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ULTRASONOGRAPHIC MONITORING OF EARLY PREGNANCY DEVELOPMENT IN MURRAH BUFFALO HEIFERS (*BUBALUS BUBALIS*)

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ABSTRACT

The aim of the current study was to monitor the early pregnancy development in buffalo and establish biometric threshold of different gestational vesicle and embryo/fetal parts from the day of diagnostic to the 70th day of pregnancy. Serial daily sonographic examinations were carried out on eleven pregnant buffalo-heifers, during which the gestational vesicle diameter, amniotic vesicle diameter, crown-rump length, trunk diameter and biparietal diameter were measured. Embryo/fetal sex characteristics were also determined during sonographic examination. The gestational vesicle, embryo and its heartbeats were assessed, respectively, by gestational days (gd) 20.55 ± 2.34; 25.18 ± 1.91 and 25.27 ± 3.58 of pregnancy. The liquid column in non pregnant horn and amniotic vesicle were detected, respectively, by days 31.0 ± 3.83 and 31.64 ± 2.34 of pregnancy. Subsequently were sequentially detected the neural tube (gd 33.0 ± 3.51), umbilical chord (gd 40.25 ± 2.76), early limbs, (gd 41.67 ± 4.85), embryos proper movement (gd 46.25 ± 2.36), vertebral column (gd 47.33 ± 4.9), body organization (gd 47.48 ± 5.61), genital tubercle (gd 44.5 ± 2.39), fetal bones (gd 56.56 ± 1.16), brain (gd 57.5 ± 1.2), urinary bladder (gd 57.88 ± 3.08), stomach cavities (gd 59.17 ± 4.36), brain ventricles (gd 61.09 ± 2.34), end of migration of genital tubercle in males (gd 58.4 ± 2.2) and in females (gd 60.4 ± 4.2), skull acoustic shadow (gd 59.66 ± 3.13), vertebra acoustic shadow (gd 62.14 ± 3.13), ribs acoustic shadow (gd 64 ± 2.65) and limb bones acoustic shadow (gd 65 ± 1.58) and liver (gd 66.63 ± 3.54). High regression and correlation coefficients were found between different studied parameters and gestational age, where the highest correlation was found with the biparietal diameter and crown-rump length. In conclusion, the overall data indicated the feasibility and value of ultrasonographic embryo, fetal and vesicle characteristics and embryo/fetometry in buffaloes for the evaluation of fetal development and estimation of gestational age during the first 70th pregnancy days.

Key words:

INTRODUCTION

Since its introduction into human obstetrics in the late 1950s, ultrasound has played an increasing important role in the characterization of normal embryo and fetal growth and development and the detection of intrauterine growth retardation. Improvements in the image quality and scanning capability have permitted visualization of greater anatomical details which, in turn had led to more sophisticated analyses of growth processes in humans (Deter *et al*., 1981, De Vries & Fong, 2006), bovine (White *et al*., 1985; Curran *et al*., 1986a,b; Szenci *et al*., 1988; Curran *et al*., 1989; Kähn, 1989a; Kastelic & Ginther, 1989; Wideman *et al*., 1989; Ogata *et al*., 1999; Ali, 2004; Rosiles *et al*., 2005) and equine (Ginther, 1984a; Ginther, 1984b; Pipers *et al*., 1984; Ginther, 1985; Leith *et al*., 1985, Matsui *et al*., 1985; Woods *et al*, 1985; Meira *et al*., 1998).

In Buffaloes, however, there are only two ultrasonographic studies monitoring the normal embryo/fetal development in the literature (Pawshe *et al*., 1994; Ali e Fahmy, 2008).

Therefore, the aim of the current study was monitoring the early pregnancy development in buffalo and establish biometric threshold of different gestational vesicle and embryo/fetal parts from the day of diagnostic to the 70th day of pregnancy.

MATERIALS AND METHODS

Eleven adult clinically healthy Murrah heifers weighing 350 – 375 Kg were daily examined by transrectal ultrasonography from days 15 – 70 of pregnancy. The ultrasound examinations were performed using a real-time, B-mode, diagnostic scanner equipped with a 5 or 7.5 MHz linear array transducer (Aloka SSD 500 - Tokyo, Japan). The buffaloes were restrained in a chute in a shed where the light was dim.

At each examination, an attempt was made to record the first detection of gestational vesicle, embryo proper, heartbeats, liquid column in non pregnant horn, amniotic vesicle, neural tube, umbilical chord, early limbs, embryo proper movements, vertebral column, body organization, genital tubercle, fetal bones, brain, urinary bladder, stomach cavities, bone acoustic shadow and liver.

Once detected it was also recorded the gestational vesicle diameter (GVD, the widest diameter), amniotic vesicle diameter (AVD, the widest diameter), crown-rump length (CRL, a straight line between the fetal crown and the origin of the tail), trunk diameter (TD, the widest diameter or at level of umbilical cord attachment) and biparietal diameter (BPD, the widest diameter).

Regression and correlation coefficients of the embryo/fetal parameters (GVD, AVD, CRL, TD and BPD) were calculated depending on days of gestation using standard linear statistical (Quinn & Keough, 2002). A 5% of significance level was used. The BioEstat pack (Ayres *et al*., 2000) was used in the analyses.

The data related the first detection of vesicle and embryo/fetal structures were presented as mean and standard deviation.

RESULTS

The first sign of pregnancy establishment was the detection of gestational vesicle on day 20.55 ± 2.34 (fig. 1a). The embryo proper within the gestational vesicle (fig. 1b) and it is heartbeats were detected, respectively, by days 25.18 ± 1.91 and 25.27 ± 3.58 of pregnancy. The liquid column in non pregnant horn and amniotic vesicle were detected, respectively, by days 31.0 ± 3.83 and 31.64 ± 2.34 of pregnancy.

The gestational vesicle initially displayed a spherical shape with distinct outline limits showing a slightly irregular inner surface. However, from 25th gestational day some endometrial bulges were detected (fig. 1f) as an early sign of uterine segmentation that was obvious by 36 (fig. 1g) and achieved its maximum magnitude by days 67-70 (fig. 1v).

At the first observations the embryos proper were always very near or in contact with the uterine wall and appeared as a elliptical structure of 0.67 ± 0.1 cm of length and 0.45 ± 0.16 cm of width (fig. 1b).

The first ultrasonographic sign of embryonic organization, detected by day 33.0 ± 3.51, was a hipoecogenic line longitudinally oriented that was recognized as the neural tube (future spinal cord).

The second ultrasonographic sign of embryonic organization was the detection of the umbilical cord by day 40.25 ± 2.76. During this period it was also observed an anecogenic vesicle located at the abdominal end of umbilical cord (fig. 1h), that was indentified as a vestige of vitelline sac.

Sequentially after day 40 important signs of embryo development were firstly detected: thoracic and pelvic limbs by day 41.67 ± 4.85 (fig. 1j), embryos proper movement by day 46.25 ± 2.36, vertebral column (vertebrae) by day 47.33 ± 4.9, and the anechoic area of the developing eye by day 47.78 ± 5.61 (fig. 1j). These ultrasonographic finds gave conceptus an organized appearance (head, body, limbs, and tail) (fig. 1k).

The genital tubercle was firstly observed as a hyperechoic bilobed structure between the pelvic members by day 47.2 ± 3 (fig. 1j), and, respectively, in males close to umbilical cord attachment and in females close to de basis of the tail by days 58.4 ± 2.2 (fig. 1u) and 60.4 ± 4.2 (fig. 1t).

Sequentially after day 50, it was firstly detected the split hooves by day 49.67 ± 5.05 (fig. 1n), the bone organization of the fetal skeleton by day 56.56 ± 1.67, brain and its hemispheres by day 57.5 ± 1.2 dg (fig. 1q), urinary bladder by day 57.88 ± 3.08 (fig. 1m), stomach cavities by day 59.17 ± 4.36, including omasum (fig. 1s), skull acoustic shadow by day 59.66 ± 3.13 (fig. 1r), cerebral ventricles by day 61.09 ± 2.34 (fig. 1p), vertebra acoustic shadow by day 62.14 ± 3.13, rib acoustic shadow by day 64 ± 2.65, limb bones acoustic shadow by days 65 ± 1.58 (fig. 1s) and liver by days 66.63 ± 3.54 (fig. 1x).

Two females, one on day 53 and another on day, showed fetuses with prolonged periods of hiccups, characterized by performing repetitive rhythmic motion of small amplitude involving the whole body.

All the correlations between gestational age and each of the studies parameters were highly significant ($p<0.0001$) and depicted in fig. 2. All the regressions were also highly significant ($p<0.0001$) and the calculated coefficients for TD, CRL, BPD, GVD, AVD were, respectively 0.92, 0.96, 0.98, 0.87 and 0.9.

DISCUSSION

In the present study the gestational vesicle and embryo first detection, respectively, on days 20.55 ± 2.34 and 25.18 ± 1.91, were delayed when compared with the results obtained by Pawshe *et al*. (1994). They reported the detection of the gestational vesicle and embryo on days 19 ± 2.1 and 19 ± 1.6 respectively. However, while these authors had detected the embryo's heartbeat only on day 29.6 ± 1.57, in the present study the detection occurred on day 25.27 ± 3.58, which happened to be by the same moment that the embryo was visualized.

The great difficulty of early identification of the embryo in the present study was due its position, which was very close to or in contact with the uterine wall. this was also reported in cattle by Kähn (1994) and in order to overcome this problem, bovine ultrasonographic pregnancy diagnosis is considered positive only when gestational vesicle and embryo, together with its heartbeats can be detected (Kastelic *et al*., 1989). The observation of similar phenomenon in buffalo heifers signalizing that the embryo's heartbeats detection is also an important step in ultrasonographic pregnancy diagnosis, as it allows us to differ the embryo from the uterine wall.

Taking into consideration the heartbeat detection as the moment in which the gestational diagnosis presents 100% of sensitivity in the present study it happened on gestational day (gd) 25.27 ± 3.58. Hence it was earlier than the one showed by Pawshe *et al*. (1994).

It was also earlier than the ones in other buffalo gestational ultrasonographic studies (30-55 days, 28-35 days and 31-35 days, by, El-Shahat *et al*., 2004; Glatzel *et al*., 2000; Karen *et al*., 2007, respectively). In studies performed in the farm the 97% sensitivity diagnosis only occurred on days 30-45 (Bhosreker *et al*., 2000).

All in all, the other findings of this study were similar to the ones from Pawshe *et al*. (1994) and this is true for the moment of the amniotic vesicle detection (31.64 ± 2.34 x 33.4 ± 1.64), neural tube (33 ± 3.51 x 35.8 ±2.5), embryo's own movement (46.25 ± 2.76 x 49.4 ± 2.3), hoof structures (49.67 ± 5.05 x 46 ± 2.66) and bone detection (56.56 ± 1.67 x 59.8 ± 2.3). The only discrepancies appeared when the diagnosis of limbs (42.7 ± 5.62 x 34.6 ± 1.34) and the optical areas (47.78 ± 5.61 x 38.2 ± 2.39) were taken into consideration. As for the first one, the difference seems to be only connected to some nomenclature differences, since what Pawshe *et al*. (1994) called as limb was in fact the limb bud; the present study only considered the observed structure as a limb when they were well developed and very similar to the definitive organ.

When it comes to the analysis of the second difference, the lack of details in the study from Pawshe *et al*. (1994) and the inexistence of further ultrasonographic studies showing details of this gestational period in buffalos hinder any possible explanation. Other findings in this study such as initial diagnosis of gestational vesicle in the non-pregnant horn, umbilical cord, genital tubercle between rear limbs, brain and its hemispheres, urinary bladder, end of the genital tubercle migration phase both in males and females, stomach, acoustic shadow of fetal bones, uterine segmentation, brain ventricles, and liver may be considered original information regarding the buffalo embryo development.

The ultrasonographic findings described by Ali & Fahmy (2008), such as the embryo organization into head, body and limbs (gd 42 - 49), detection of bones (gd 56 – 70), diagnosis of fetus gender (gd 56 – 70) and omasum (gd 70), are similar to the ones found in the present study. Nevertheless, due to the weekly or biweekly assessment adopted by these authors it is not possible to compare results in an appropriate way. Apart from the placentomes, optical area, and lengthy of uterine segmentation, the initial moment of ultrasound detection of gestational vesicle, embryo and buffalo fetus were similar to the ones found in the bovine (Pierson & Ginther, 1984; Kähn *et al*., 1989b; Kähn, 1994). The placentomes that were not seen until the 70th gd in buffaloes could be noticed on gd 30-40 in the bovine (Curran *et al*., 1986b). As for the optical area it was only noticed on day 47.78 ± 5.61 in the present study, which could be noticed on gd 40 in the bovine (KÄHN *et al*., 1989b). The uterine segmentation was kept evident until the end of the experimental period in the buffalo cows, but for the cow this was less intense by the 70th day of pregnancy (Kähn *et al*., 1989b).

In humans, the eighth pregnancy week represents the last phase of the embryo period. During that week the fingers, which still present webbing between digits, separate via a process that is commanded by apoptosis; the head, which is about half of the embryo size, draws attention to itself due to the development of the neck, and on the head it is possible to recognize lips, nose, eyes and ears; external sexual organs are not differentiated thus they can become masculine or feminine. At the end of that week, on the 56th pregnancy day, limbs are well defined and the first own movements are observed. From this moment onwards the new being starts to be called as a fetus (Adé-Damilano *et al*., 2009).

Taking into consideration the morphological and functional criteria that are adopted to define the transition between embryo and fetus in humans, the detection of the embryo closure and the umbilical cord formation (dg 40.25 ± 2.76), the detection of embryo's own movements (dg 46.25 ± 2.36), visualization of the genital tubercle between the rear limbs (dg 47.2 ± 3.00) and the hoof structures (dg 49.67 ± 5.05) in the buffalo embryos suggest that this is the last phase from the embryo period in buffaloes. Thus, at the end of this period (after gd 50) the new being could be called as a fetus.

The human fetus performs a series of diaphragm movements, from which we can highlight the breathing, sighing and hiccupping. The hiccupping is the most precocious movement of the three since it appears at the ninth week. Together with the other two movements and the detection of embryo's own movement, the hiccup is considered a signal that there had been a neuromuscular development and also of the fetal central nervous system (De Vries & Fong, 2006). The detection of hiccup in two buffalo fetuses on days 53 and 56 of pregnancy confirm that on this phase the embryo period had already finished.

Taking into account the coefficients of correlation and regression gathered in this study, the best variables to estimate the pregnancy time were: the crown-rump length (r=0.989) and biparietal diameter(r=0.971). Ali & Fahmy (2008) had found different results. These authors did not observe a better adjustment for the variables related to embryo/fetus apart from the crown-rump length (r=0.94). The variables such as gestational vesicle diameter (r=0.96) and amniotic vesicle diameter(r=0.97) presented better adjustments to the ones observed for the biparietal diameter (r=0.89) and trunk diameter (r=0.86). However, as this study was performed with weekly observations of these variables it is not possible to make more accurate comparisons between the two studies.

As far as the bovine pregnancy is concerned, there is the report that the measurement of the diameter of the gestational vesicle and the amniotic vesicle present a good variability inter and intraindividuals if repetitions of assessments are performed at different points of the vesicle (Kähn, 1994). Similar results were seen by Rosiles *et al*. (2005), when they studied the pregnancy development in *Bos taurus indicus* until 40th day after artificial insemination.

In conclusion the overall data indicated the feasibility and value of ultrasonographic embryo, fetal and vesicle characteristics and embryo/fetometry in buffaloes for the evaluation of fetal development and estimation of gestational age during the first 70th pregnancy days.

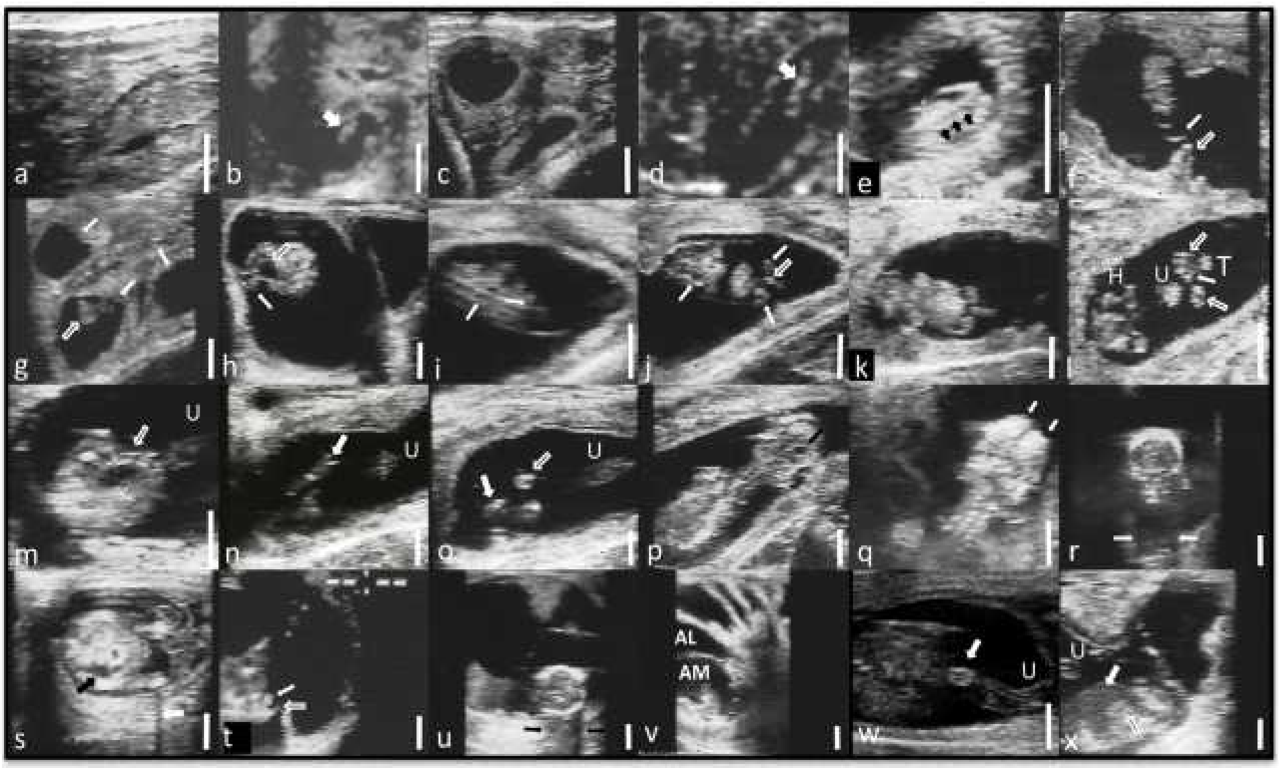

Figure 1. Ultrasonography of buffalo gestational vesicles (a) longitudinal section - day 23 (d23); (b) longitudinal section: embryo proper (arrow) - d27; (c) transversal and oblique section - d28; (d) longitudinal section: embryo proper (arrow) – d28; (e) embryo horizontal section: neural tube (arrows) - d29; (f) embryo sagittal section, amniotic membrane (white arrow) and endometrial bulge (black arrow) - d25; (g) segmentation of gestational vesicle (white arrows) and embryo proper (black arrow) - d36; (h) embryo transversal section: umbilical cord (white arrow) and vitelline sac vestige (black arrow) - d45; (i) embryo horizontal section: vertebral spine (arrow) - d45; (j) embryo horizontal section: developing eye (white arrow) and genital tubercle (black arrow) between pelvic limbs; (k) embryo horizontal section: body organization into head, body, limbs buds and tail– d48; (l) embryo horizontal section: head (H), umbilical cord (U) e genital tubercle (white arrow), pelvic limbs (black arrows) and tail (T) - d50; (m) fetal oblique transversal section at abdominal region: urinary bladder (black arrow) and umbilical cord (U) - d60; (n) pelvic limb longitudinal section: split hoof (white arrow) and umbilical cord (U) – d60; (o) pelvic limbs transversal section (black arrow): female genital tubercle and umbilical cord (U) - d60; (p) fetal horizontal section at head and neck regions: cerebral ventricles (black arrow) - d60; (q) head and neck horizontal section: cerebral hemisphere – d62; (r) head horizontal section: skull acoustic shadow (arrows) – d66; (s) abdomen transversal section: stomach cavities (black arrow), omasum and limb bone acoustic shadow (white arrow) – d66; (t) sagittal section of pelvic region: tail (black arrow) and female genital tubercle (white arrow) - d67; (u) head sagittal section: acoustic shadow (arrows); (v) intense segmentation of gestational vesicle – allantoic (AL) and amniotic (AM) vesicles - d67; (w) transversal abdominal section: umbilical cord (U) and male genital tubercle (arrow) - d70; (x) abdominal sagittal section: liver (white arrow) and omasum (black arrow) - d70.

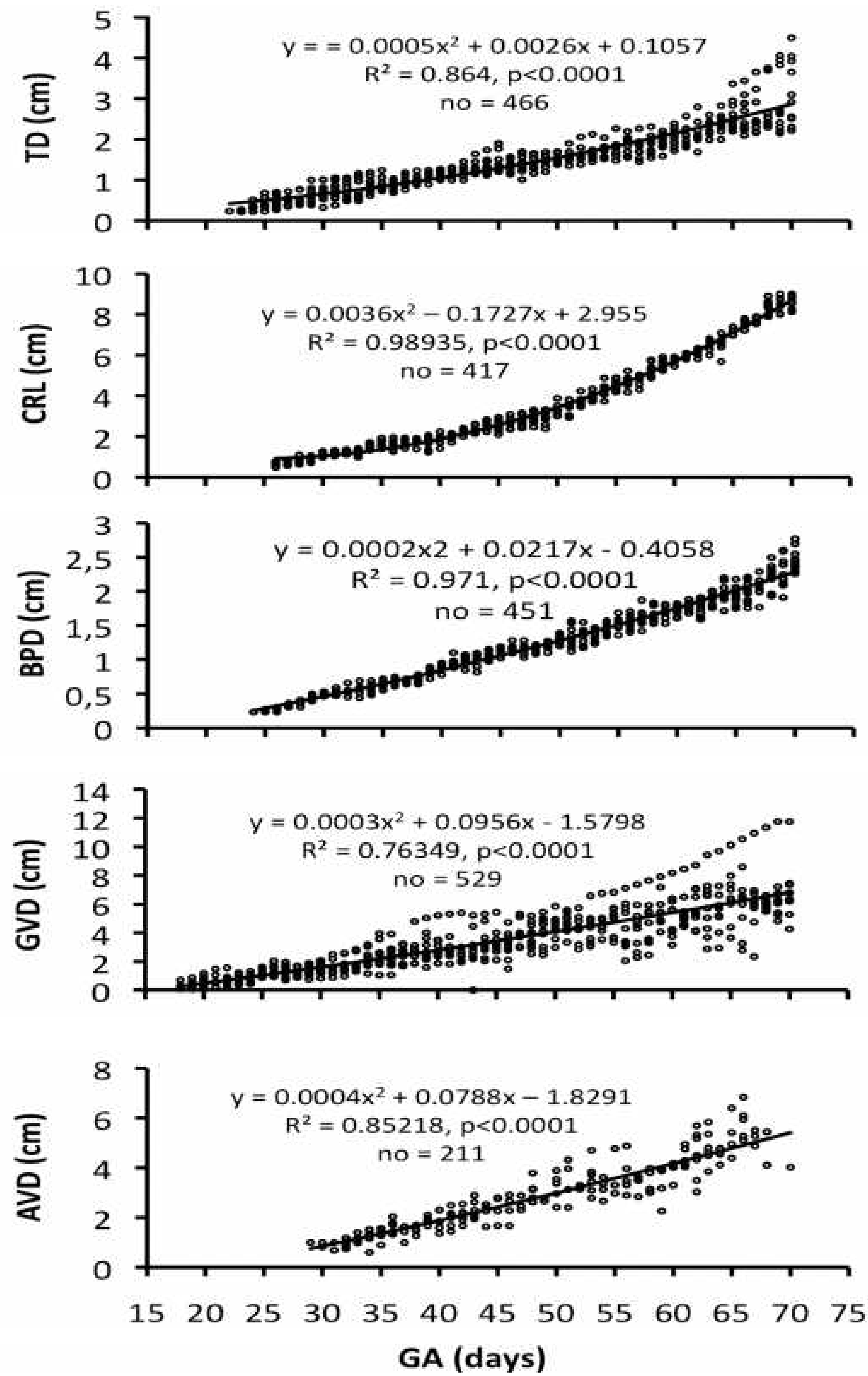

Figure 2. Linear regression between trunk diameter (TD), crown-rump length (CRL), biparietal diameter (BPD), gestational vesicle diameter (GVD), amniotic vesicle diameter (AVD) and gestational age (GA) of buffalo heifers (n=11). (no = number of observations).

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