ESTIMATION OF GESTATIONAL AGE BY THE USE OF FETAL PARAMETERS; PLACENTOME, FEMUR LENGTH, AND BIPARIETAL DIAMETER

A dissertation presented in partial fulfillment of the requirements for the degree of Master of Veterinary Studies at Massey University

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Dedicated to Wilson Bunyaga, Theresia Kigadye, Mary Bunyaga and Zilatashe Bunyaga

Abstract

The research was conducted at (LATU) Large Animal Teaching Unit, Massey University, New Zealand. The study involved 23 beef cows and 60 dairy cows. The aim of the study was to test and assess the agreement between actual gestational age and that predicted using a model developed in previous studies based on placentome length (gestational age = placentome*2.88 - 6.11; Adeyinka et al., (2014) and to compare this agreement with that obtained using fetal measurements of femur length and head size (biparietal diameter). The research commenced on February 2015 and July 2015 for beef and dairy cows respectively and ended June 2015 and September 2015 for beef and dairy cows respectively. Beef cows were scanned transrectally every after three (3) weeks while dairy cows were scanned every after one (1) week.. Overall there was no evidence of bias in all the parameters studied. The strongest association (R^2=0.85) was seen in dairy cattle when biparietal diameter was measured and the weakest was placentome size in dairy cattle (R^2=0.39). This is the first study that has specifically focused on the agreement between estimates of gestational age from fetal size and actual gestational age. This study has shown biparietal diameter to be a better predictor of gestational age than mean placentome size across the range of gestational ages found in this study. However, because the limits-of-agreement for biparietal diameter increase as gestational age increases, by 120 days of gestation the difference between the limits-of-agreement for biparietal diameter and those for mean placentome size are much smaller than in early gestation, and the limits-of-agreement for biparietal diameter are similar to those previously reported where data from all placentomes measured were used to predict gestational age rather than just a single mean placentome size. At 120 days of gestation, placentomes can be measured in a much higher proportion of cattle than biparietal diameter, and even when the latter can be measured, placentome measurements are markedly easier to obtain. Femur length was by far the most difficult parameter to measure and especially by 120 days was much less precise than mean placentome size or biparietal diameter. Therefore, femur length should be restricted to use only in early gestation and then should be used alongside other parameters like biparietal diameter.

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List of Abbreviations

AI Artificial insemination

BPD Biparietal diameter

CL Corpus luteum

CRL Crown Rump Length

FTAI Fixed-time artificial insemination

GH Growth Hormone

IGF 1 Insulin-like growth factor 1

IVF In vitro fertilization

LATU Large animal research unit

MHz megahertz

mm millimetres

NT Nuclear transfer

P P-value

R^2 Squared correlation, R-squared

SPSS Statistical Packages for Social Sciences

Vs Versus

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1: Literature review of fetal and placentome growth

1.1 Introduction

Gestation is the period between conception and birth, and gestational age is the time since conception. In cattle, gestational age is most commonly estimated using rectal palpation or transrectal ultrasonography. In the present study, the focus is on the use of transrectal ultrasonography. This is a quick, safe and non-invasive technique that can be used to diagnose pregnancy as early as 28 days after conception (Racewicz and Jaskowski, 2013). Transrectal ultrasound can also be used to determine gestational age, based on the relationship between time since conception and size of the conceptus (Varol *et al*., 2001), and fetal viability, based on the presence of a beating heart (Lamb *et al*., 2015). Importantly transrectal ultrasound has not been found to affect embryonic or fetal viability (Ball and Logue, 1994; Kahn, 1992).

Why should we be concerned about gestational age?

The key rationale for estimating (or confirming) gestational age is that doing so will allow prediction of expected calving date; in dairy cattle this prediction can be used to identify drying-off date and to plan for calving (e.g. identifying labour requirements) (Doize *et al*., 1997). In extensive systems, drafting cows based on fetal age at particular time points during the year may be easier than trying to locate and remove all bulls from a paddock (Jephcott, 2009).

A huge range of parameters are potentially measureable using transrectal ultrasound including biparietal diameter, crown-rump length, femoral length, thoracic diameter and abdominal diameter and non-fetally-based measures such as placentome diameter or corpus luteum size.

1.2 Fetal growth

To properly understand the value of fetal measurement as a measure of gestational age, it is necessary to understand fetal growth and the factors that affect it. Fetal growth and development are complex processes that involve interactions of genetic and environmental factors (Bellows *et al*., 1993). In cattle, genetic factors influencing fetal growth include breed-level effects and genotype effects within breeds, while environmental factors can include maternal effects such as nutrition, lactation status, parity and size (which can also be influenced by genetics). Placental development and blood flow also influence fetal development; these are influenced, like fetal growth itself, by a combination of maternal and fetal factors. Much of the impact of environmental and genetic factors on fetal growth is mediated by hormones such as thyroid hormone, growth hormone, and insulin.

Fetal growth is strongly influenced by the genetic potential of the fetus as well as maternal genetics (Bellows *et al*., 1993; Ferrell, 1991). Fetal genotype seems to be most important during the early and mid-gestation whereas maternal genotype is most important during late gestation when most of the growth occurs (Greenwood *et al*., 2010).

Breed	**Body weight (kg) at gestational age of**		
	3 months	**6 months**	**9 months**
German Angus	0.22±0.04	8.70±1.41	34.17±4.34
Galloway	0.11±0.07	6.75±2.15	29.60±6.92
Holstein Friesian	0.32±0.03	8.81±1.05	46.34±6.84
Belgian Blue	0.24±0.05	8.39±1.55	46.34±6.28

Table 1: Illustration of the effect of breed on fetal growth (body weight) (data from Mao *et al*., 2008).

The physiological status of the mother may also affect fetal growth; for example, in early pregnancy, fetal development in lactating Holsteins is slower than in cows which were dried off at calving (Green *et al*., 2012); however, there are no published studies which compare this in detail. In contrast, in Belgian Blue dams, growth of the dam seems to be a more important limiting factor for fetal growth than lactation. Across all breeds significant variation is noted between heifers and cows (Roberts, 1986). This may be related to placental development as, compared to mature cows, heifers have a smaller total cotyledonary surface (Van Eetvelde *et al*.,2016).

Another crucial factor influencing fetal growth rate is fetal gender, with differences between male and female fetuses apparent from 100 days of pregnancy (Eley *et al*., 1978). Gestational age also influences fetal growth rates; fetal growth rates increase with time until a maximum is reached at ~230 days of gestation (Ferrell *et al*., 1976; Eley *et al*., 1978). These effects are illustrated in Figure 1. Eley *et al*., (1978) suggested that the slowing in the growth rate of the fetus in the third trimester is the result of placental capacity being unable to increase sufficiently rapidly to support the very high growth rates seen before 230 days of gestation.

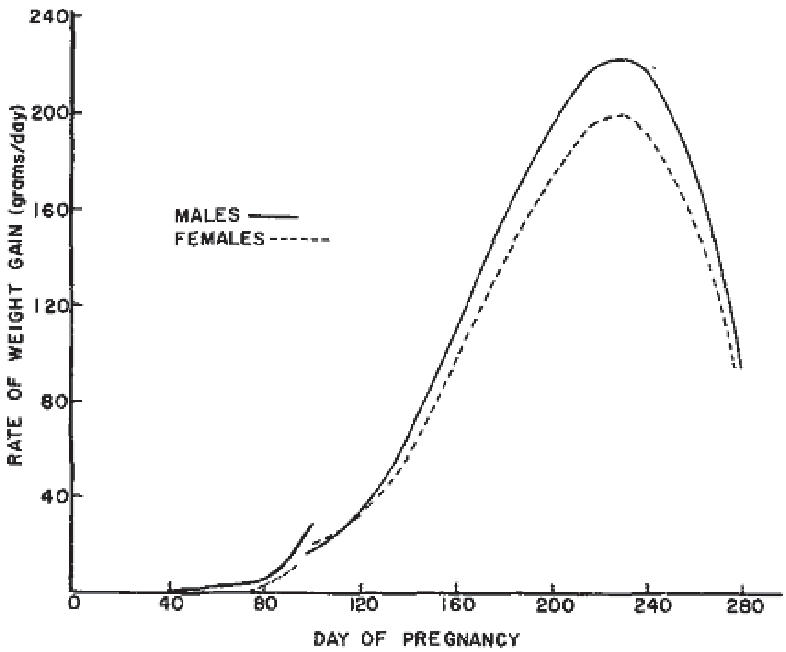


Figure 1: Effect of age and sex on fetal growth rates (adapted from Eley *et al*., 1978)

(Note: the kink at ~100 days represents a demarcation between two groups of fetuses, one measured from 40 to 100 days of gestation and the other measured from day 100 to day 280).

1.2.1 Fetal growth – data from post-mortem studies

Post-mortem studies have provided valuable information on fetal development, identifying stages such as implantation (~day 30), forelimb bud and tail development (days 24-29) and hind limb development (30-36 days) (Alberto *et al*., 2013). As seen in Figure 2, the curve for fetal growth has dominant, positive linear components with negative quadratic ones (O' Rourke *et al*., 1991).

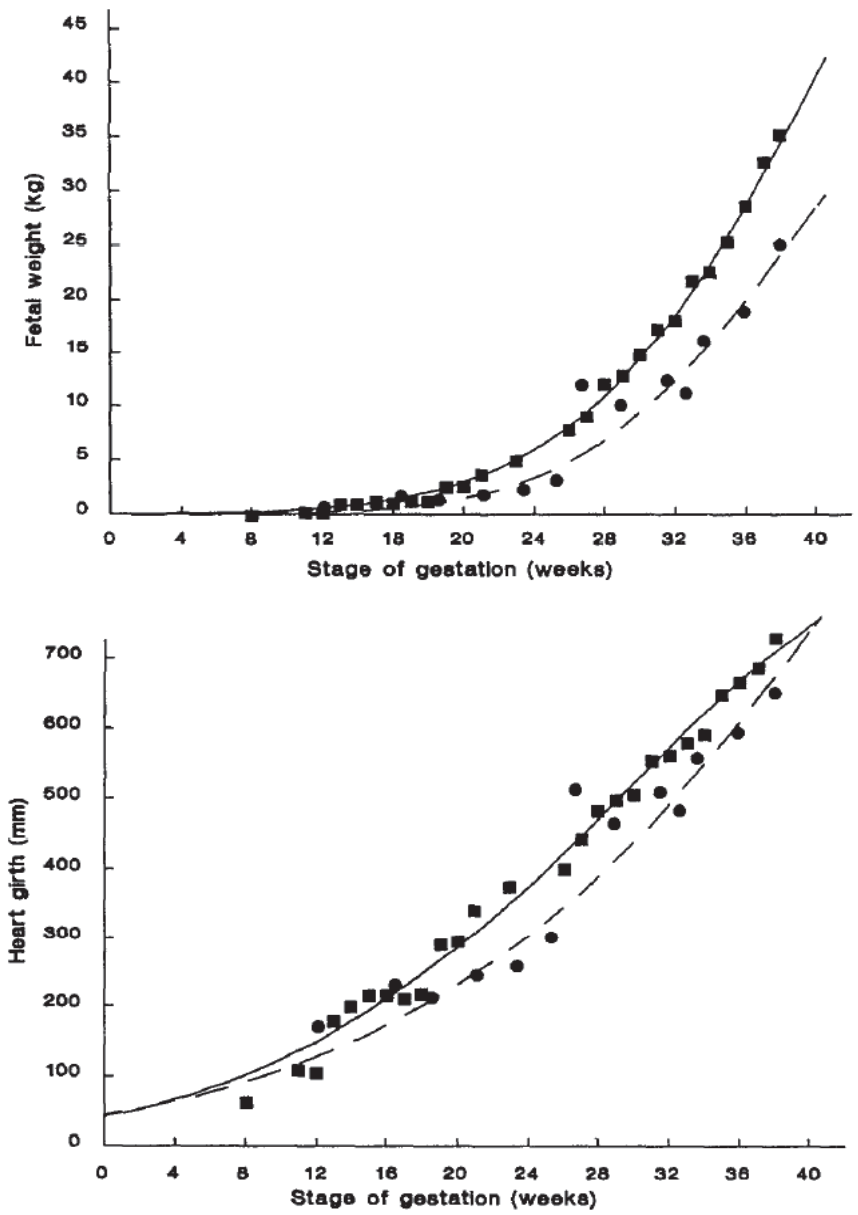


Figure 2: Growth curves and stage means for fetal weight (kg) and heart girth against gestational age (weeks). Data from *Bos taurus* (squares) and *Bos indicus* (circles) (from Orourke *et al*., 1991)

Post mortem studies have shown that the growth patterns of different fetal parts vary. For instance, compared to the head, the forelegs grow very slowly in the first trimester but in the second trimester foreleg growth is faster than head growth (O' Rourke *et al*., 1991).

1.2.2 Fetal growth – data from rectal palpation

Rectal palpation of the uterus and fetus can be used to estimate gestational age reasonably accurately (Matthews and Morton, 2012), but, as the technique does not lend itself to accurate measurement, fetal size is often reported as a vague estimate rather than an exact size. For example, Roberts (1986) stated that when gestational age is 2 months, the fetus is like a mouse, at 3 months like a rat, at 4 months like a small cat, at 5 months like a large cat and at 6 months like a beagle dog.

1.2.3 Fetal growth- data from in vivo ultrasound studies

Ultrasonography is probably the most versatile method of pregnancy diagnosis in cattle and is the method of choice when determining gestational age as it can be used to accurately measure parameters such as embryo width, biparietal diameter and crown-rump length as well as identifying key stages in fetal/embryo development such as limb bud formation, and differentiation of the head and abdomen.

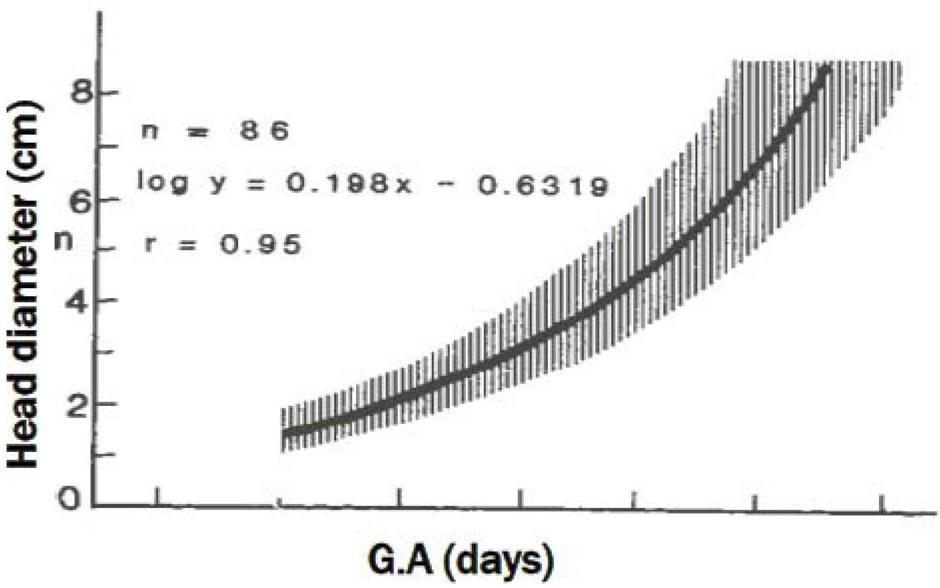
In early gestation, combining fetal/embryo measurements with data from key stages is extremely accurate in determining gestational age. Fitzgerald *et al*., (2015) found that the correlation between actual gestational age (based on most recent service recorded) and that estimated using ultrasound was extremely strong (r=0.99) and the mean difference between actual and predicted gestational age was 0.51 ± 0.04 days, with a standard deviation of 3.39 days. However, they also reported a larger mean difference when only data from fetuses >42 days of age were included (0.81 ± 0.13), and that accuracy of age determination declined markedly after 60 days of gestation.

Fitzgerald *et al*., (2015) identified several factors in addition to gestational age which influenced the difference between actual and estimated gestational age: parity of the cow (cows with parity 5+ had smaller conceptuses than those in their first parity), sex of the calf and herd-year-season. Interestingly, fetal growth up to day 95 of gestation is not affected by twinning (Echtemkamp, 1993).

White (1985) found the overall accuracy of pregnancy diagnosis to be 98.3%, and reported the correlation between head diameter and gestational age was 0.95 (see figure 3). They reported that crown-rump length provided a good estimate of gestational age with a residual standard deviation (i.e. the standard deviation of the differences between observed and predicted values) of 4.5 days (i.e. 95% of predictions were within 9 days of the observed result) whereas diameters of the head, trunk and nose had residual standard deviations of 6.9-8.7 days. Wright *et al*., (1988) also reported a residual standard deviation of 6.9 days for head diameter. They also found an overall bias of 0.9 days, i.e. on average predicted gestational ages based on multiple parameters were 0.9 days greater than actual gestational ages, but when head diameter alone was used the bias was 1.4 days.

Probe frequency has been suggested as important when determining accuracy of pregnancy diagnosis and gestational age. Kahn, (2004) suggested that if the fetus was very close to the probe then a 5 MHz probe was suitable, but if the fetus was further away from the probe then a 3.5 MHz one was preferred. Arthur *et al*., (2009) recommended that a 7 MHz linear transducer be used for detecting early pregnancy and a 3.5-5.0 MHz probe for late pregnancy. Transducers with lower frequencies provide better tissue penetration but give poorer resolution (because of their longer wavelength). Despite these recommendations, there are no data showing that changing probe frequency alters the accuracy of estimating gestational age.

Figure 3: Correlation of the head diameter and gestational age (G.A) in cows, showing very strong correlation. Shaded area marks 95% confidence interval. Source: White *et al*., (1985)



1.2.4 Fetal measurements

A wide range of parameters that can be measured by ultrasonography have been shown to be highly correlated with gestational age, including: crown rump length (r=0.91), head (biparietal) diameter (r=0.95), head length (r=0.94), trunk diameter (r=0.95), nose diameter (r=0.95), uterine diameter (r=0.93) (all results from White et al., 1985) and femur length (r=0.99; Kahn, 1989).

These measurements are illustrated in Fig 4 and 5. Femur measurement is considered to be accurate only when the image shows two blunted ends, i.e. the extension to the greater trochanter and the head of the femur are not included.

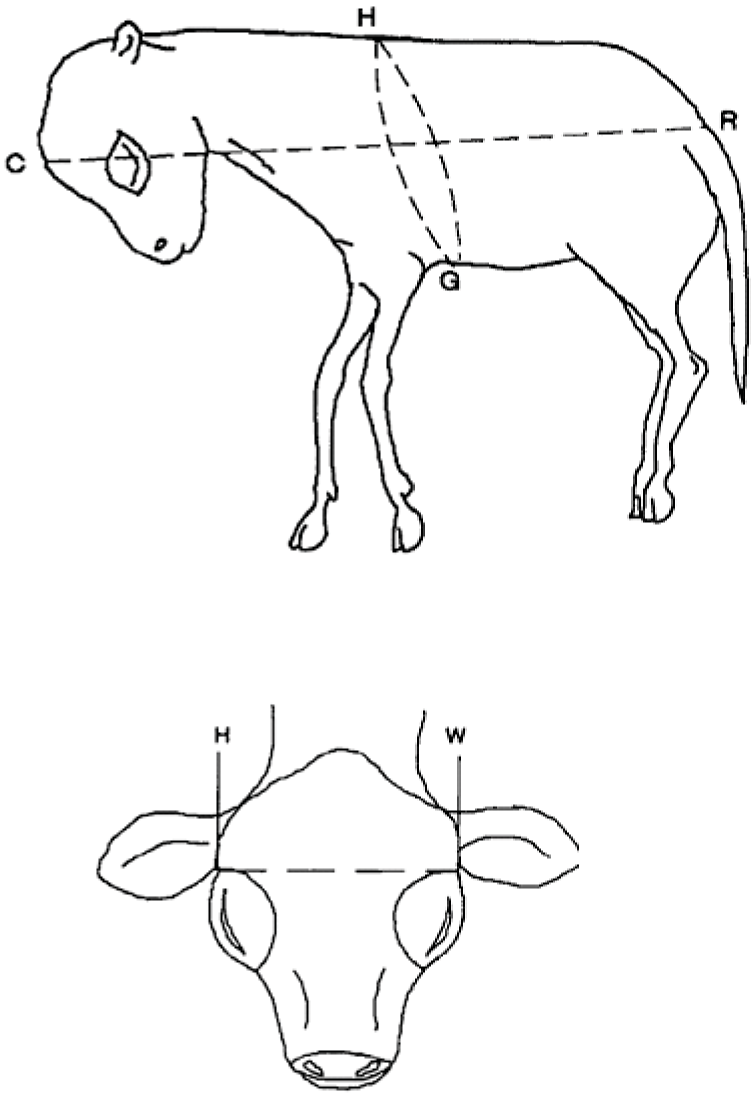


Figure 4: Illustration of reference points for three fetal measurements which can be made using transrectal ultrasound

CR – crown-rump length

GH – trunk diameter

HW – head (biparietal) diameter

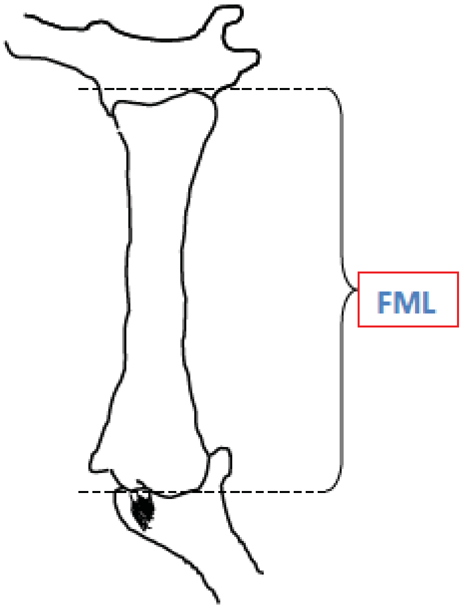


Figure 5: Representation of reference points for femur length (FML) used when measuring using ultrasound

1.2.5 Biparietal diameter

The biparietal diameter, the widest distance between the outer borders of the cranium at an angle of 90° to its long axis, is a commonly used measurement of fetal size. Reports in a wide range of species have shown a significant correlation between BPD and gestational age e.g. Ferreira *et al*., (2012) in Murrah buffalo (see Figure 6), goats (Haibel and Perkins, 1989; Abdelghafar *et al*., 2011), sheep (Sergeev *et al*., 1990) and hyena (Place *et al*., 2002) as well as cattle (White *et al*., 1985).

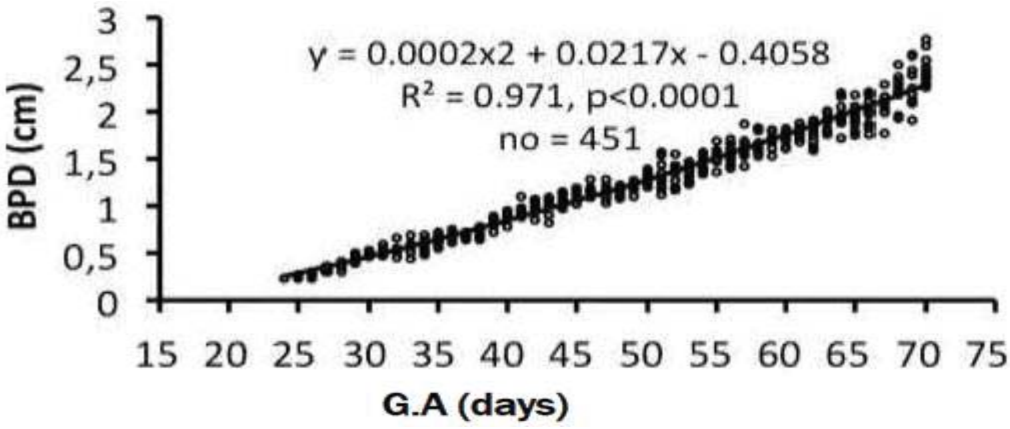


Figure 6: Association between biparietal diameter (BPD) and gestational age (G.A) in Murrah buffalos showing strong correlation in early gestation (from Ferreira *et al*., 2016)

Another advantage of using BPD is the strong correlation between this parameter and crown-rump length (Riding *et al*., 2008; Ferreira *et al*., 2012). The latter parameter is useful in vivo in early gestation and also ex vivo, but in later gestation can be impossible to measure as fetal size increases. In addition BPD is simple to measure and has markedly lower measurement error than other fetal parameters (Wu *et al*., 2012). Other advantages include an apparent lack of impact of fetal sex, and, in Neroli dams, no impact of fetal sire (Bergamaschi *et al*., 2004). Finally, as Kahn 1989 showed, although the proportion of scans when BPD measurement is achievable does decrease with gestational age (from >95% in months 3 and 4 to ~50% in months 9 and 10), throughout gestation BPD remains the fetal parameter which is most frequently possible to measure.

The head is one of the body parts of the embryo which can be identified early in gestation, with a clear differentiation between the head and the rest of the body being demonstrable at the fifth week of pregnancy. The dark area of the developing eye, which is a key landmark for measuring biparietal diameter, can be recognized about day 40 of gestation (Kahn, 2004). BPD measurement can thus be used across a wide range of gestational ages.

1.2.6 Femur length

The femur is is the most proximal bone in the hind limb of the cow, articulating with the acetabulum in the pelvic bone to form the hip joint whereas distally there is a knee joint. As for BPD, studies in multiple species have shown a very strong correlation between gestational age and femur length including goats (Rihab *et al*., 2012), sheep (Noia *et al*., 2002), buffalo (Terzano *et al*., 2012) and hyena (Place *et al*., 2002) as well as cattle (Kahn, 1989) (see Figure 7). In cattle the association is seen in all breeds though there can be significant differences between them (Table 3)

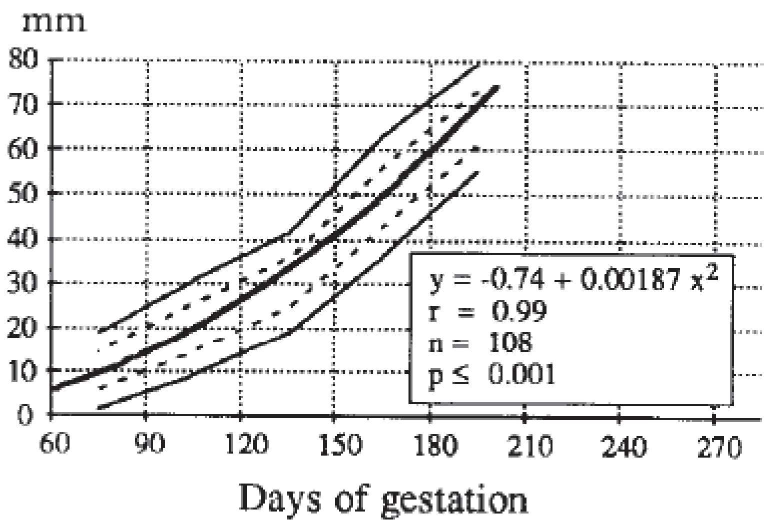


Figure 7: Association between femur length and gestational age in cattle. Solid outer line – 2*SD. From Kahn (1989)

	Gestational age		
Breed	**3 months**	**6 months**	**9 months**
German Angus	4.3	19.7	32.7
Galloway	3.2	17.1	33.3
Holstein Friesian	5.6	21.3	38.5
Belgian Blue	4.6	19.2	36.4

Table 2: Effect of breed on change in leg length (cm) with gestational age (adapted from Mao *et al*., 2008).

Post mortem data has shown that significant cartilaginous development of the long bones has begun by ~7 weeks of gestation with ossification beginning a few weeks later being detectable from around 74 days of gestation (the first identification of calcium phosphate deposits), with ossification centres being seen in all bones by day 81 (Trujillo *et al*., 2011). So although femur measurement is possible from 49 days, measurement using bony landmarks is only feasible only from ~80 days onwards

Compared to BPD, femur measurement is much less practicable in older fetuses. Kahn (1989) reported that by month 4 of gestation only 60% of fetuses could have measurements made of their hind limb area. By month 7 this figure had decreased to 25%, while in months 9 and 10 no measurement of the hind limbs was possible. This means that femur measurement as an estimate of gestational age needs to be restricted to fetuses <160 days.

1.3 Placentome

1.3.1 Introduction

One key issue with estimating gestational age using fetal measurement is the difficulty of finding the fetus in later gestation, particularly >day 120 (Kahn, 1989). In cattle, one potential alternative measurement is to measure the bovine placentome (Adeyinka *et al*., 2014) as the mean size of placentomes is significantly correlated with gestational age (Laven and Peters, 2001).

Placentomes are the functional units of the bovine placenta that are responsible for mediating maternal-fetal gaseous, nutrient and metabolic waste exchange (Leiser *et al*., 1997; Schlafer *et al*., 2000; Hashizime, 2007) as well as production of hormones and other active chemicals (Hoffmann and Schuler, 2002). In the cow the number of placentome ranges from 70 to 140 (Anderson, 1927; Laven and Peters, 2006). Placentomes form as a result of the attachment of the chorioallantois to the uterine epithelium; this occurs at around 30 days of pregnancy (Schlafer *et al*., 2000). Placentome growth is thus a combination of growth of maternal (caruncular) and fetal (cotyledonary) tissues.The growth rates of these two tissues are not the same; Reynolds *et al*., (1990) reported that at day 100 of gestation the weight of the caruncle was equivalent to that of the cotyledon, but by 250 days of gestation, caruncular weight was more than twice that of the cotyledonary tissue (Figure 8b). However, this has no direct relevance to gestational aging as differentiation of fetal and maternal tissue is not required as part of that process. Placentome growth is significantly slower than fetal growth; between day 100 to day 250 fetal weight increases by 73 times whereas placentomal weight increases 16-fold (Figure 8a).

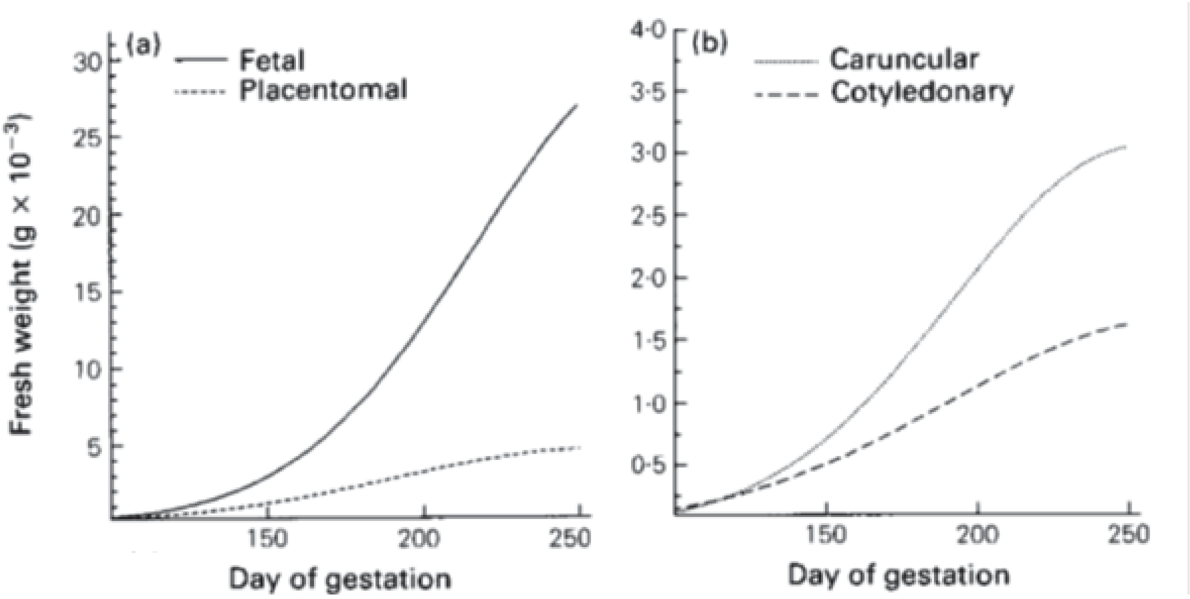


Figure 8: Relationship between a) fetal and placentomal weight and gestational age and b) caruncular and cotyledonary weight and gestational age (Adapted with modification from Reynolds *et al*., 1990).

Both mean placentome weight and length increase significantly throughout the gestation with a relatively linear increase in placentome length as pregnancy advances (Laven and Peters, 2001). Some studies have reported that placentome growth continues throughout the gestation (Abdel-Raouf and Badawi, 1966; Reynolds *et al*., 1990); however, Laven and Peters (2001) reported that it ceased around day 200 of gestation, as did Liu (2010) (see Fig 9).

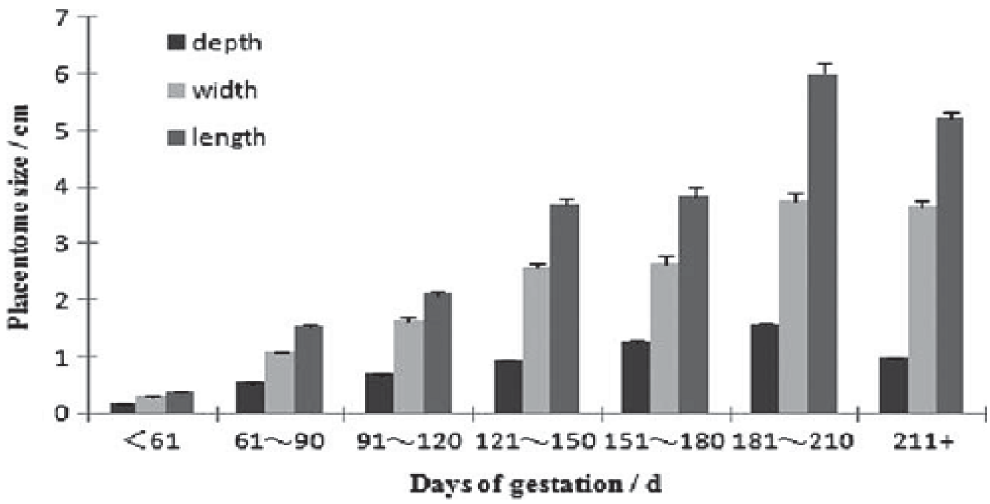


Figure 9: Change in mean placentome depth, width and length during gestation (from Liu, 2010)

Factors other than gestational age can affect placentome size. Average placentome length is significantly less in the non-pregnant horn compared to the pregnant horn (Laven and Peters, 2001). Placentome size also varies depending on the proximity of the measured placentomes to the site of fetal attachment, with larger placentome occurring nearer that area (Youngquist, 1997).

Breed also influences placentome weight and size. Reynolds *et al*., (1990) showed that placentome weight was greater in Charolais cows than Brahman cows, while Van Eetvelde *et al*., (2016) showed that caruncular surface area was greater in Belgian Blue cows than Holstein-Friesian cows. Another factor that can affect placentome growth is fetal sex with male fetuses having larger placentome than females (Arthur, 2009).

1.3.2 Placentome growth – data from rectal palpation

Placentomes are palpable per rectum from around 75-80 days of gestation (Mortimer and Hansen, 2006; Purohit, 2010). Similar to fetal size, estimation of gestational age using rectal palpation of placentomes is often based around a vague estimate. One scheme is that recommended by Mortimer and Hansen, (2006) which is based on U.S currency: a placentome is about "dime" size at 90 days, "nickel" size at around 105 days, a "quarter" at around 120 days and a "fifty cent" size at 150 day.

However, there is a significant variation in size of placentomes across the uterus with those near the fetus being most variable in size as well as being, on average, the largest placentomes (Purohit, 2010). Placentomes near the cervix tend to be smaller but the variation between placentome is less (Adeyinka *et al*., 2014).

1.3.3 Placentome growth – data from ultrasound

Currently, there are three published studies which have used placentome size as measured using ultrasonography to determine gestational age in cows: Hunnam *et al*., (2009), Adeyinka *et al*., (2014) and Lazim *et al* (2016). Hunnam *et al*., (2009) measured placentome size using trans-abdominal ultrasonography; they found no significant association with placentome height or length and gestational age. In contrast Adeyinka *et al*., (2014) who measured placentomes using transrectal ultrasonography, and Lazim *et al*., (2016) who used trans-abdominal ultrasonography found placentome size to be significantly associated with gestational age. Adeyinka *et al*., (2014) suggested that the difference between their results and those of Hunnam *et al*., (2009) was likely to be related to the consistency of placentomes that were measured. Adeyinka *et al*., (2014) measured cervical placentomes and they were thus likely to be selecting placentomes from a consistent population whereas Hunnam *et al*., (2009) were just measuring placentomes near the fetus (which have inherently more variation) and were not necessarily measuring placentomes from the same area every time. However, it is unclear why these factors did not also apply to Lazim *et al*., (2016).

Nevertheless, although they found a significant association, Adeyinka *et al*., (2014) concluded that the agreement between placentome size and gestational age was probably not sufficient for placentome measurement to be used as method of choice for age estimation, based on their limits-of-agreement being too wide at ±33 days. Lawrence *et al*., (2016) were able, by using data from multiple placentomes rather than just mean placentome size, to reduce the limits-of-agreement to ±20 days, which is much nearer to the results calculated from the residuals from the residual standard deviations reported by White *et al*., (1985). Furthermore, Lawrence *et al*., (2016) showed that their limits-of-agreement did not change significantly with gestational age, whereas the graphs of White *et al*., (1985) (see Figure 3) showed that variability did increase with age and thus using a single residual standard deviation would overestimate the variability in early gestation and underestimate it in later gestation (consistent with the findings of Fitzgerald *et al*., (2015) that fetal aging became less accurate as gestation progressed).

The aim of this study was to therefore create a dataset from two fetal measurements (biparietal diameter and femur length) and to directly compare these as predictors for gestational age with placentome size using limits-of-agreement analysis, in a population of beef cattle and a population of dairy cattle.

The current study focuses on agreement whereas previous studies (e.g. White *et al*., (1985) and Kahn (1989)) used correlation. Correlation evaluates whether there is an association between two measurements, i.e. whether as one measurement increases the other does the same. In contrast agreement evaluates how well one measurement can predict another. High correlation does not necessarily imply that there is good agreement between the two measurements (Bland and Altman 2003). Limits-of-agreement analysis will show: i) bias (prediction is systematically different from actual – i.e gestational age predicted from a parameter is 5 days less than actual age); ii) relationship between agreement and the magnitude of the measurements (i.e does agreement decrease as gestational age and fetal size increase); and iii) the accuracy of the prediction (i.e for a specific fetal size, you can say that, 95% of actual gestational ages will be between x and y days Data can be analyzed both as unit differences plot and as percentage difference plot (Bland and Altman, 2015)

2 Methodology

2.1 Materials and methods

2.1.1 Animals

The animals used in this study belonged to the large animal teaching unit (LATU) of Massey University. Twenty-three mixed age Aberdeen Angus cows and 60 non-lactating 2.5-year-old dairy cows (Friesian and Friesian cross Jersey) were used.

In order to ensure that the exact date of conception was known, all the cows used in this study had been synchronised using an intravaginal progesterone plus GnRH-$PGF_{2\alpha}$-GnRH program (Adeyinka *et al*., 2014), with pregnancy diagnosis undertaken 6 weeks after synchronisation. The beef cows were inseminated in groups between 12th December 2014 and 30th January 2015, while the dairy cows were all inseminated on 14th May 2015.

2.1.1.1 Ultrasound equipment

The uteri of the selected cows were examined transrectally, using a B-Mode real-time ultrasound scanner with a variable frequency linear probe set to 7.5 MHz (Mindray DP6600, Mindray Szechuan, China). Fetal and placentome measurement were made in the beef cattle from 18th February to 20th June 2015, while measurements were made in dairy cattle from 20th July to 1st October 2015. This meant beef cattle were scanned between days 45 to 135 of gestation, while dairy cattle were scanned between days 60 to 130. Measurements were made in the beef cows every three weeks, and weekly in the dairy cows.

2.1.1.2 Measurement of biparietal diameter

Biparietal diameter was measured in both the dairy and the beef cows, and was defined as the distance between the two lateral canthi of the eyesockets (line x to x) as shown in Figure 10. Once a suitable image was obtained it was recorded digitally before transfer to a desktop computer for image analysis using the image processing and analysis programme ImageJ (http://rsbweb.nih.gov/ij/index.html)

2.1.1.2.1 Measurement using Image J

Image J was downloaded from https://imagej.nih.gov/ij/download.html and installed. Image J was then run in the computer. Upon opening of the window the memory allocation was adjusted to 70%. The scale was then set i.e. distance displaying in pixels was adjusted by entering 10 for the known distance and mm for the unit length. Length parameter was chosen, and the stored images in the computer (from the ultrasound) were opened, then a line was drawn between the two lateral canthi of the eyesockets. After analysis and measurements the files were saved as JPEG and TIFF images

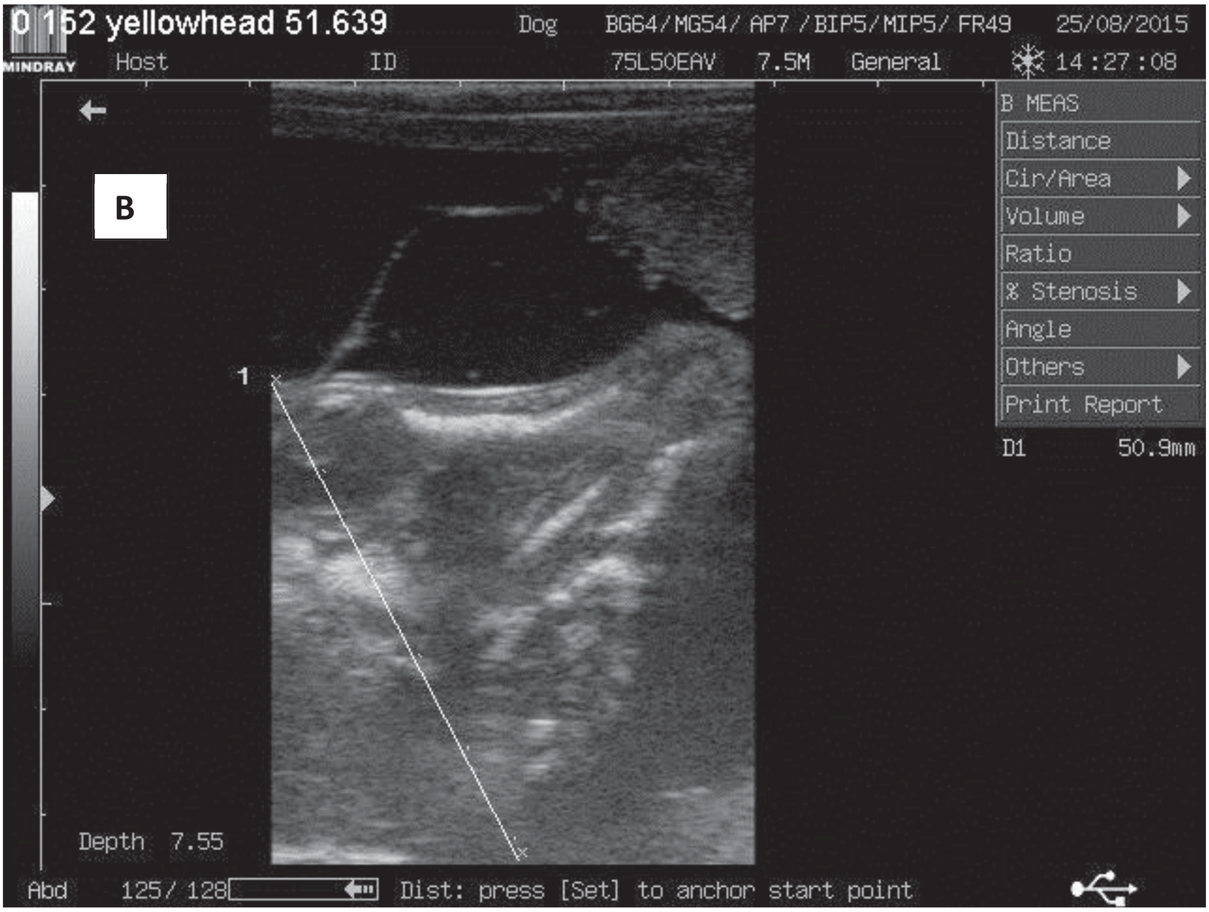


Figure 10: Example of a digital image showing measurement of biparietal diameter

2.1.1.3 Measurement of femur length

Femur length was only measured in the dairy cattle, and was defined as the length of the diaphysis of the femur (see Figure 5) diaphysis at both ends (line x to x) as shown in figure 11. Once a suitable image was obtained it was recorded digitally before transfer to a desktop computer for image analysis using the image processing and analysis programme ImageJ (see Figure 11)

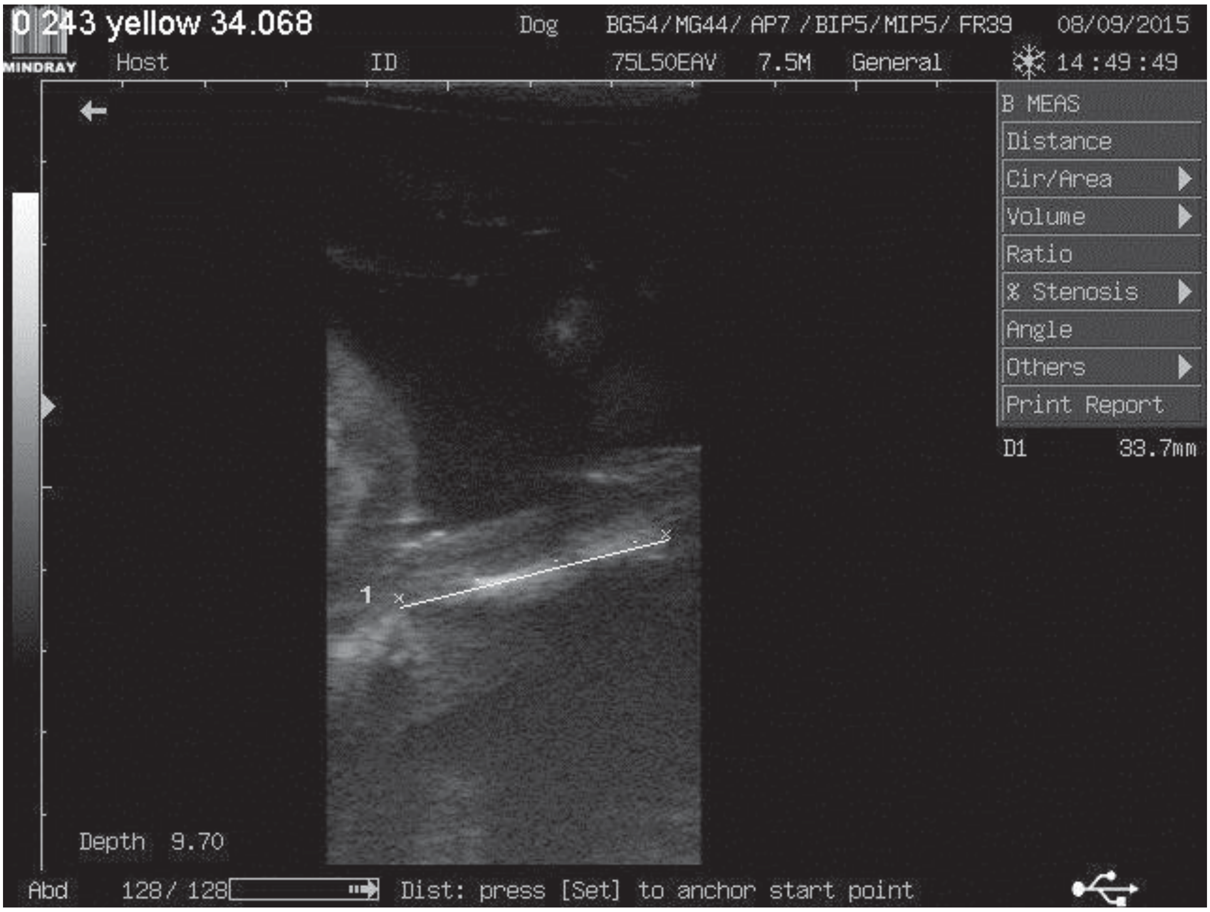


Figure 11: A digital image showing how femur length was measured by using image J software

2.1.1.4 Placentome

Placentome examination was undertaken as described by Adeyinka *et al*., (2014) On each occasion the placentomes for examination were determined by locating the cervix, using the ultrasound probe and then moving the probe one probe-length (about 6 cm) from the cervix. Two to six placentomes were examined on each occasion and were recorded as digital images for later analysis. For each image, the probe was moved to make the placentome image as circular as possible. The images were then transferred to a desktop computer for image analysis using the image processing and analysis programme Image J. Only the longest axis of the placentome was measured (see Figure 12)

Figure 12: Showing how the length of placentome was measured by using image J software

2.1.2 Statistical analysis

Unless otherwise stated all analysis were undertaken using SPSS 24 (IBM, USA)

2.1.2.1 Regression analysis

For each of the three measures, a regression analysis of gestational age against measurement was undertaken to establish the strength of the association between the measures and the best equation for predicting gestational age from the measurements. For biparietal diameter and mean placentome size the regression results for beef and dairy cattle were compared by including type in the regression alongside a dummy variable of type* size (where type=1 if beef and 0 if dairy).

2.1.3.2. Limits-of-agreement analysis

For each measure, predicted age (based on the regression equation) was calculated from the size of the measure, and a mean/difference plot (Bland and Altman, 1989) created. Regression analysis was then undertaken to identify whether there was a significant association between mean and difference (and the variance of that difference), and the limits-of-agreement then calculated (Bland and Altman, 1999). For biparietal diameter limits-of-agreement analyses were undertaken separately for beef and dairy cattle, while for placentome size data from beef and dairy cattle were amalgamated. For placentome size alone, limits-of-agreement were also created using the equation derived by Adeyinka *et al*., (2014) from their data (i.e. predicted gestational age = (mean placentome size – 6.11)/0.288) in addition to those created using the regression equation calculated from the amalgamated data

2.1.2.3 Placentome

Mean placentome length for each cow at each given time point was used to create the produced gestational age. The agreement between the predicted and the actual gestational age of both beef and dairy cows were identified by the limits-of-agreement. Two limits-of-agreement analyses were used. For the first analysis limits-of-agreement were calculated using the standard deviation of the difference between the predicted and the actual gestational age (Bland and Altman, 1999). The second analysis (Bland and Altman, 2007) accounted for the repeated measurement, taking into consideration the association between method difference and gestational age.

2.1.2.4 Biparietal diameter

Biparietal diameter for each cow at each given time point was used to create the predicted gestational age. The agreement between the predicted and the actual gestational age of both beef and dairy cows were identified by the same limits-of-agreement analyses as used for the placentome measurements.

2.1.2.5 Femur length

Femur length for each cow at each given time point was used to create the predicted gestational age. The agreement between the predicted and the actual gestational age of both beef and dairy cows were identified by the same limits-of-agreement analyses as used for the placentome measurements.

2.2 Results

2.2.1 Regression analysis

For the three measures their association with gestational age is illustrated in Figures 13-15

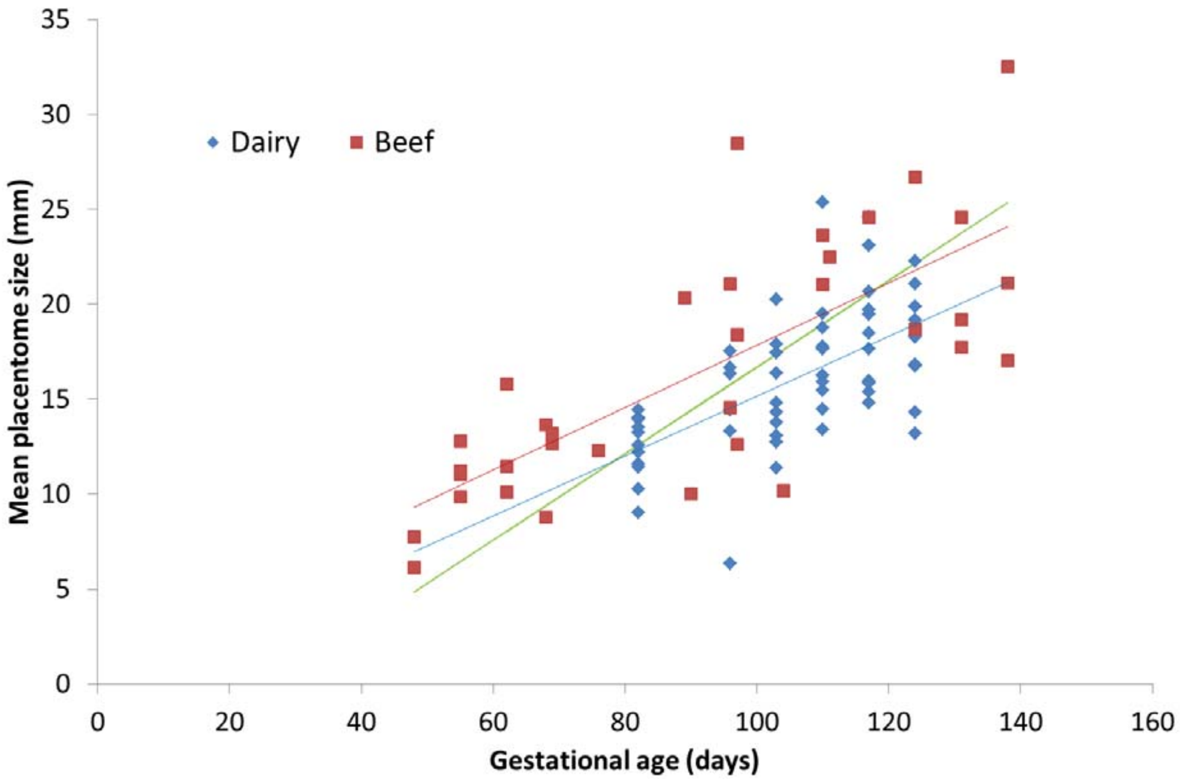


Figure 13: Relationship between placentome length (mm) and gestational age for dairy and beef cows measured using transrectal ultrasonography. Red line: line of best fit for beef cattle; blue line: line of best fit for dairy cattle; green line: mean placentome size = gestational age * 0.288 – 6.11 (prediction line from Adeyinka *et al*., 2014).

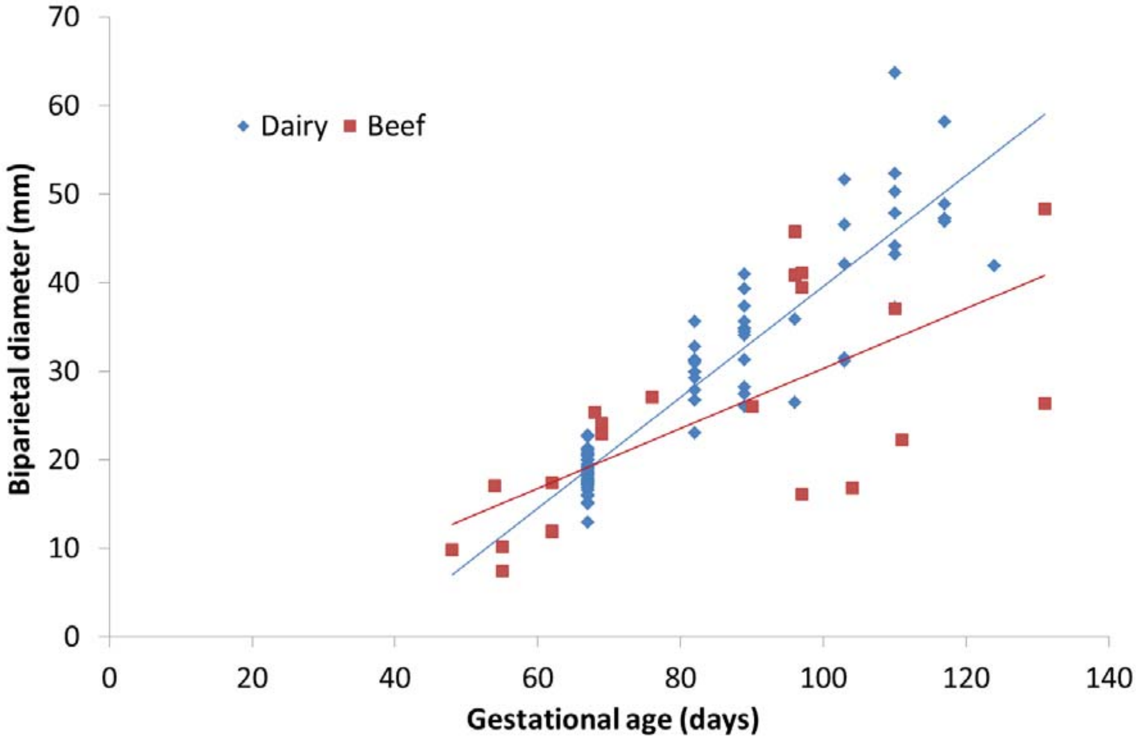


Figure 14: Relationship between biparietal diameter (mm) and gestational age for dairy and beef cows measured using transrectal ultrasonography. Red line: line of best fit for beef cattle; blue line: line of best fit for dairy cattle.

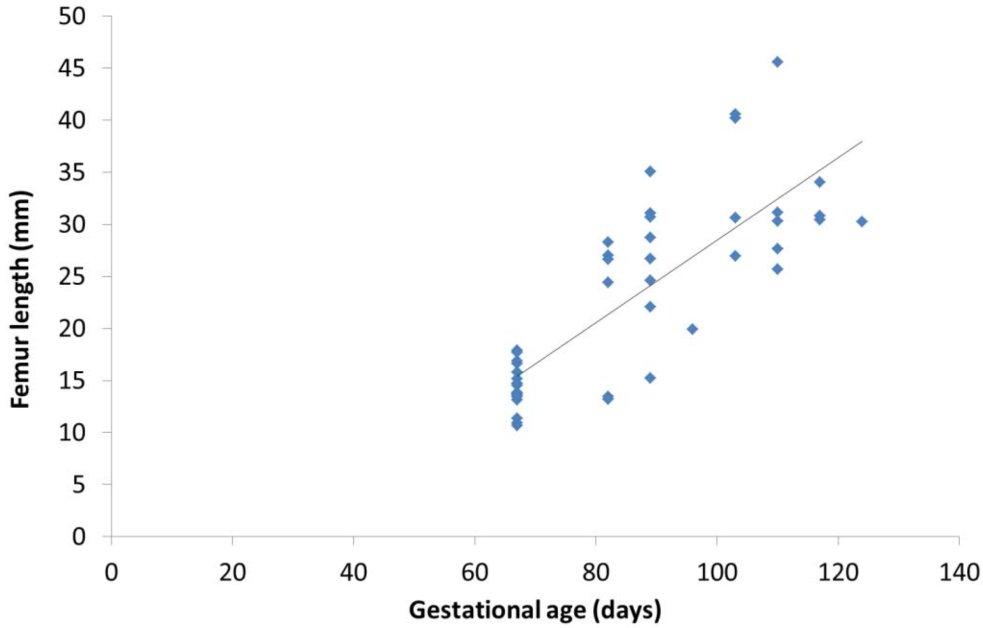


Figure 15: Relationship between femur length (mm) and gestational age for dairy cows measured using transrectal ultrasonography. Solid line: line of best fit.

The results of the five regression analyses are summarised in Table 3.

Measure	**Type**	**Prediction equation from gestational age (days)**	**R^2**
Mean placentome size (mm)	Beef	0.16 *age + 1.45	0.57
	Dairy	0.16*age - 0.63	0.39
	Both	0.15*age + 1.66	0.44
Biparietal diameter (mm)	Beef	0.34*age - 3.5	0.47
	Dairy	0.63*age - 23.0	0.85
Femur (mm)	Dairy	0.40*age - 11.1	0.64

Table 3: Association between gestational and size of three parameters measured using transrectal ultrasound

For biparietal diameter both the intercept and the slope of the regression equation were different for beef cattle compared to dairy cattle ($P<0.001$); in contrast, for placentome size neither of two parameters were different between beef and dairy cattle ($p \geq 0.59$). Thus for the subsequent limits-of-agreement analysis, beef and dairy data were separated for biparietal data but not for placentome size.

2.2.2 Limits-of-agreement analysis

The limits-of-agreement plots for predicted age from biparietal diameter and actual age are shown for beef cattle in figure 16. Overall there was no evidence of bias (mean difference [SEM] was -0.41 [5.8]), but mean and difference were moderately associated (R^2 = 0.22; p = 0.029), and as mean increased the difference went from negative to positive. However the variance of the difference and mean were not associated (R^2 = 0.11; p = 0.161), so limits-of-agreement were calculated accounting for the association between mean and difference only. The limits-of-agreement analysis suggests that at 80 days, 95% of the differences between predicted and actual gestational age will be between -26 and +22 days whereas at 120 days the equivalent figures will be -8.6 and +40 days. So, relative to the line of best fit, ~67% of differences will be ≤10 days.

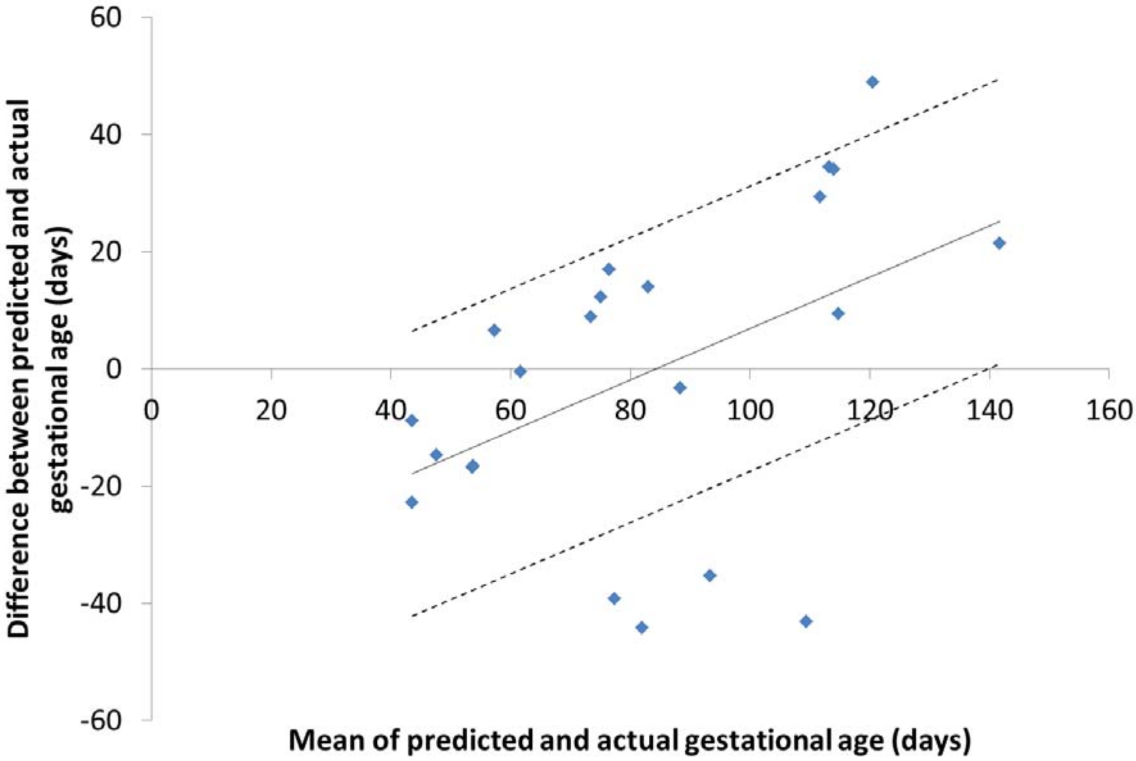


Figure 16: Limits-of-agreement for predicted gestational age (from biparietal diameter) and actual gestational age for Angus cattle. Solid line is association between mean and difference, dashed lines are limits-of-agreement.

The limits-of-agreement plot for dairy cattle is shown in Figure 17. Overall there was no evidence of overall bias (mean difference [SEM] was -0.55 [0.75]). Mean and difference were moderately but not significantly associated (R^2 = 0.19; p = 0.078), but there was a stronger association between mean and variance of the difference (R^2 = 0.31; p <0.001). The limits-of-agreement analysis thus took account of the latter effect only, and suggests that at 80 days, 95% of the differences between predicted and actual gestational age will be between -9.8 and +9.8 days whereas at 120 days the equivalent figures will be -22.3 and +22.3 days. So at 80 days >95% of differences will be ≤10 days, whereas at 120 days ~67% of differences will be ≤10 days

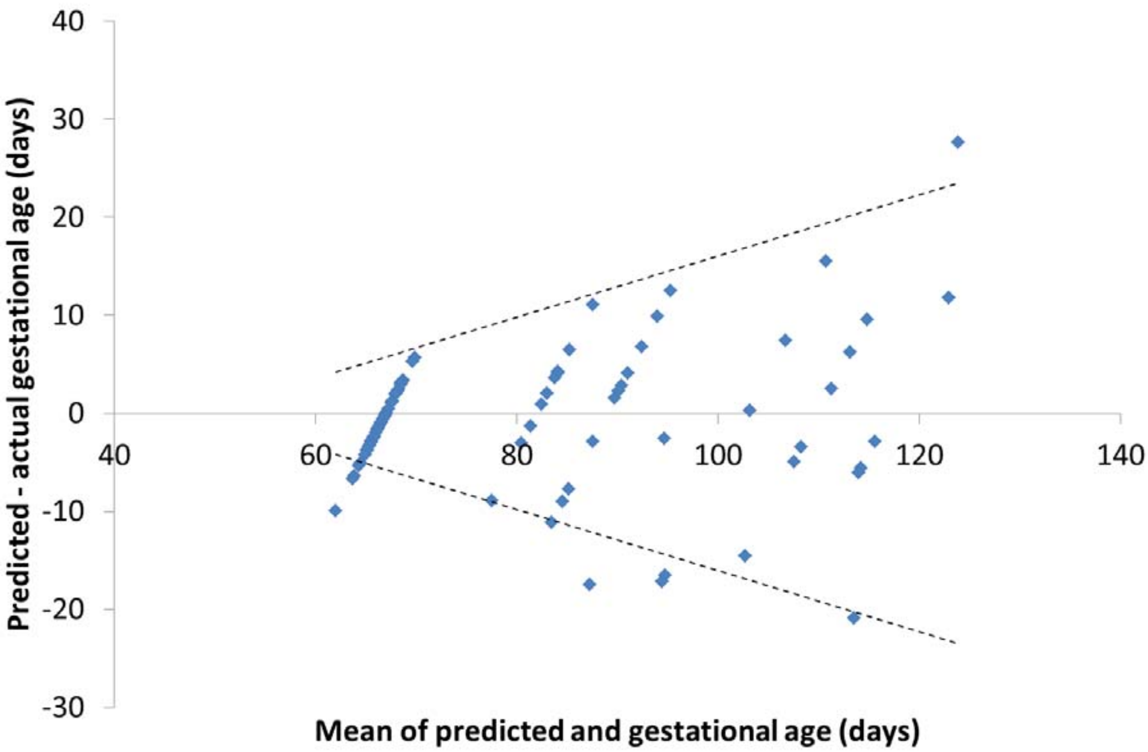


Figure 17: Limits-of-agreement for predicted gestational age (from biparietal diameter) and actual gestational age for dairy cattle. Solid line is association between mean and difference; dashed lines are limits-of-agreement.

The limits-of-agreement plot for mean placentome size based on the regression equation from this study (see Table 3) is shown in Figure 18. Overall there was no evidence of overall bias (mean difference [SEM] was -0.0025 [0.05]). Mean and difference were moderately associated (R^2 = 0.24; p <0.001), but there no association between mean and variance of the difference (R^2 = 0.09; p = 0.358). The limits-of-agreement analysis thus took account of the association between mean and difference and suggests that at 80 days, 95% of the differences between predicted and actual gestational age will be between -55 and +31 days whereas at 120 days the equivalent figures will be -41 and +45 days, with 35% of the differences being between ±10 days.

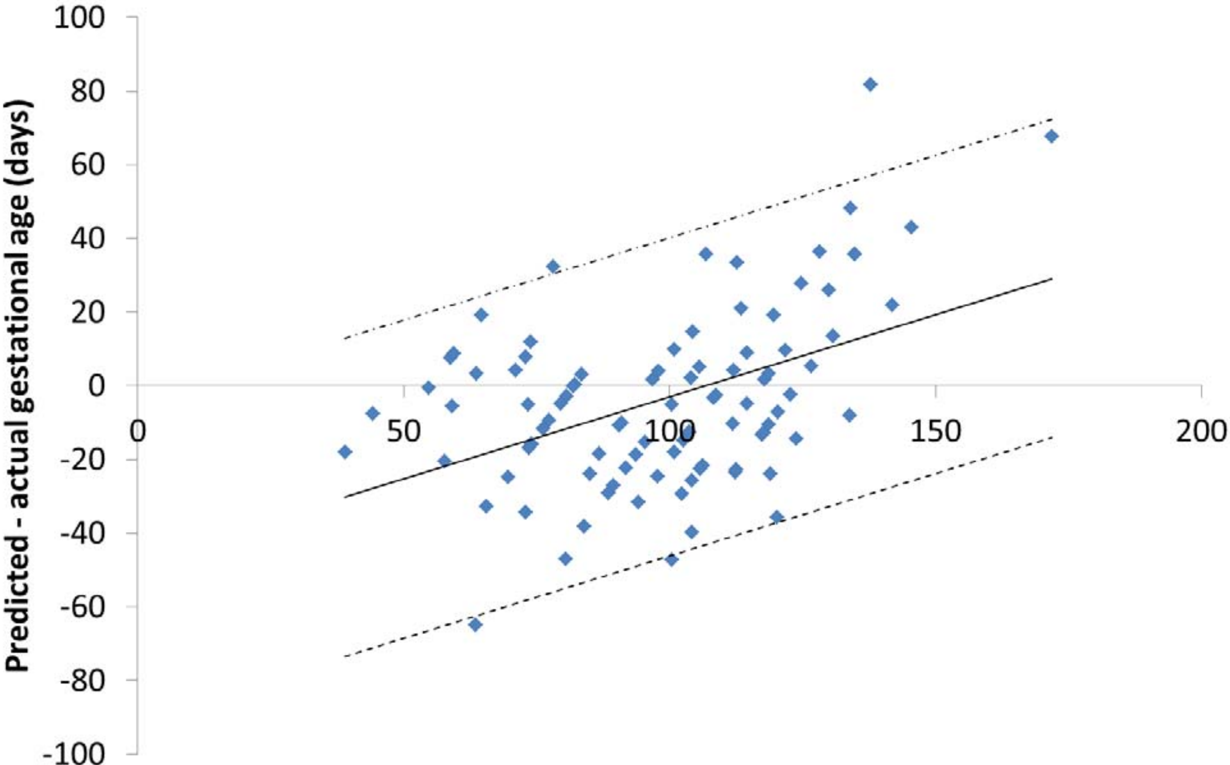


Figure 18: Limits-of-agreement for predicted gestational age (from placentome size) and actual gestational age for dairy cattle. Solid line is association between mean and difference; dashed lines are limits-of-agreement.

The limits-of-agreement plot for mean placentome size based on the regression equation from Adeyinka *et al*., (2014) is shown in Figure 19. Overall there was no evidence of bias (mean difference [SEM] was -2.66 [1.85]). There was no association between mean and difference or between mean and variance of the difference ($R^2<0.03$; $p>0.15$). The limits-of-agreement were thus parallel to the x-axis and predict that 95% of the differences between predicted and actual gestational age will be between -35 and +35 days, with 37% of differences being between ±10 days.

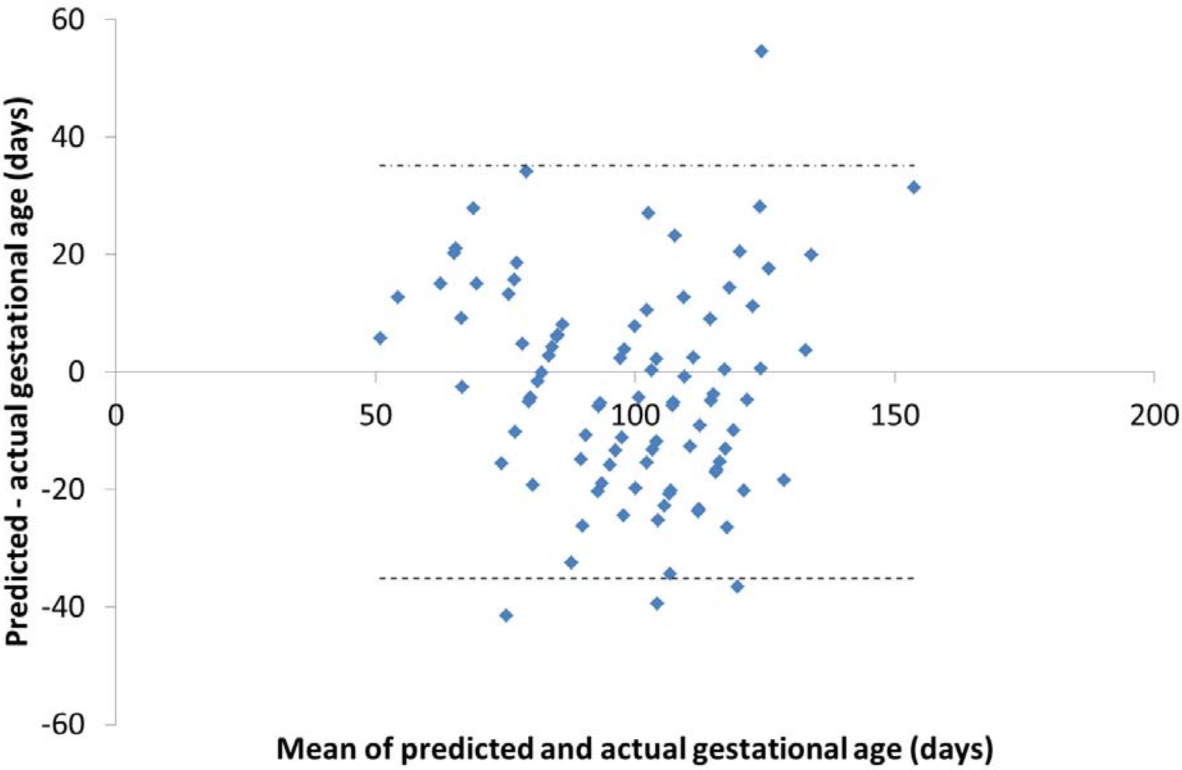


Figure 19: Limits-of-agreement for predicted gestational age (from placentome size using regression equation from Adeyinka *et al*., 2014) and actual gestational age for all cattle. There was no association between mean and difference; dashed lines are limits-of-agreement.

The limits-of-agreement plot for mean placentome size based on the femur size is shown in Figure 20. Overall there was no evidence of bias (mean difference [SEM] was -0.025 [2.05]). There was a moderate association between mean and difference ($R^2=0.124$; $p=0.018$) and a strong association between mean and variance of the difference ($R^2=0.447$; $p<0.001$). The limits-of-agreement analysis thus took account of these associations and suggests that at 80 days, 95% of the differences between predicted and actual gestational age will be between -19 and +16 days whereas at 120 days the equivalent figures will be -29 and +46 days. So at 80 days ~74% of differences will be ≤10 days from the line of best fit, whereas at 120 days ~21% of differences will be ≤10 days

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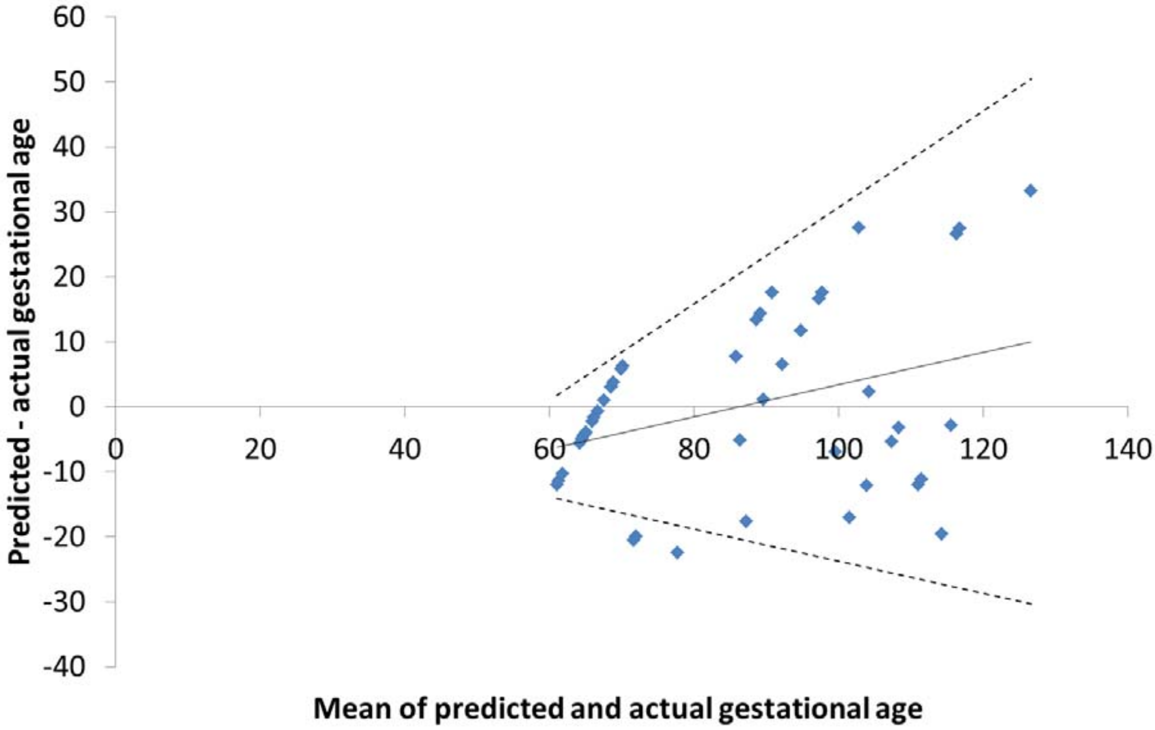

Figure 20: Limits-of-agreement for predicted gestational age (from femur size) and actual gestational age for all cattle

2.3 Discussion

In cattle, measurement of fetal size is the most commonly used method of estimating gestational age; however very few studies have properly assessed the agreement between fetal size and gestational age. Many studies have focussed on correlation and relationship between means which are not appropriate for assessing agreement as they ignore the variation between individual results (variance of the difference) and the change in that variance as measurements increase

Measuring placentome size has also been recommended as a method of estimating gestational age (Zemjanis, 1962); however it is less commonly used than fetal measurements. Adeyinka *et al*., (2014) undertook a limits-of-agreement assessment of placentome size and concluded that based on their limits-of-agreement placentome measurement was unlikely to produce estimates of gestational age that were likely to be useful in practice. However, they also noted that they were unable to compare their results with those of other parameters as no equivalent assessment had been undertaken for fetal measures. This study was therefore undertaken to replicate the assessment undertaken by Adeyinka *et al*., (2014) with placentomes and, at the same time to compare these results with two fetal measures – biparietal diameter and femur length.

For all three measures there was a significant association with gestational age. The strongest association (R^2=0.85) was seen in dairy cattle when biparietal diameter was measured and the weakest was placentome size in dairy cattle (R^2=0.39). The association for the two fetal measures, were poorer than some previous results (e.g. Kahn 1989, White *et al*., 1989), but other studies reported similar values (e.g. Terzano (2012) for femur length and Kramer *et al*., (2016) for biparietal diameter). It is not clear why correlations vary between studies. In this study, in an attempt to mimic what was feasible under New Zealand conditions, measurement was undertaken as soon as the measure could be identified, so it is likely that measurement time was short relative to previous studies. In addition, the beef cattle used in this study were unused to handling, so were difficult to scan safely, again increasing the likelihood of errors. Another possibility which could have decreased correlations in this study is the relatively high gestational age of many of the fetuses when they were first scanned which would again reduce association.

For placentome data, the association between mean placentome size and gestational age was similar to that reported by Adeyinka *et al*., (2014) (r =0.66) but lower than Lazim *et al*.,

(2016) (r=0.88), even though the latter used transabdominal ultrasonography which Hunnam *et al*., (2009) had previously used and found no significant association between placentome size and gestational age. The reason for the differences between studies, particularly the two transabdominal ones, is unclear.

This is the first study that has specifically focussed on the agreement between estimates of gestational age from fetal size and actual gestational age. Previous papers (e.g. White *et al*., 1985 and Kahn, 1989), have principally focussed on correlation and when they have included measures of agreement have not taken account of change in agreement with time; e.g. White *et al*., (1985) reported residual standard deviations ranging from 4.5 days for crown-rump length to 12.6 days for uterine diameter, but did not take into account the increase in standard deviation with time shown on their graphs (see Figure 3). The impact of time on agreement is particularly important under New Zealand conditions as, although in intensive systems most cows are scanned before 42 days (Fitzgerald *et al*., 2015), in New Zealand the majority of pregnant cows are >80 days when scanned with many cows scanned at a later stage (Brownlie *et al*., 2015).

The limits-of-agreement analysis showed that for both femur length and biparietal diameter (in dairy cows) there was a large increase in the predicted differences with increasing gestational age; i.e. estimation of gestational age became less precise as pregnancy progressed. In contrast for placentome size the width of the limits-of-agreement did not increase as gestational age increased; i.e. precision of the estimate did not depend on gestational age. This is consistent with the results shown by previous studies of fetal size such as Khan (1989) and White *et al*., (1985) where graphical representation of the data from the fetus showed that variance increased with gestational age, and Adeyinka *et al*., (2014) who found no effect of gestational age on the precision of its estimation using placentome length. This lack of association was observed irrespective of whether the regression equation from this dataset or that from Adeyinka *et al*., (2014) was used. As Adeyinka *et al*., (2014) stated, the lack of an association between agreement and gestational age is a significant advantage of using placentome measurement as an estimator of gestational age in later gestation. Another advantage is the ease of finding placentomes. In all cases at least three placentomes were found during the scanning process; in contrast the two fetal measurements, particularly femur size, became increasingly difficult to access as gestational age increased.

However, these two advantages are irrelevant if the level of agreement between predicted and actual gestational age is not good enough. The results of the limits-of-agreement analysis for each of the three measures are compared in Table 4.

Table 4: Comparison of limits-of-agreement analysis for the estimates of gestational age for fetal and placentome measurements

Measure	**Type**	**Diff ∝ mean**	**Width of LOA***		**% of differences ≤10%**
			80 days	**120 days**	
Biparietal diameter	Beef	Yes	48	48	67
	Dairy	No	20	45	95/67[†]
Placentome	All[a]	Yes	86	86	35
	All[b]	No	70	70	37
Femur length	Dairy	No	35	75	74/21[†]

*, limits-of-agreement; [†], at 80 and 120 days, respectively; [a], using regression equation from current dataset; [b], using regression equation from Adeyinka *et al*., (2014)

The results in this table show that at 80 days both femur length and biparietal diameter were more precise than placentome measurement, whereas at 120 days only biparietal diameter was more precise. In particular the proportion of differences ≤10 days (the target recommended by Funnell (2015)) was much lower for femur length than either of the placentome measurements. This highlights the importance of testing agreement rather than simply correlation, as the latter does not reflect heteroscedasticity, i.e. the association between error and magnitude. Limits-of-agreement analysis tests for heteroscedasticity and therefore, in contrast to correlation, does not assume that precision of the estimate from fetal or placentome size stays the same over the whole of gestation

Nevertheless, despite the heteroscedasticity, this study has shown that biparietal diameter is a better predictor of gestational age than mean placentome size across the range of gestational ages found in this study. However, this analysis has also validated the equation from Adeyinka *et al*., (2014) as a standard equation for predicting gestational age from placentome size (see Figure 19 which shows a lack of significant bias or heteroscedasticity); furthermore the limits-of-agreement in Figure 19(±35 days) are similar to those reported by Adeyinka et al for their data (±33 days). This means that it is likely that the conclusions from Lawrence et al., (2015) who reanalysed the dataset used by Adeyinka *et al*., (2014) also apply more generally. They reported that using data from all placentomes measured rather than a single mean placentome size improved agreement to ± 25 days. At 120 days this level of agreement is similar to that recorded in this study for biparietal diameter. Thus, in later gestation using placentomes may be both easier (as biparietal diameter is more difficult to measure than placentome size) and more accurate, provided multiple placentome results are used. Using multiple placentome measurements is computationally more difficult than a simple conversion of mean size to gestational age, but could be feasible with a bespoke computer programme

2.4 Conclusion

Measurement of gestational age is a crucial part of the pregnancy diagnosis process. This study is the first to compare the agreement between predicted and actual gestational age for fetal parameters and placentome size. It has also extended the previous results in dairy cattle relating to placentome size and gestational age to beef cattle and shown that breed differences are, at most, of limited importance over the range of ages in this study (i.e. ~60 to 130 days)

Of the three measures the most precise predictions came from biparietal diameter in dairy cattle, followed by the same measure in beef cattle. However this study has confirmed that the precision of these measurements decreases significantly as gestation progresses and by 120 days of gestation, the precision of biparietal diameter is equivalent to that of measuring multiple placentomes. Thus at this stage measuring placentomes is both easier and, probably, more accurate. Measuring femur length was by far the most difficult measure and by 120 days was much less precise than mean placentome size or biparietal diameter. Femur measurement should be restricted to use only in early gestation and then it should be used alongside biparietal diameter measurement.

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