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ORIGINAL ARTICLE

Evaluation of early embrionic development after natural mating using ultrasonography in *bos indicus* cows in the humid tropics of Costa Rica

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Abstract Fifty-eight nursing Zebu cows averaging 5922 days postpartum were used to evaluate the early embryonic development by ultrasound under a natural mating system (bulls A to F). Three consecutive mating periods of 21 days were established. Sixty two percent of the animals were diagnosed as pregnant (36/58). During the first period (AB) 76% (27/36) of the animals become pregnant, 17% (6/36) during the second (CD) and 9% (3/36) in the last mating period (EF). Sixty two percent of the animals were true positives (animals found pregnant diagnosed by ultrasound (US), and conforming with the embryonic scale proposed by Rosiles et al. (2006); 12% (n=7) of false negatives (pregnant cows by US but not conforming with the scale); 2% (n=1) of false positives (pregnant cows, evaluated with US and conforming with the embryonic scale but not pregnant at the end of the study); and a 26% (n=15) of true negatives (not pregnancy evaluated with US). The sensitivity of the test was 97%, with a specificity of 68% with a positive predictive value of 83% and a negative predictive value of 6%. Relation between the measuring scale of embryo development and the results obtained by ultrasonography was k=0.69. No significant correlation were observed between body condition score (BCS) evaluation and

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body fat (BF) measurement at any of the three evaluations performed after mating, however, measurements of BCS and BF among time showed a positive correlation (0.48 $P < 0.05$, 0.29, $P < 0.07$, respectively) from the beginning of the evaluation period to the end of the trial. Ultrasonography to monitor embryonic development to determine the early establishment of pregnancy in natural mating reproductive programs can be a valuable technique to monitor reproductive efficiency.

Keywords Ultrasonography · Early embryonic development · Zebu cattle

Introduction

Animals raised in the humid tropics show diverse limitations such as slow growth rate, low average daily gain and poor feed efficiency which consequently is reflected in low reproductive performance. Additionally, the tropical regions are characterized by lack of technological livestock practices and also short of accurate reproductive records, all of these constraints make in it difficult to organize and examine reproductive events in the herd. Recent studies have demonstrated that using ultrasonography, pregnancy and embryonic development may be detected and evaluated with safety and efficiency, for example, Gregory et al. (1996) and Galicia et al. (1999) evaluated the embryonic mortality in *Bos indicus* cattle and reported that 10 to 15% of the animals detected as pregnant were found non-gravid when pregnancy was assessed via rectal palpation. Many factors may come to affect normal embryonic development and therefore cause embryonic mortality. Yavas and Watson (2000) suggested that prior the establishment of normal ovarian activity, post partum cows may have short cycles (8–12 days). Sreenan and Diskin (1983) pointed out that early embryonic death could be caused by a progesterone circulation failure due to the above mentioned short cycles, while others suggest that intra uterine and embryonic factors could lead to embryo death (Li et al. 2005). Rosiles et al. (2005) used ultrasonography from day 20 to 40 post insemination to evaluate the embryonic development in order to identify the size of the embryonic structures in *Bos indicus* cattle, and develop a scale to predict the embryo age when the exact date of the insemination or mating was not recorded.

Therefore, the present study aims to assess the early embryonic development and a secondary objective was to implement the scale for embryonic progress proposed by Rosiles et al. (2005) in order to validate it as a tool to estimate the pregnancy rate of bulls in a rotational sire mating system.

Materials and methods

Location

The study was performed in the Bovine Production Unit of the Agronomy School of the Costa Rica Technological Institute, in San Carlos, Alajuela (10°25′N and 84°32′W). The area has an altitude of 172 meters above sea level. The climate of the region is classified as humid tropics, with an annual average precipitation of 3062 mm, an average temperature of 27.3°C and 85.3% relative humidity. Throughout this study, all animals were kept at pasture

in a combined field of African Star grass (*Cynodon nlemfuensis*), Pará (*Brachiara mutica*) and Ratana (*Ischaemum indicum*).

Animals and mating system

Fifty-eight suckling Zebu cows with an average of 59±22 days post partum and six bulls evaluated for breeding soundness according to the recommendation proposed by Chacón et al. (1999) were used. The mating period consisted of three weeks of exposure to the females for each pair of bulls and resulting in three periods of mating, period 1 (AB), period 2 (CD) and period 3 (EF).

Ultrasonography

In order to evaluate the reproductive condition of the females two weeks before their exposure to the males, ultrasonography evaluations were performed twice a week to determine the percentage of animals in the herd with a corpus luteum in the ovaries. At the same time, blood samples were obtained from all cows by venipuncture of the coccigeal vein or artery, samples were kept in ice, and all the samples were centrifuged at 7000 rpm per minute, labeled with the animal number, dated and frozen at −20°C for further processing. The progesterone serum values were analyzed using solid phase radio-immunoanalysis and a pregnant animal was considered when the three or more serum progesterone values were above 1 ng/ml (Pulido et al. 1991). Additionallly, ultrasonography was performed 21 days after the females were exposed to each pair of males and every four days to measure the development of the embryo according to the procedure described by Rosiles et al. (2005). A linear probe (7.5 MHz) was used to determine the presence of embryonic tissue and amniotic fluid in the uterine horns every seven days starting at 21 days from the start of period AB. Pregnancy was confirmed by serum progesterone levels when three or more consecutive values of progesterone were above 2ng/ml and estimation of the date of conception was established according to the scale proposed by Rosiles et al. (2005) considering the measurement of the embryo vesicle.

Embryonic death (EMD) was considered when two subsequent evaluations the measurements of the embryo were unchanged or a decrease in embryonic size was detected in comparison with the previous evaluation.

Evaluation of the Nutritional Status

In order to determine the nutritional status of the animals, body condition score (BCS) was evaluated every 7 days using a score of 1 to 5 Pullan (1978) where 1 is an emaciated animal and 5 obese. Parallel to the body condition scoring assessment, on each animal ultrasonograpic evaluation of the back fat thickness (BF) was taken using a 3.5 MHz linear probe according to the methodology published by Silva et al. (2005).

Statistical analysis

First three embryo measurements on each pregnant cow were analyzed by ANOVA using the SAS procedure PROC MIXED for repeated measures (SAS Institute Inc. 2004) to determine differences in the size of the embryo. Additionally, correlations between BCS

and BF were determined using the SAS procedure PROC CORR (SAS Institute Inc., 2004). The model tested was:

$$Y_{ijk} = \mu + T_i + h_{j:I} + D_K + (TD)_{ik} + e_{ijk}$$

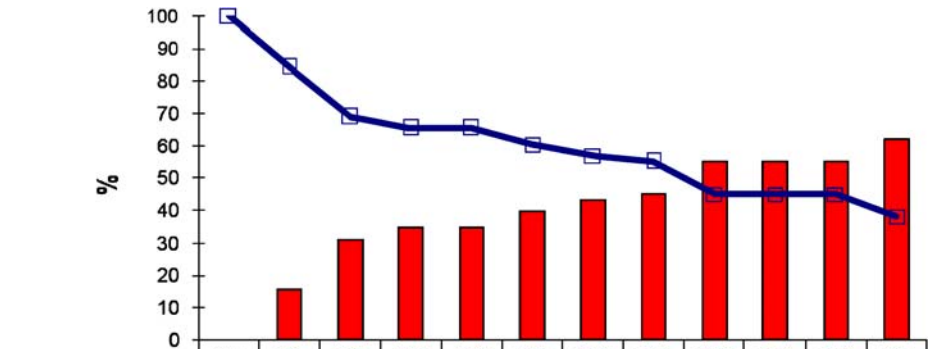
Where:

Y_{ijk} Observation of the *j*th embryo in the *i*th mating period on the *k*th measurement,
μ Overall mean,
T_i *i*th Mating period,
$h_{j:i}$ Random effect of the *j*th embryo within the *i*th mating period ($hj:i \sim N[0, \sigma 2\ h]$),
D_k *k*th measurement,
$(TD)_{ik}$ Interaction between mating period and measurement,
e_{ijk} error

The Kappa test (Feinsten 1985) was used to establish the difference and predictive values between radioimmunoanalysis and ultrasonogrphy, as well as their predictive values for each technique.

Results

At the beginning of the trial, a functional corpus luteum (CL) was present in 58% (34/58) of the animals based on the progesterone values and the presence of the ovarian structure. At the end of the trial, 62% of the animals were diagnosed as pregnant (36/58) (Fig. 1), of which 6% (2/36) showed embryonic death on ultrasonography. In addition, 79% (27/34) of the animals that cycle at the beginning of the trial become pregnant at the end of the three mating periods. During the first mating period (AB) 76% (27/36) of the animals become



	D0	D3	D9	D11	D21	D31	D38	D45	D52	D59	D66	D73
Pregnant cows	0.0	15.5	31.0	34.5	34.5	39.7	43.1	44.8	55.2	55.2	55.2	62.1
Open cows	100.	84.5	69.0	65.5	65.5	60.3	56.9	55.2	44.8	44.8	44.8	37.9

Fig. 1 Overall percentage of animals becoming pregnant throughout the experiment related to the rate of cycling animals which were considered at risk to becoming pregnant. D0=21 days later after starting of the AB mating period

pregnant, 17% (6/36) during the CD period and finally, 9% (3/36) become pregnant in the last mating period (bulls E and F).

The embryos that showed ultrasonographic characteristics of embryonic death displayed a decrease in size and subsequently the disappearance of the embryonic structures (Fig. 2). As can be seen the embryonic vesicles showed an increase in size, whereas in the animals that experienced embryonic death the embryo was not growing. Finally, 19% (n=11) had a functional CL and 7% (n=4) were in anestrus. Likewise, 12% (n=7) were found pregnant, however the evaluation of the age of the embryo as well as the progesterone values were out of the range described by Rosiles et al. (2005).

Sixty two percent of the pregnant animals were true positives (animals found pregnant diagnosed by US, and conforming with the scale proposed by Rosiles et al., (2006); 12% (n=7) of false negatives (pregnant cows diagnosed by US but not conforming with the scale); 2% (n=1) of false positives (pregnant cows, evaluated with US and conforming with the scale but not pregnant at the end of the study); and a 26% (n=15) of true negatives (not pregnancy evaluated with US). The sensitivity of the test was 97%, with a specificity of 68% with a positive predictive value of 83% and a negative predictive value of 6%.

In order to determine the relation between the measuring scale of embryo age and the results obtained by ultrasonography the Kappa test was analyzed (k=0.69), indicating a strong relationship between the predicted age based on the ultrasound findings and the mentioned scale. Likewise, using the ultrasonographic evaluation, the number of animals pregnant per week of the ones at risk was calculated for the three pairs of bulls giving 3.7 cows per week.

No correlation was observed between BCS evaluation and BF measurement at any of the three evaluations performed after mating, however, measurements of BCS and BF among time showed a positive correlation (0.48 $P<0.05$, 0.29, $P<0.07$, respectively) from the beginning of the evaluation period to the end of the trial. Finally, BCS throughout the study showed that the animals established successful pregnancy 88% (32/36) maintained or gained body condition, in contrast just 12% (4/36) of the pregnant animals lost body condition. Dorsal back fat (DF) measurements indicated that 62% of the animals with 0.3 to

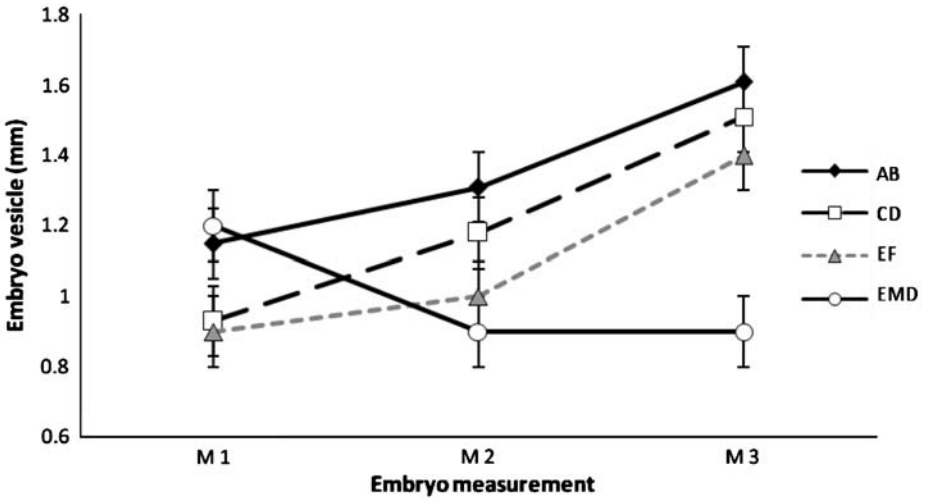


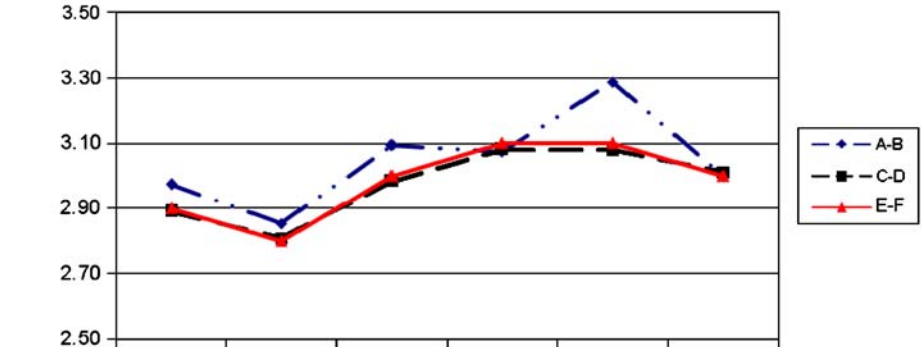
Fig. 2 Early embryonic development during mating periods. LSM±SEM

0.5 cm of body fat and 38% between 0.6 and 0.8 cm. When the trial was over, 72% of the animals had 0.3 to 0.5 cm. of BF and 28% from 0.6 to 0.8 cm. The BF results indicate that 30% of the cows pregnant from the first couple of bulls A-B gained DF, 35% maintained DF and 35% had lost DF. From the cows pregnant from the C-D group of males, 20% had an increase of DF, 40% maintained it and a decrease was observed in the other 40%. Contrary to this, none of the cows pregnant from bulls of group E-F gained BF, but 33% of them maintained it and the other 67% had a decrease. Finally, as can be seen in Fig. 3, the BCS on each group of cows that become pregnant was similar between periods.

Discussion

One of the common methods to breed animals in the tropics is the use of natural mating with one or several bulls (Molina et al. 2002). In traditional systems, postpartum cows are exposed to the bulls and the mating occurs naturally (Chenoweth 2004). The use of reproductive programs based on natural mating is relatively simple and easy to apply. However, the evaluation of the efficiency of this program, and most of all, the estimation of the breeding capacity of the bulls is somewhat variable. Additionally, these systems present several disadvantages, such as poor selection of animals, low reproductive performance, and lack of accurate reproductive records (Molina et al. 2003a). Nonetheless, evaluation of mating programs using the bull or assisted techniques such as artificial insemination requires the early diagnosis of the pregnant females. Generally, this evaluation is carried out by rectal palpation around 40 days after the end of the mating season. Therefore, the use of US can speed up the detection of non pregnant animals facilitating prompt reproductive intervention.

As reported in the present study, the proportion of females pregnant at the end of the trial was 62% and in accord with previous experiments (Galicia et al. 1999; 51.3% and Molina et al. 2002; 56%). As indicated earlier (Menendez et al. 1979) when using natural mating programs, the pregnancy rate (number of animals pregnant from the total exposed to the male) is around 60%. All these experiments indicate that when the mating period concludes, the pregnancy will be similar; the difference is established by the cumulative



	D-21	D-5	D10	D25	D40	D55
A-B	2.98	2.85	3.09	3.08	3.29	3.00
C-D	2.89	2.81	2.98	3.08	3.08	3.01
E-F	2.90	2.80	3.00	3.10	3.10	3.00

Fig. 3 BCS evaluation prior mating periods and throughout the experiment

percentage of pregnant cows which relates to the speed which bulls can breed and fertilize cows. Data from this study suggests that bulls impregnated 3.7 cows per week. This result is at odds with similar evaluations (Galicia et al. 1999; Webb et al. 2004) where bulls settled 0.67 and 2.0 cows per week respectively. Once more, the rate which cows became pregnant is most probably derived from the number of animals cycling once the mating program began, and not likely from the bull capability to serve females. Interestingly, at 60 days postpartum on average, 58% of the animals were cycling and this fact could affect the establishment of the pregnancy during the first mating period. In contrast studies performed in the tropics evaluating male rotational systems, have shown that during the last periods of mating an increase in the number of pregnant animals occurs. In fact, Molina et al. (2003b) reported that during the first mating period just 32% of the cows become pregnant whereas 68% of the pregnant animals where observed in subsequent mating periods. This difference could be attributed to many factors, the proportion of animals cycling at the beginning of the study, the effect of the male stimulating and inducing the onset of estrus (Berardinelli and Joshi et al. 2005, Vishwanath 2003) and finally the BCS of the cows at the beginning of each mating period could influenced positively the establishment of pregnancy. Webb et al. (2004) observed that the animals maintaining or gaining body condition when mated have a greater probability of becoming pregnant. The results of the present experiment, confirm the conclusions of Selk et al. (1988) suggesting that it is necessary an adequate energy balance returning to normal to initiate the ovarian activity.

No significant relationship was found in the present study when comparing BCS and BF in spite that dorsal back fat is a good indicator of body condition and also represents a realistic measure of the energy status of the animal. However, the constant changing of the metabolic state of the dam makes the results rather difficult to interpret. The value obtained in a given day, is the reflection of an event that probably happened earlier as the animal is probably gaining or loosing BF but this episode will be reflected days later and could change due to energetic intake of the animal during this period (Mösenfechtel et al. 2002; Schröder and Staufenblel 2006). Therefore, the need for intensive measurements is in demand in future studies. The rate of animals having embryonic death was low 6% (n=2). However, the relatively low incidence of embryo death could suggest that the majority of cows were on a positive energy balance. Using ultrasonography, it was obtained a 97% of sensibility, 68% of specificity this resulting in a positive predictive value of 83% and 6% of the negative predictive value for early pregnancy detection. According to the data published by Rosiles et al. (2005) the embryo vesicle at 24 days of development is 0.95±0.31 cm, and by 1.62±0.64 cm. The first embryo measurement on each mating ranged between 0.8 to 1.2 cm, however and the age predicted was ranged between 21 and 27 days, the embryos that continued with their development the final measurement (at approximately 34 days of development) ranged between 1.2 and 1.6 cm, similarly, Rosiles et al. (2005) reported that at 34 days of development the embryo vesicle was 1.53±0.31 cm. The results on the present study tend to be in accord with the measurements of the embryo when the date of conceptions was known. Results regarding embryo development suggest that the ultrasonography can be used to predict the age of the embryo based on the measurement of the embryonic vesicle even when the date of conception is uncertain. In addition, Kastelic et al. (1989), Oltenacu et al. (1990) indicate that with the use of ultrasonography it is possible to estimate the age of the embryo with a certain degree of confidence.

In conclusion, the present study reinforces the value of using ultrasonography as a reliable technique for taking expeditious, accurate, evaluation of the early embryonic development using natural mating systems.

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