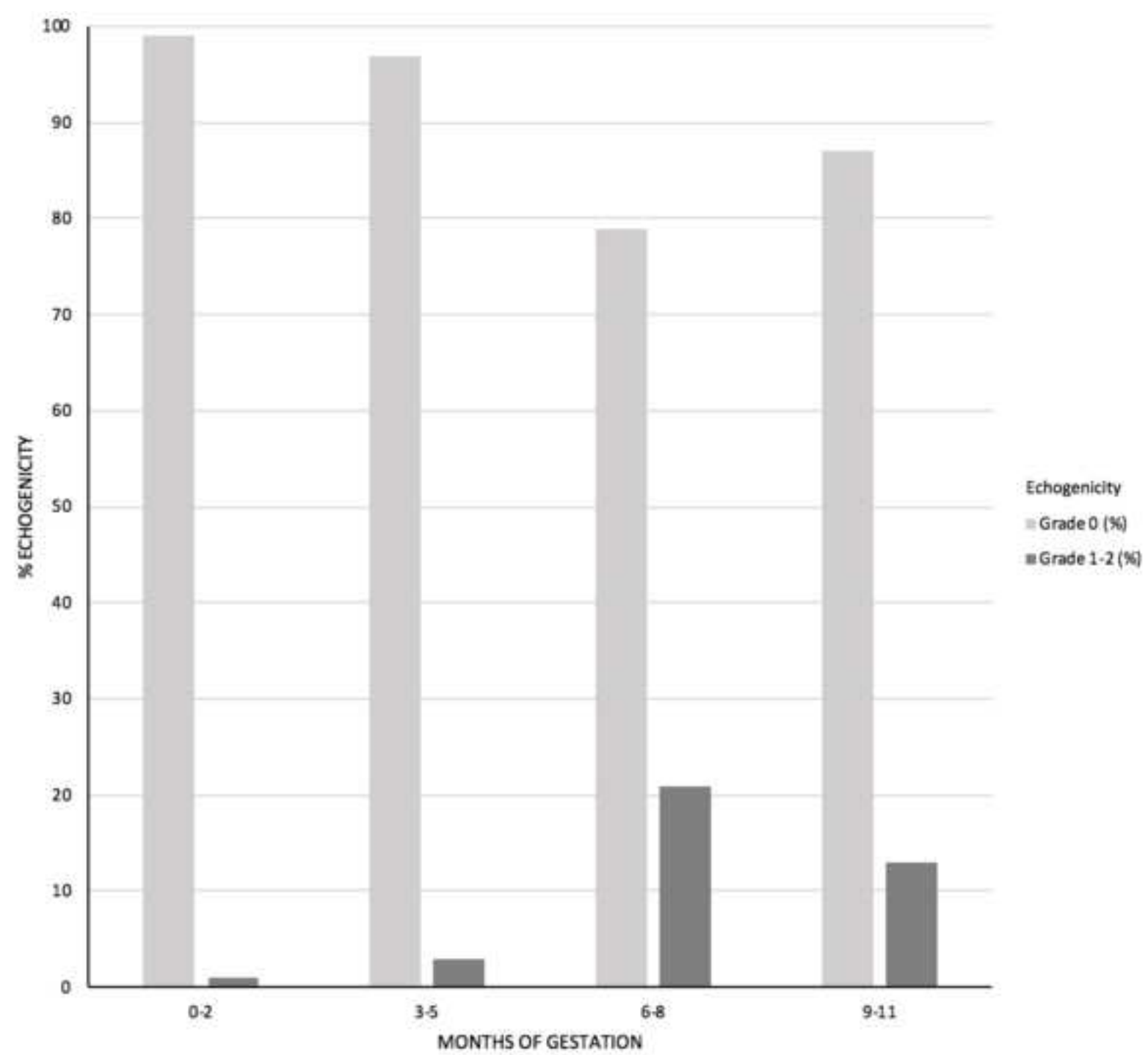
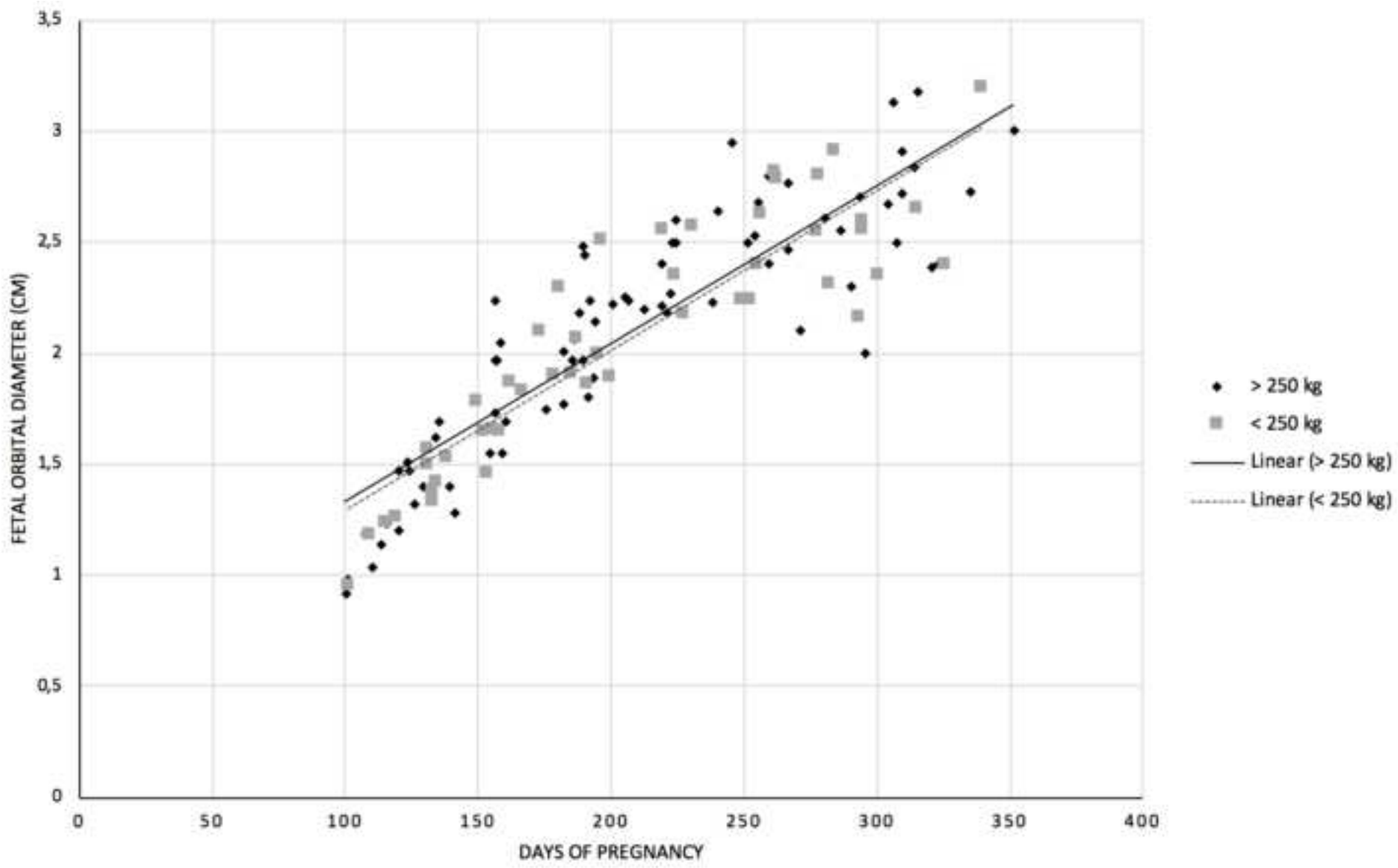


Figure 10





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

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