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# Review: Current status of corpus luteum assessment by Doppler ultrasonography to diagnose non-pregnancy and select embryo recipients in cattle

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ABSTRACT

A number of potentials uses of Doppler ultrasonography have been explored in the last decades, both as research tools in reproductive physiology investigations and for the reproductive management of farm animals. The objective of this review was to address some of the recent strategies developed in fixed-time reproductive programs and resynchronization of ovulation in cattle, based on the evaluation of corpus luteum function by color-Doppler ultrasound imaging. Recent studies in dairy and beef cattle pointed out to a high accuracy when Doppler ultrasonography is used to assess the functionality of the corpus luteum and identify non-pregnant females at 20–24 days after breeding. Therefore, super-early resynchronization programs starting in the second week after timed-artificial insemination or embryo transfer have been developed and are being implemented in commercial assisted reproduction programs; thus, anticipating conception with proven semen or genetically superior embryos. In addition, assessment of corpus luteum blood perfusion can be used for identifying high fertility embryo recipients in fixed-time embryo transfer programs.


## Implications

Current alternatives to evaluate corpus luteum function by color-Doppler ultrasound imaging pointed to new tools that could improve reproductive performance and profitability of dairy and beef cattle operations. Defined methods to determine luteal blood perfusion allowed the estimation of luteal function at different phases of the estrous cycle and early pregnancy and its association with circulating progesterone profile. This review provides an overview of the technical and physiological concepts underlying the use of Doppler ultrasonography for the assessment of corpus luteum blood perfusion and highlights the applications in the reproductive management in order to build applied uses in cattle.

## Introduction

The basic principle of the Doppler ultrasound is the Doppler effect, a shift in the frequency of the sound wave reflected by an object moving toward or away from the probe. In medical ultrasound, this shift is observed in the echo from blood cells and is, thus, used to depict blood flow within a tissue or organ (Ginther, 2007). The Doppler ultrasound provides important information about the functional status of a tissue or organ, leading to its adoption in many areas of human and veterinary medicine. In cattle reproduction, Doppler ultrasound has been used for over two decades for the study of vascular perfusion in the ovaries, uterus and vagina, as well as fetuses and placenta (reviewed by Herzog & Bollwein, 2007; Matsui & Miyamoto, 2009; Bollwein et al., 2016).

In spite of the undeniable gains for diagnostic quality and accuracy, devices equipped with Doppler mode and respective licenses are usually significantly more expensive than their conventional, B-mode-only counterparts. This price difference hampered a broader adoption of the technology by field practitioners in the past. A turning point was the demonstration that Doppler ultrasound could be used as a practical tool for the reproductive management of herds, particularly for the early diagnosis of non-pregnancy based on corpus luteum (**CL**) blood perfusion (**CLBP**) (Siqueira et al., 2013; Pugliesi et al., 2014). The early detection of non-pregnant cattle is critical for the development of strategies

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to reduce the number of days open and calving intervals, thus improving reproductive performance of cattle herds (Gnemmi et al., 2022). The development of practical applications of Doppler ultrasound in reproductive management ultimately led to a substantial increase in the interest on the technology, as seen by the number of scientific papers published in this subject over the past 10 years by crossing related key words in databases such as PubMed (https://pubmed.ncbi.nlm.nih.gov/).

The aim of this review is to present the technical and physiological concepts underlying the use of color-Doppler ultrasonography for the assessment of CLBP, and subsequent applications in the reproductive management of dairy and beef herds.

## Corpus luteum assessment by Doppler ultrasonography

The Doppler signal can be displayed at the ultrasound device screen as spectral graphs or color-flow images. The spectral mode presents the Doppler signal as a time × velocity graphic, depicting changes in blood flow associated with the cardiac cycle (Ginther, 2007). Blood velocities and the pulsatility and resistance indexes can be obtained from the spectral graph. However, such measurements are calculated for an individual artery and, thus are more useful if a single, large vessel, accounts for most of a tissués or organś blood supply (e.g., the uterine artery; Herzog & Bollwein, 2007). Moreover, immobility is required to obtain a good spectral graph, particularly for small vessels, making the exam time-consuming and frequently not feasible in large animals. Therefore, most studies on CLBP in cattle were performed based on the analysis of the colored area displayed in the color-flow Doppler image.

The color-flow mode generates real-time and easy-to-interpret images of the blood perfusion, which depicts the vascularization of the CL. The use of color-Doppler data for research or as a diagnostic tool, however, usually requires a comparative analysis considering changes over time, or individual differences among animals, or even relative to cutoff values. Therefore, after visual assessment, CLBP still needs to be quantified. In this regard, one must consider that the CL is surrounded by complex plexuses of tortuous capillaries and small vessels (Macchiarelli et al., 1998), making the correct estimation of its vascularization a major challenge. Different approaches have been proposed to evaluate CLBP, taking into account not only accuracy but also the magnitude of expected differences, the possibility of real-time decisions, and feasibility under field conditions.

The most straightforward way to characterize CLBP is the subjective scoring by the evaluator of the amount of colored area within the Doppler image (Siqueira et al., 2013; Guimarães et al., 2015) (Fig. 1). This approach has the advantage to account for the whole scanning of the tissue, because the score is given based on a mental 3D reconstruction of the organ. Moreover, it allows real-time diagnosis and rapid decision-making, which is frequently required for a practical use of the technology. As for any other subjective scoring system, it relies on the evaluatorś experience and consistency tend to increase over time. The agreement rate between evaluators with similar knowledge of the technology is very high though, and the correlation with objective evaluations is high (Siqueira et al., 2013).

Although the technician can perceive small differences in blood perfusion, the scoring system limits our capacity to discriminate them. In this regard, the accuracy of the diagnosis depends on the expected difference among categories. For instance, CLBP dramatically changes between pregnant and non-pregnant cattle from day 16 after AI onwards (Siqueira et al., 2019), and a subjective score can be effectively used to predict non-pregnant status (Siqueira et al., 2013; Pugliesi et al., 2014). On the other hand, association studies such as those to determine the relationship between CLBP and progesterone (**P4**) production may require the discrimination of minor changes in CLBP (Herzog et al., 2010; Pugliesi et al., 2019b).

The colored area or the number of colored pixels (RGB value ≠ 0) within an image could be objectively measured using internal calipers of the ultrasound device or assisted by an image analysis software (Herzog et al., 2010; Siqueira et al., 2019) (Fig. 2). The results are presented as a continuous variable, expressed as absolute (e.g., $mm^2$) or relative (% of tissue) values. The use of relative indexes is interesting because it takes into account the total volume of tissue supported by a certain amount of blood supply and thus provides a better idea of its physiological status. In fact, Siqueira et al. (2019) obtained a slightly higher area under curve for CLBP adjusted by CL size than for CLBP alone (0.81 vs 0.78, respectively) in the Receiving Operator Curve analysis used to determine the relationships between each CL endpoint and pregnancy status.

Images generated with either color-flow or power Doppler can be used to measure blood perfusion. Depending on the device settings, however, the colored signal in images generated with power Doppler may have blurry edges, compared with those from color Doppler (Fig. 2A and B), thus making it difficult to delimitate a specific-colored area. On the other hand, flow intensity information provided by power Doppler would be more useful than flow direction, typically assessed by color Doppler. Nevertheless, differences in mean pixel color are more complex to analyze and are currently not taken into account in most studies.

For research purposes, the calculation of blood perfusion area can overcome the intrinsic limitations related to score subjectivity and to the lack of statistical power associated with analysis of discrete variables. This approach, however, requires the selection of a representative image frame, which end up creating a certain degree of subjectivity as well. Frequently, this selected image is

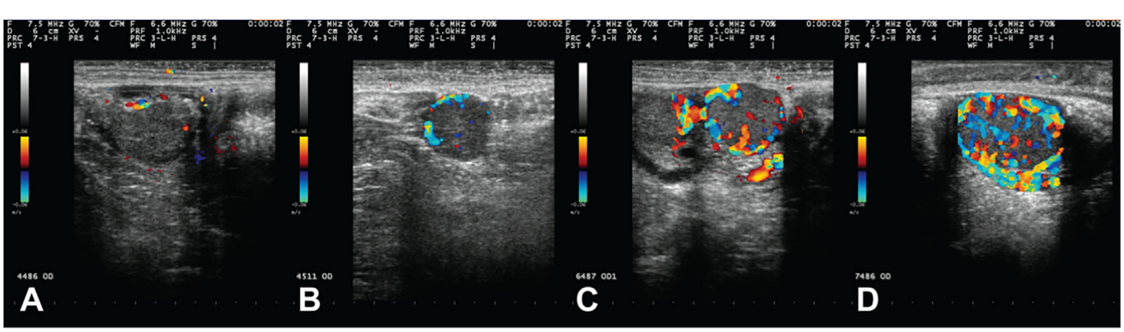


**Fig. 1.** (A–D) Color-Doppler images of bovine corpora lutea showing different degrees of CLBP (increasing from A to D). Panel A also represents non-pregnant females diagnosed based on the CLBP to detect luteolysis (CL with ≤25% of blood perfusion and without color signals in the center of the luteal tissue). Panels B, C and D also indicate, respectively, low, medium and high CLBP. Abbreviations: CLBP = corpus luteum blood perfusion; CL = corpus luteum.

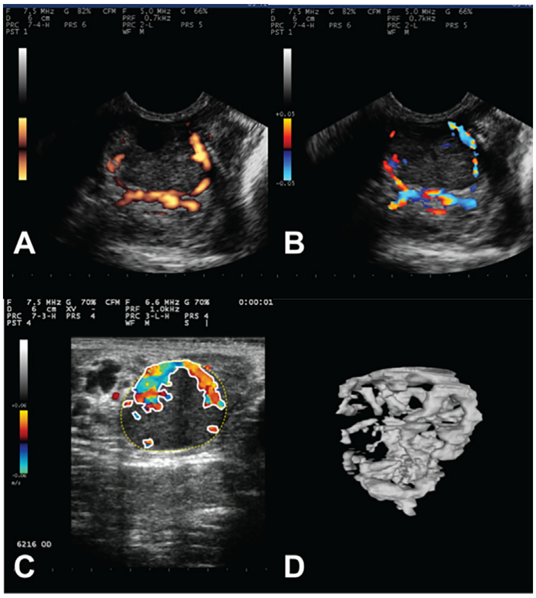


**Fig. 2.** (A–D) Different approaches to evaluate Doppler images of bovine corpora lutea. (A, B) subjective evaluation of images generated with power- (A) or color-flow (B) Doppler; (C) objective quantification of the colored area within a CL assessed by color-flow imaging. Colored pixels are delimited by a white line and the entire CL area by a yellow-dotted line; (D) Three-dimensional reconstruction of CLBP. Abbreviations: CLBP = corpus luteum blood perfusion; CL = corpus luteum.

arbitrarily defined as the cross-section frame of the central area of the CL at its maximum diameter or the frame with the greater amount of Doppler signal (Viana et al., 2013). Nevertheless, due to the uneven distribution of vascularization around follicles and CL, even minor differences in probe position or angle may cause bias in measurements, as observed for ovarian follicles (Arashiro et al., 2013).

Objective measurement of the color-Doppler signal usually requires postacquisition image processing and analysis. Fortunately, the current trend in biomedical imaging is that computer-assisted image analysis tools will become progressively available as embedded technology in ultrasound devices, allowing real-time diagnosis. In general, single-image analysis does not demand a great computer processing capacity and many software for image analysis, including some license-free or open-source, are widely available.

Volumetry is currently used in internal medicine imaging to measure structures of irregular shape and could be an alternative to assess CLBP. The vascularization of the CL is typically branched and tortuous, and the three- or four-dimensional (**3D/4D**) reconstruction is likely to provide the best representation of the vascular architecture of the tissue (Viana et al., 2013). In fact, this approach takes into account multiple frames representing the whole organ, while providing an objective measurement of the Doppler signal (Scully et al., 2014). The limitations for the use of volumetry for CLBP evaluation are mostly technological. Ultrasound built-in 3D/4D measurement tools require substantial data processing capacity. In addition, postprocessing image analysis using recorded videoclips is labor-intensive and demand-specific 3D reconstruction software (Viana et al., 2013; Scully et al., 2014). Most 3D/4D ultrasound devices currently available are either too expensive and unsuitable for use in the field or lack processing capacity to avoid image delay and associated distortion due to organ movement during scanning. Nevertheless, technological advances may turn volumetry into an important tool for the study of CL hemodynamics in the future.

## Luteal blood perfusion during the estrous cycle and early pregnancy

The CL is a transitory endocrine gland that secretes primarily P4, which is essential for pregnancy establishment in ruminants and other farm animals (Mann and Lamming, 1995). Development of the CL after ovulation is often divided into three main phases: early luteal phase or luteogenesis; mid-luteal or static phase; regression or luteolysis (Niswender et al., 2000). The maternal recognition of pregnancy is associated to the maintenance of a fully functional CL, secreting adequate amounts of P4 to support early embryonic development (Lonergan, 2011).

Due to its transitory nature, the CL must develop physically and functioning very rapidly just so an eventual gestation can be established or, in case of conception failure, a new estrous cycle begins. These particular features of the CL are mainly governed by intense angiogenesis (Acosta and Miyamoto, 2004) for appropriate development of luteal cells during luteogenesis (early luteal phase), and nutrients and steroid precursors delivery to support P4 secretion into the bloodstream (mid-luteal phase). Because of this highly angiogenic and vascular nature of the CL (Fraser and Wulff, 2003), the use of Doppler ultrasonography has become a reliable and useful tool to indirectly assess CL function by imaging blood perfusion into the gland (Bollwein et al., 2016).

In this scenario, color-Doppler flow imaging has long been successfully used to quantify blood flow to the ovaries of ruminants during a regular estrous cycle (Honnens et al., 2008; Herzog et al., 2010). Blood perfusion in the ovary bearing a CL dramatically increases from estrus to mid-cycle (diestrus) in ewes (Niswender et al., 1975), cattle (Herzog et al., 2010) and other species (Miyazaki et al., 1998) followed by a decrease at the end of the estrous cycle (Siqueira et al., 2019). Besides the regular pattern of increase-plateau-decrease blood perfusion during CL development in the estrous cycle, studies have also demonstrated a progressive increase in CLBP in pregnant females (Herzog et al., 2011; Siqueira et al., 2019) perhaps as a consequence of the presence of the conceptus and the secretion of luteotropic factors.

If conception fails, luteolysis takes places and is driven by uterus-borne luteolysins, primarily prostaglandin $F_{2\alpha}$ (Niswender et al., 2000). Some authors have divided luteolysis into two main events: functional luteolysis (loss of function) and structural luteolysis (luteal tissue physical regression). First, the drop in CLBP (Siqueira et al., 2019) almost immediately suppresses P4 production and secretion. Then, luteal cells go into apoptosis, followed by necrosis and tissue remodeling (Pate, 1994). These two major events of the luteolysis process occur at different timepoints, i.e., there is a temporal difference between loss of function (reduction in circulating P4) and CL physical regression (reduction in size) (Siqueira et al., 2009a; 2019). Therefore, CLBP is highly correlated with plasma P4 concentrations, whereas correlations between area of luteal tissue and plasma P4 were not as high during static and regression phases (Herzog et al., 2010). Likewise, circulating P4 is correlated to blood flow to the ovary bearing a CL in cattle (Herzog et al., 2010).

There is strong evidence to support the idea that higher CLBP is positively correlated with an increased odd of successful pregnancy establishment. In a study on vascular and morphological features of the bovine CL, Siqueira et al. (2019) reported a progressive and consistently greater CLBP from day 16 to day 20 after timed-artificial insemination (**TAI**) in pregnant compared with non-pregnant females. Area of luteal tissue, however, only differed between pregnant and non-pregnant on day 20, at the very end of a normal estrous cycle length. Likewise, other CL characteristics such as circulating P4 and CL echotexture were not good predictors of the odds of getting pregnant. A progressive increase in CLBP

(Herzog et al., 2011) and uterine blood flow in the first weeks of pregnancy (Honnens et al., 2008) have also been previously reported and the latter might be indirectly involved in the increased blood perfusion of the CL-bearing ovary.

## Interrelationships among circulating progesterone, luteal tissue area, echotexture, and blood perfusion

The CL development and capacity to produce and secrete P4 rely on the amount of steroidogenic luteal cells (small and large) and the vascular support to provide nutrients and precursors for steroid secretion. Therefore, luteal tissue area not surprisingly has a high, positive correlation with plasma P4, mainly during early diestrus (Niswender et al., 2000; Siqueira et al., 2009a; Scully et al., 2014). During this phase, luteogenesis and CL development are responsible for a gradual increase in P4 secretion and concentration in plasma. At mid-diestrus, CL size (luteal area) achieves its maximum values, followed by a peak in circulating P4 a few days later (Siqueira et al., 2009a).

Extensive angiogenesis and the distribution of luteal cells within the CL are sonographic visualized as a hypoechoic image pattern and changes in this pattern might reflect distinct luteal phases and function (Tom et al., 1998). In this regard, previous studies have investigated the correlations between plasma P4 and quantitative CL echotexture (defined as the patterning of echogenicity). Results were, however, rather inconsistent (Siqueira et al., 2009a; 2019). In general, it seems that pixel brightness (mean pixel value) within CL tissue has limited value for inferences about CL function (plasma P4 secretion). In contrast, the variation in pixel brightness within the luteal tissue, i.e., pixel heterogeneity, appears to be a good indicator of luteal function and has a fair-to-high correlation with plasma P4 during luteolysis (Siqueira et al., 2009a). In other words, an ultrasonic image of a heterogeneous luteal tissue is often observed in early luteal phase (metestrus) and after onset of luteolysis, reflecting the ongoing organization (metestrus) or disorganization (luteolysis) of the CL itself (luteal cells, connective tissue, and blood vessels). An ultrasound image with low pixel heterogeneity values, however, is observed during diestrus, concomitantly with a high-circulating P4 scenario, evidence of a homogeneous luteal tissue mostly covered by small and large luteal steroid-secreting cells (Niswender et al., 2000).

## Use for early detection of pregnancy status

Ultrasound-based pregnancy diagnosis is usually performed by B-mode and only recommended after 28–30 days of pregnancy (Pieterse et al., 1990), as at this period, the accuracy in conceptus visualization reaches 100% (Nation et al., 2003). Yet, the detection of spontaneous CL regression by ultrasound in non-pregnant cows could allow an earlier assessment of gestational status (Pugliesi et al., 2013; Pugliesi et al., 2014; Scully et al., 2014; 2015; Siqueira et al., 2019).

Evaluation of CL size has been used with high accuracy to identify non-pregnant cattle between after day 20 of gestation (Kastelic et al., 1991), but plasma/serum P4 concentrations have a greater correlation with CLBP than CL size during the luteolytic period in ruminants (Herzog et al., 2010; Balaro et al., 2017; Rocha et al., 2019). This indicates that a decrease in CLBP occurs prior to structural luteolysis and has been observed as early as day 16 after TAI in dairy cows and heifers (Siqueira et al., 2019). Also, a considerable rate of false negative results is observed when using only CL area for detection of luteolysis in non-pregnant beef cows, as some animals have a small but functional CL for pregnancy maintenance (Pugliesi et al., 2018).

To our knowledge, the first attempt to diagnosis pregnancy by luteolysis detection using Doppler imaging was reported by Utt et al. (2009) in beef recipient cows; however, the method used to determine CLBP was time-consuming and resulted in low accuracy (Table 1). Thus, more in-depth studies during spontaneous luteolysis characterized the CLBP changes between pregnant and non-pregnant cows (Matsui and Miyamoto, 2009; Pugliesi et al., 2014). The results allowed the definition of CLBP during luteolysis and served as a basis in pioneered investigations in dairy (Siqueira et al., 2013) and beef (Pugliesi et al., 2014) for the conception of applied criteria to identify a functional or non-functional CL at early pregnancy. In dairy females, a high accuracy and negative predictive value (**NPV**) was obtained by Siqueira et al. (2013) at 20 days post-TAI using only CLBP. In beef cows, 91% accuracy and 100% NPV were obtained to determine pregnancy status on day 20 post-TAI (Pugliesi et al., 2014). Although different definitions were used to identify a non-functional CL in these studies, the criteria are similar to detect non-pregnant females and the early diagnoses were compared with the gold standard method on day 30 of pregnancy. Both methods use a subjective and real-time evaluation (Fig. 1) that is simpler and more practical than using pixels counting to determine colored area and classify the CL as functional or not (Pugliesi et al., 2018).

Because these methods do not allow visualization of the embryo, one can state that color-Doppler ultrasound technique allows the detection of luteolysis for early indirect identification/-diagnosis of non-pregnant females. Other studies (Table 1) were performed in different breeds and parity classes and confirmed a high accuracy and null or extremely low rate of false negative results using the proposed cut-offs in dairy and beef cattle. Altogether, these outcomes support that determination of CLBP is an accurate diagnosis method for detection of pregnancy status, as there is a low likelihood of incorrectly detecting a pregnant female as non-pregnant, resulting in high NPV when applied after 19 days of pregnancy.

Nonetheless, proportion of false-positive (**FP**) results (cows diagnosed as pregnant by Doppler, but actually not confirmed as pregnant by the gold standard diagnosis) is the main factor differing among the studies (Table 1). The FP results may occur due to different reasons that result in the presence of an active CL on the day of CLBP-based diagnosis. Late ovulation in timed-programs and variations in time of physiological luteolysis among breeds and parity order are possible explanations, but the greatest part to blame for FP is apparently related to the rate of early embryonic loss between the Doppler and the confirmatory diagnosis. More recently, different studies in Nelore (Dalmaso de Melo et al., 2020), *Bos taurus* beef (Holton et al. 2022a; 2022b) and Holstein (Madoz et al., 2022) females have indicated by evaluating the expression of interferon-stimulated genes in circulating immune cells and/or pregnancy-associated proteins, that at least one third of the FP results are occurring due to early embryonic loss between days 19/20 and 30–34 of pregnancy. Collectively, these results indicate that most FP results are probably a result of prolonged luteal phase rather than embryonic mortality beyond maternal recognition of pregnancy. This is supported by the rate of 10–15% of Nelore heifers and cows synchronized for TAI, but not inseminated, that did not undergo luteolysis up to 22 days after arbitrary estrus (Ataíde et al., 2021).

In addition, it is noteworthy that no significant differences in CLBP on days 20 and 22 were observed between females diagnosed as true positive and FP (Holton et al., 2022a), but CLBP and plasma P4 concentrations on day 20 were lesser in females with indicative of embryonic mortality between days 20 and 25 of pregnancy (Dalmaso de Melo et al., 2020). Also, we performed a recent analysis (unpublished; Fig. 3) which indicates that the proportion of CLBP at day 20 is negatively associated with the rate of FP results.

**Table 1**
Studies comparing the use of Doppler ultrasonography to detect luteal function for diagnosis of pregnancy status between day 17 and day 22 and the B-mode ultrasonography for detection of embryo after day 28 of pregnancy in beef and dairy cows and heifers.

| Authors | Type/breed | Parity order | Type of service | Moment of gestation, d | Animals, n | Conception rate, % | Accuracy, % | False positive, % (total) | False negative, % (total) | PPV, % | NPV, % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Utt et al., 2009 | Beef (Crossbreed) | Cows | TET | 17 | 50 | 46 | 60 | 30.6 | 9.4 | 54.4 | 73.3 |
| | | | | 19 | | | 68.8 | 24.6 | 6.6 | 61.4 | 82.9 |
| | | | | 21 | | | 71.4 | 21 | 7.6 | 64.8 | 81.5 |
| Siqueira et al., 2013 | Dairy (Holstein-Gir) | Parous cows | TAI | 20 | 317 | 46.1 | 74.6 | 24.6 | 0.7 | 64.8 | 97.7 |
| | | Heifers | | | 209 | 47.4 | 76.6 | 22.7 | 0.6 | 67.2 | 98 |
| Pugliesi et al., 2014 | Beef (Nelore) | Parous cows | TAI | 20 | 111 | 37.8 | 91 | 9 | 0 | 80.8 | 100 |
| Guimarães et al., 2015 | Beef (Nelore) | Parous cows | TET | 21 | 163 | 43.6 | 88.3 | 11.7 | 0 | 78.9 | 100 |
| Scully et al., 2014[1] | Dairy (*Bos taurus*) | Parous cows | AI after estrus | 18 | 80 | 52.8 | 68.1 | 20.9 | 11.1 | 66.6 | 70.3 |
| | | | | 19 | 80 | 56.8 | 82.4 | 13.5 | 4.1 | 79.5 | 88 |
| | | | | 20 | 90 | 42.2 | 77.7 | 17.3 | 4.9 | 70.8 | 87.8 |
| | | | | 21 | 94 | 45.2 | 87.1 | 11.8 | 1.1 | 78.8 | 97.5 |
| Pugliesi et al., 2018 | Beef (Nelore) | Parous cows | TAI | 22 | 246 | 49.6 | 94.7 | 5.3 | 0 | 90.4 | 100 |
| | | Heifers | | | 231 | 41.6 | 90 | 10 | 0 | 80.5 | 100 |
| Ataíde et al., 2018 | Beef (Nelore) | Parous cows | TET | 22 | 221 | 35.5 | 83.8 | 16.2 | 0 | 68.8 | 100 |
| Andrade et al., 2019 | Beef (Nelore) | Heifers | TAI | 21 | 113 | – | 87.8 | 12.2 | 0 | 77.3 | 100 |
| Dalmaso de Melo et al., 2020 | Beef (Nelore) | Parous cows | TAI | 20 | 144 | 58 | 93 | 7 | 0 | 89 | 100 |
| | | Heifers | | | 100 | 52 | 88.3 | 11.7 | 0 | 81.8 | 100 |
| Wellert et al., 2020 | Beef (Angus-cross) | Parous cows | TAI | 21 | 84 | – | – | – | 0 | 89.4 | 100 |
| | | Heifers | | | 25 | | | | 0 | 75 | 100 |
| Dubuc et al., 2020 | Dairy (Holstein) | Parous cows | AI | 21 | 1 632 | 22 | 62.1 | 37.5 | 0.4 | 52 | 98.1 |
| Holton et al., 2022a | Beef (*Bos taurus*) | Parous cows | TAI | 20 | 208 | 52.9 | 87 | 13 | 0 | 80 | 100 |
| | | | | 22 | 209 | 52.6 | 92 | 8 | 0 | 85 | 100 |
| Holton et al., 2022b | Beef (*Bos taurus*) | Heifers | TAI | 20 | 183 | – | 90 | 10 | 0 | 86 | 100 |
| | | | | 22 | | – | 92 | 8 | 0 | 90 | 100 |
| Madoz et al., 2022 | Dairy (Holstein) | Parous cows | TAI | 19/20 | 131 | 37.4 | 74.8 | 24.5 | 0.7 | 60 | 98.2 |
| | | Heifers | | | | | | | | | |
| Ferraz et al., 2022 | Dairy (Holstein) | Parous cows | TAI | 21 | 140 | 28.6 | 53 | 46.3 | 0.7 | 38 | 98 |
| | | Heifers | | | 32 | 31.3 | 66 | 34 | 0 | 48 | 100 |

Abbreviations: PPV = positive predictive value; NPV = negative predictive value; TET = timed-embryo transfer; TAI = timed-artificial insemination; AI = artificial insemination.
[1] This study also considered the uterine echotexture and corpus luteum size as additional criteria to the luteal blood perfusion for diagnosis of non-pregnant animals.

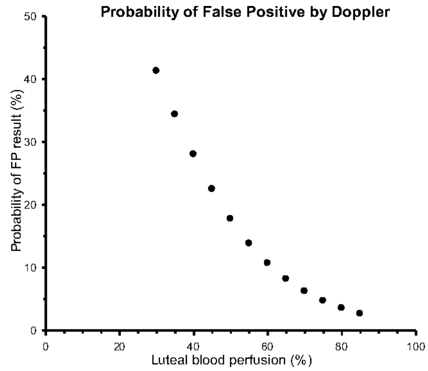


**Fig. 3.** Probability of false-positive result (females identified with a functional CL on day 20 post-TAI by Doppler ultrasonography, but non-pregnant on day 30) according to the CLBP at the Doppler evaluation in beef cattle (n = 159). Probability of FP = exp(−0.0591*x* + 1.4201)/1 + exp(−0.0591*x* + 1.4201); *P* = 0.008. Abbreviations: CLBP = corpus luteum blood perfusion; CL = corpus luteum; FP = false positive; TAI = timed-artificial insemination.

So, the probability to have a FP result is greater than 25% in females with ≤40% of CLBP, and less than 5% in females with ≥75% of CLBP at time of pregnancy diagnosis.

By comparing the results related to accuracy in the different studies (Table 1), it is possible to observe a greater FP rate and lesser positive predictive value and accuracy in dairy compared with beef cattle, in which pregnancy losses are normally lower, but other factors may be also involved as moment of ovulation after TAI and time of luteolysis (Reese et al., 2020). On the other hand, an influence of parity (nulliparous vs multiparous) on FP rate seems to occur only in beef animals. In dairy, FP rates did not differ between lactating cows and heifers (Siqueira et al., 2013; Ferraz et al., 2022) whereas in beef, studies (Dalmaso de Melo et al., 2020; Holton et al., 2022a) reported increased FP rate and reduced accuracy in heifers compared with suckled cows. At least in *Bos indicus* beef animals, this difference is probably caused by a postponed spontaneous luteolysis in Nelore heifers than in suckled cows (Ataíde et al., 2021).

Although there are well-known intrinsic differences in ovarian physiology between *B. indicus* and *B. taurus* females (Sartori et al., 2016), the accuracies of pregnancy diagnosis by Doppler on days 20 and 22 post-TAI in *Bos taurus* beef cattle (87 and 92%, respectively; Holton et al., 2022a), were similar to those observed in *B. indicus* (Dalmaso de Melo et al., 2020). Finally, it is important to note that the accuracy increases over time after service (Pugliesi et al., 2014; Scully et al., 2015; Siqueira et al., 2019), mainly because a greater proportion of non-pregnant cows are detected around luteolysis. In this regard, Holton et al. (2022a) reported a 40% reduction in FP rate when the diagnosis was performed on day 22 compared with day 20 post-TAI in beef cows. In general, studies have indicated that non-pregnancy diagnosis based on CLBF is a feasible and accurate method only when performed after day 20 of pregnancy in cattle.

In small ruminants, the subjective CLBP assessment using color-Doppler ultrasonography to distinguish pregnant and non-pregnant animals also resulted in high accuracy (>85%) and 100% of NPV (Arashiro et al., 2018; Cosentino et al., 2018). In goats, Cosentino et al. (2018) demonstrated that pregnancy diagnosis could be accurately performed beginning 21 days postbreeding. In ewes, the luteolytic period is earlier than goats and cows, and subjective CLBP assessment was considered a highly efficient method as early as 17 days after breeding (Arashiro et al., 2018).

In most studies, inferences about CL functional status and prediction of non-pregnancy were based on the quantification of the colored area within the CL Doppler image. In this regard, differences in settings of the ultrasound device that changes the amount of signal displayed as colored pixels can potentially affect accuracy of a given exam by increasing the proportion of FP or FN. During B-mode ultrasonography, most settings (transducer frequency, overall gain, gray-scale curve) can be intuitively adjusted by an experienced examiner to obtain a clear image. Moreover, although echotexture attributes such as mean pixel value or pixel heterogeneity can be used to evaluate the CL (Siqueira et al., 2009a), in routine gynecologic exams, most inferences on CL function are based only on its presence or size (area or diameter). Thus, small differences in B-mode image settings are less likely to interfere on the interpretation of scanning.

On the other hand, changes in color-Doppler settings might have a deeper impact on exam accuracy. The pulse repetition frequency (**PRF**) is of particular importance, as it defines the capacity of the device to display the Doppler signal. The higher the PRF, the lesser the signal displayed as colored areas in the image (Fig. 4). Yet, the use of a very low PRF (and thus set to a high sensitivity scenario) may increase the chance of occurring flash artifacts due to minor movements during scanning, such as those caused by breathing (Fig. 5).

In a recent study (unpublished data), we scanned cows 20 days after TAI and recorded three images of each CL using PRF values of 0.7, 1.0, and 1.5 KHz, respectively. The stored images were later used to predict non-pregnant status by a second evaluator who was blinded of the differences in PRF and the fact that more than one image referred to the same cow. Results were compared to those of pregnancy diagnosis at day 30, as previously described (Siqueira et al., 2013). As expected, reducing PRF from 1.0 to 0.7 KHz (and thus increasing the colored area in the image) decreased PPV and specificity (79.2 and 72.2% vs 67.9 and 50.0%, respectively), whereas increasing PRF from 1.0 to 1.4 KHz decreased NPV and sensitivity (100 and 100% vs 81.3 and 84.2%, respectively). These results demonstrate that minor differences on Doppler settings may lead to image misinterpretation and, thus, the accuracy of non-pregnancy diagnosis based on color-Doppler evaluation of

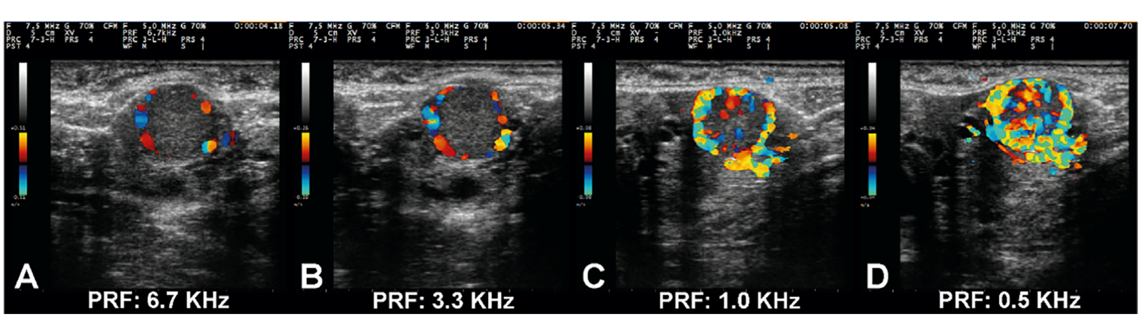


**Fig. 4.** (A–D) Color-Doppler images of the same bovine CL generated using decreasing PRF values. (A) PRF set at 6.7 KHz, (B) PRF set at 3.3 KHz, (C) PRF set at 1.0 KHz, (D) PRF set at 0.5KHz. Abbreviations: CL = corpus luteum; PRF = pulse repetition frequency.

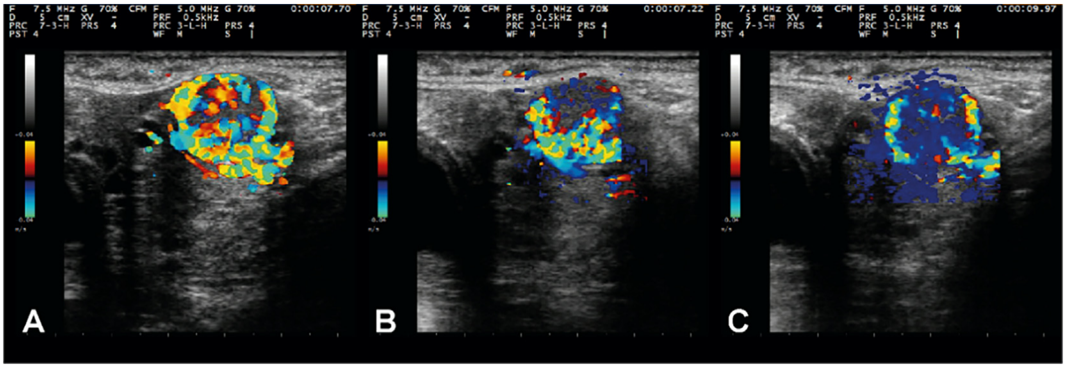


**Fig. 5.** (A–C) Effect of movement during acquisition of an image of a bovine CL using color-Doppler set to low (0.5 KHz) PRF value. (A) Image without flash artifacts, (B) Image with minor artifacts, (C) Image with major artifacts. Abbreviations: CL = corpus luteum; PRF = pulse repetition frequency.

CLBP requires the use of consistent standard settings throughout exams.

## Application in fixed-time programs in cattle

Resynchronization protocols provide a chance for a second round of TAI or timed-embryo transfer (**TET**) and may increase the reproductive efficiency of beef and dairy cattle (Baruselli et al., 2017). Due to the high efficiency of detecting non-pregnant females earlier by using Doppler imaging, new resynchronization protocols for the anticipation of the second TAI or TET were developed (Pugliesi et al., 2018). Therefore, it is possible to perform two TAIs or TETs within 22–24 days compared with 40 or 32 days when using the conventional or early resynchronization protocols, respectively (Fig. 6). For this, the so-called super-early resynchronization protocols must begin in all females between 12 and 18 days after TAI or estrus, regardless of gestational status. Alternatively, CLBP evaluation can be performed to resynchronize only females without an active CL. In this regard, (Guimarães et al., 2015) performed the detection of non-pregnant recipients by Doppler on day 21 after previous estrus (14 days after TET) and obtained 79.3% of non-pregnant animals resynchronized earlier than the conventional management.

The onset of the super-early resynchronization protocol may vary according to the time of Doppler exam and duration of the protocol, but it usually precedes the period of maternal recognition of pregnancy (days 12–17 after estrus). Thus, the association of drugs used for these protocols must not jeopardize the ongoing pregnancy. Considering that estradiol is involved in CL regression (Araujo et al., 2009), administration of exogenous estradiol esters in the second week of pregnancy is controversial. Initial studies comparing the use of estradiol to resynchronize bovine females from the second week postbreeding had varying results according to parity, type (dairy or beef), breed, dose, and time of treatment (El-Zarkouny and Stevenson, 2004; Colazo et al., 2006; Machado et al., 2008). In the first study to evaluate the association of super-early resynchronization and Doppler diagnosis, a negative effect on pregnancy rate was observed in cows receiving 1.5 mg estradiol benzoate (**EB**) on day 13 post-TAI (Vieira et al., 2014). On the other hand, negative effects on pregnancy rates were not

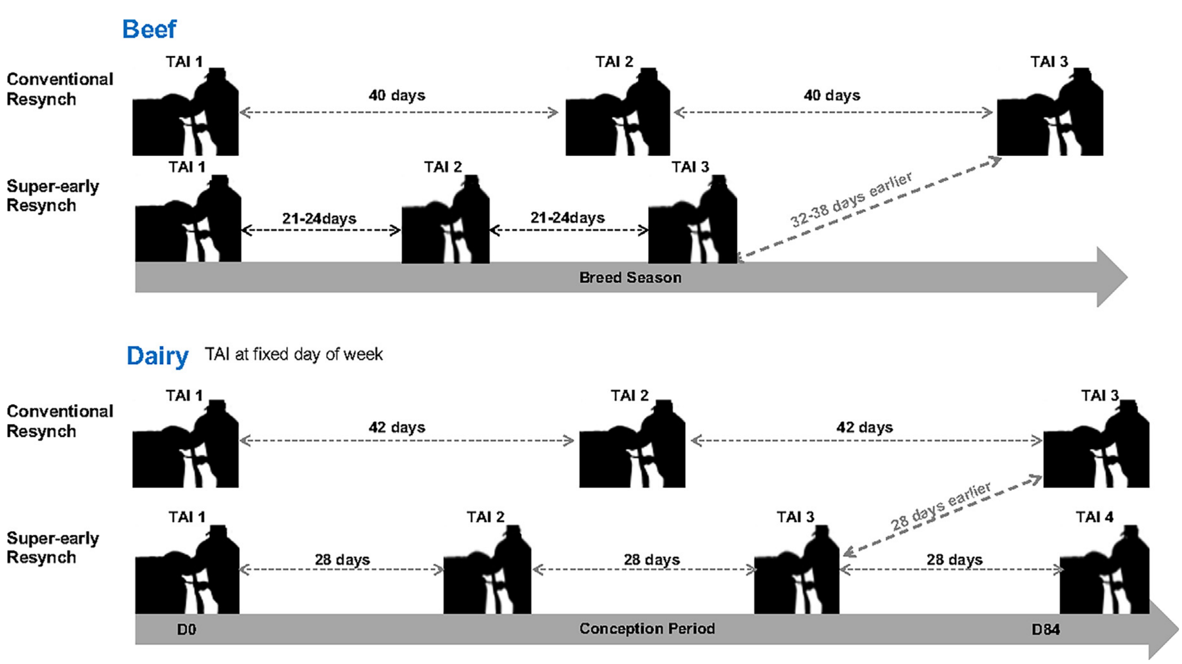


**Fig. 6.** Schematic illustration of the super-early resynchronization strategy compared to the conventional resynchronization in TAI programs in beef and dairy cattle. In dairy, the TAI is usually performed on the same day of week. Super-early resynchronization may start 12–15 days after TAI in beef and 17–19 days after TAI in dairy, resulting in an interval between TAIs of 21–24 days in beef and 28 days in dairy cattle. Conventional resynchronization is performed after pregnancy diagnosis on days 28–32 of pregnancy, resulting in an interval of 40–42 days in beef and dairy herds. Abbreviations: TAI = timed-artificial insemination; Resynch = resynchronization.

observed when EB treatment (1 mg) was administered 13 days after first TAI in beef cows (Stevenson et al., 2003) or lactating dairy cattle (El-Zarkouny and Stevenson, 2004). Therefore, our group and others have been dedicated to develop different strategies for super-early resynchronization protocols without using EB or limiting its use to a maximal dose of 1 mg.

In beef cattle, initial studies were carried out in heifers to evaluate the use of 1 mg EB (Motta et al., 2020) and to compare with the administration of injectable P4 (Vieira et al., 2021) in the resynchronization protocols initiated 14 days after TAI. In both studies, the Doppler diagnosis for evaluation of CLBP was performed on Day 22 and heifers diagnosed as non-pregnant were re-inseminated on Day 24 after first TAI. Although Motta et al. (2020) reported that the additional treatment with estradiol or EB associated with a P4 device anticipated luteolysis in non-pregnant heifers, no difference was observed in pregnancy rates from first TAI among experimental groups. This indicated that there is no harmful effect on the pre-existing pregnancy when using 1 mg EB or estradiol in *Bos indicus* and crossbred heifers. In fact, the additional treatment with 1 mg EB 14 days post-TAI resulted in greater pregnancy rates (Motta et al., 2020; Vieira et al., 2021). Vieira et al., (2021) compared pregnancy rates in beef heifers resynchronized 14 days after the first TAI using a P4 intravaginal device associated with either long-acting P4 or 1 mg EB, and observed that the EB treatment resulted in greater resynchronization pregnancy rates than injectable P4 treatment.

In agreement with what has been observed for beef heifers, Palhão et al. (2020) reported that the use of 1 mg EB to resynchronize suckled beef cows on Day 13 post-TAI did not compromise pregnancy rate to first TAI. However, Silva et al. (2022) compared the use of 1 or 2 mg EB 14 days after TAI and reported a reduction in pregnancy rate at first TAI in *Bos indicus* beef cows resynchronized with 2 mg EB. Furthermore, cows receiving 2 mg EB had a two-fold greater risk of early pregnancy loss compared with cows that received 1 mg EB. Together, these studies indicate that the use of 1 mg EB associated with a P4 device is safe and efficient for super-early resynchronization of beef heifers and suckled beef cows.

Because of the controversial results of the first studies using estradiol or EB, alternatives were also concomitant investigated. Thus, considering that high plasma P4 concentrations have a potential inhibiting effect on the growth of luteinizing hormone- and follicle-stimulating hormone-dependent follicles in cattle (Cavalieri, 2018), the effects of short-acting or long-acting injectable P4 at different doses have been evaluated for super-early resynchronization in heifers and cows (Pugliesi et al., 2019a; Ataíde et al., 2021). Studies comparing the use or not of the additional treatment with injectable P4 at doses greater than 75 mg indicated a positive effect on conception rates of resynchronized beef cows and heifers (Pugliesi et al., 2019a; Ataíde et al., 2021). In Pugliesi et al. (2019a), the interval between the first and second TAI was only 22 days and resulted in a 75% pregnancy rate after two TAIs. In Ataíde et al. (2018), Nelore beef females were submitted to two consecutive resynchronizations, allowing three TAIs in 48 days, and the cumulative pregnancy rate reached 83.3%. Thus, the treatment with injectable P4 in its different presentations for super-early resynchronization offers the potential to increase pregnancy outcomes.

In *Bos indicus* beef cattle, where postpartum anestrous is a great issue, super-early resynchronization may have a greater impact on reproductive performance compared to *Bos taurus* beef breeds. The implementation of super-early resynchronization protocols has the potential to increase the number of cows becoming pregnant within the first month of the breeding season, as it allows for two services (TAI or TET) at 21- to 24-day intervals. In this regard, Ojeda-Rojas et al. (2021) compared using a simulation model different types of resynchronization strategies in *Bos indicus* beef cattle and reported that the scenario of two TAIs within a 24-day interval represents the reproductive program with the best technical performance. Moreover, three TAIs at a 24-day interval lead to a greater number of births early in the calving season and heavier calves at weaning, when compared with only natural mating or other resynchronization strategies (Ojeda-Rojas et al., 2021). Considering that this result was based on a model rather than an actual controlled study, further studies for investigation of impact of super-early resynchronization on the calving distribution are encouraged. In a preliminary study using controlled data (unpublished) obtained from two consecutive breeding seasons with the same group of Nelore females, we have also observed an earlier calving after using three TAIs at a 24-day interval. A 43% increase in births at the first month of calving season (218 vs 152 births) and 9% increase in total number of calves born (394 vs 361 calves) were observed after the super-early resynchronization was adopted compared to previous season using only TAI and cleanup bulls. In addition, a retrospective analysis in order to evaluate calving distribution indicated that calves born in the first three weeks of birth season were ∼22 kg heavier than those born in the last three weeks.

In dairy cattle, investigations on super-early resynchronization protocols have been scarcer when compared with beef cattle. One of the factors that contributes for the reduced number of studies in dairy and the application of these protocols is the fit of a Doppler diagnosis into the schedule of weekly reproductive management visits by the veterinarian in dairy operations (Fig. 6). Another factor to be considered for the development of resynchronization protocols for dairy cattle was the greater proportion of FP results reported in all studies, mainly for lactating dairy cows (Siqueira et al., 2013). Nevertheless, a recent study by our group (Neto et al., 2022) reported that starting resynchronization 17 days after TAI increases pregnancy rate within the first 84 days after the end of the voluntary waiting period compared, with the traditional resynchronization starting 31 days after TAI in dairy cows. Also, the use of 1 mg EB associated with a P4 device at the onset of the protocol did not impact pregnancy from first TAI and reduces by half the proportion of FP results (cows with functional CL at 24 days after previous TAI). The association of a low EB dose given at a later timepoint relative to the period of maternal recognition of pregnancy, as performed by Neto et al. (2022) and Freitas et al. (2022) on 17–19 days after TAI, may have an even lower risk to harm a pre-existing pregnancy due to greater conceptus' signaling to maintain the CL active at this period. In countries where estradiol drugs are not allowed, the super-early resynchronization can be performed using only the P4 device or the P4 device associated to injectable P4 (if allowed), but conception rates are approximately 12% less when EB or injectable P4 is not used to synchronize follicular wave emergence.

## Use of Doppler for recipient selection

A correct selection of eligible recipients is one of the leading objectives for an embryo transfer program in cattle. Among the several characteristics of the reproductive tract, the presence of an evident, active CL is considered one of the most important factors to determine whether a healthy recipient should be used to reach a high likelihood of pregnancy (Phillips and Jahnke, 2016). Usually, luteal size is assessed by palpation or by gray-scale ultrasonography per rectum and a larger CL is preferable over a smaller CL. Picking recipients founded only on CL size may result in transfer of embryos to recipients with a non-adequate uterine environment associated to less functional CL (Pinaffi et al., 2015).

Indeed, a pioneered study in cattle (Pinaffi et al., 2015) classifying recipient cows as having low (≤40%) or high (>40%) CLBP at the time of embryo transfer (day 7) indicated a significant greater pregnancy rate in those with high CLBP compared with low CLBP (48% vs 0%). In agreement, CLBP on the day of embryo transfer was greater in pregnant than non-pregnant Holstein recipients (Kanazawa et al., 2016). But, the CLBP was determined in this later report based on the area of CLBP and the time-averaged maximum velocity at the base of the spiral artery, which have low applicability in large-scale operations since they are time-consuming.

In this regard, we have studied (Pugliesi et al., 2019b) the association between CL characteristics assessed by ultrasonography at the time of embryo transfer and pregnancy rate in beef recipients, using real-time evaluations to determine CLBP. In this study, we used the two most practical approaches to subjectively evaluate CLBP (i.e., estimation of the proportion of tissue with colored signals [0–100%] and a scoring system (depicted in Fig. 1)). Thus, beef recipients (n = 444) were retrospectively divided into three subgroups according to CL area and three subgroups based on CLBP [low (≤40%), medium (45–50%) or high (≥55%)]. Results indicated that CLBP determined by color-Doppler ultrasonography was the most important factor evaluated at embryo transfer that affected pregnancy outcome. Our results demonstrated a positive and linear association between the proportion of CLBP and pregnancy outcomes in synchronized beef recipients. Latter studies (Aragon et al., 2018; Munhoz et al., 2018; Silva et al., 2018) have also evaluated CLBP using different real-time criteria. Although not significant, pregnancy rates per embryo transfer in recipients classified as presenting high/excellent CLBP were approximately 1.26–2.33-fold greater compared with those females with low/bad CLBP in Holstein cows (42 vs 18%; Aragon et al., 2018), Holstein-Gir cows and heifers (51.8 vs 33%; Munhoz et al., 2018) and Angus heifers (60.6 vs 48.1%; Silva et al., 2018).

Consistent with the results in cattle, our group (Morelli et al., 2022) recently detected that CLBP in recipient mares was the only luteal sonographic characteristic that affected pregnancy establishment after transfer of *in vivo* equine embryos (low CLBP: 65.5% [38/58] vs high CLBP: 88.4% [38/43]). This also reflected a progressive increase in the pregnancy rate associated with increased CLBP. In addition, in buffalo, pregnancy rates were 2.5-fold greater in recipients with high CLBP (≥50%) than those with low CLBP (<50%) at the time of embryo transfer (Silva et al., 2018). This indicated that CLBP could be used as a main criterion to select highly receptive recipients for different large animal species.

Considering that the number of recipients at the time of embryo transfer in fixed-time programs in cattle is limited and usually lesser than the number of embryos, one strategy for using Doppler in recipient selection is rejecting females with a non-functional yet palpable CL. This represented 9.5% of the females bearing a CL at time of embryo transfer (Pugliesi et al., 2019b). In addition, if an excessive number of recipients were available, CLBP evaluation could be used to prioritize the transfer of embryos only into recipients with medium or high CLBP (>40%). Consequently, embryos would not be transferred to females with a non- or sub-functional CL identified by Doppler imaging (Pugliesi et al., 2019b). Alternatively, embryos of high generic merit could be rather transferred in recipients ranked by high CLBP.

Although all the studies have been pointing to the same positive effect of CLBP on pregnancy rates in recipients, the mechanisms associated with this effect to favor pregnancy establishment are still not fully understood. The hypothesis that high CLBP could be associated with embryotropic effects of circulating P4 during embryo development seems not to be supported by results of recent studies, because correlations with circulating P4 are weak for CLBP and moderate or strong for luteal size at early diestrus (Rocha et al., 2019). In addition, Siqueira et al. (2009a and 2009b) and Chagas e Silva et al. (2002) observed that circulating P4 concentrations on the day of embryo transfer are of limited practical use for recipient selection in dairy and beef cattle. Alternatively, an increased CLBP could be associated with a greater resistance of the CL to undergo regression or with a greater vascularization to the reproductive tract. These alternative hypotheses, however, still remain to be investigated in further studies for a better understanding of the mechanisms involved in the positive effects of CLBP on pregnancy establishment in cattle.

## Final considerations

Along the last decades, the cumulative knowledge about CLBP determined by color-Doppler ultrasonography indicated that this luteal characteristic is a practical and real-time tool to evaluate CL function. Based on the high correlations between circulating P4 vs CLBP compared with P4 vs CL size, the evaluation of CLBP during the phase of luteal regression has been used to anticipate the detection of non-pregnant cattle in TAI and TET programs. Several studies have used a subjective and real-time evaluation of CLBP in beef and dairy cattle. This method has shown a high accuracy and low or near to zero false negative results and, consequently, very high NPV. In beef females, when the diagnosis is performed 20–22 days post-TAI, the accuracy compared with the gold standard diagnosis on the 30th day of gestation exceeded 90%.

Although CLBP assessment can detect non-pregnant females, CL function is not a specific biomarker of pregnancy; therefore, a considerable percentage of false-positive results (12–30%) may occur, mainly in dairy cows and heifers. The detection of non-pregnancy by evaluation of CLBP earlier than the conventional methods (visualization of embryo and heartbeat) allowed for the development of new strategies of resynchronization of ovulation in a super-early manner, resulting in the possibility of two services (TAI or TET) within a 22- to 24-day interval. It is worth mentioning that such methods to evaluate CLBP and their practical applications boosted the commercial use of Doppler ultrasound for early detection of non-pregnant bovine females in an unprecedented and pioneering way in South America, mainly in Brazil. Finally, a greater CLBP at the day of embryo transfer in prospective pregnant females and other findings indicating that CLBP may be a better indicator of luteal activity in studies in cattle and horses suggest that evaluation of CLBP at embryo transfer is a more valuable information to estimate the probability of pregnancy success than systemic P4 concentrations in embryo recipients. It is noteworthy, however, that in order to obtain these great advances, training and understanding of the imaging principles and equipment is required, because the device's settings must be properly regulated and the operator very well prepared to carry out the assessments correctly.

## Ethics approval

The authors did not use any live animal to conduct this review.

## Data and model availability statement

Data or models were not deposited in an official repository. No new datasets were created.

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**Amanda Guimarães Silva:** writing original draft, review and editing.

**Joao H. M. Viana:** conceptualization, writing original draft and editing.

**Luiz Gustavo B. Siqueira:** conceptualization, writing original draft and editing.

## Declaration of interest

The authors have declared that no conflict of interest exists.

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