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REVIEW

Pregnancy monitoring in mares: Ultrasonographic and endocrine approaches

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Abstract

Methods to diagnose and monitor equine pregnancy continue to advance with improved instrumentation enabling the development of novel, non-invasive approaches to assess fetal well-being and viability using ultrasound and endocrine testing. From early embryonic loss to placentitis, that is typically encountered later in gestation, fetal viability and development as well as placental function can be evaluated using two fundamentally different, structural and functional, approaches. Ultrasound provides structural information on embryonic and fetal growth using such parameters as combined thickness of the uterus and placenta (CTUP), visual assessment of fetal fluids, activity, heart rate and multiple biometrics involving the fetal head and eyes, limbs and joints among many others, depending on the stage of gestation. Endocrine profiles that include progesterone and 5α-dihydroprogesterone, other metabolites, androgens and estrogens can be evaluated simultaneously using liquid chromatography–tandem mass spectrometry (LC–MS/MS) providing more functional information on fetal and placental competence and development. Endocrine information can be used in making clinical decisions including the need for progestin supplementation or when it can cease, and even estimating gestational stage in mares that cannot be easily palpated or scanned, as with mini-breeds or rancorous animals most notably. When used together, monitoring gestation by ultrasound and hormonal analysis provides unusual insight into feto-placental well-being and the progress of pregnancy, helping to identify problems needing therapeutic intervention.

KEYWORDS
equine, fetus, hormone, ultrasound

1 | INTRODUCTION

Most pregnancies result in the birth of a healthy foal, but pregnancy losses remain a concern especially early in gestation and to a lesser degree during mid to late gestation. For example, twin pregnancies should be detected and reduced early to minimize the likely associated negative outcome and placentitis later in pregnancy poses an ever-present threat. Therefore, monitoring pregnancies is an important clinical consideration with the potential to improve pregnancy outcomes. Ultrasonography and endocrine testing are non-invasive tools available for routine pregnancy diagnosis and evaluation. The objectives of this paper are to describe recent advances in each, as well as their comparative value in diagnosing pregnancy and in assessing feto-placental well-being, fetal age and even predicting parturition date.

2 | PREGNANCY DIAGNOSIS AND VIABILITY

2.1 | Ultrasonographic approach

Pregnancy diagnosis can be performed as early as 9 days post ovulation using the transrectal approach but, in practice, mares are diagnosed pregnant at 14 days when an anechoic round vesicle about 16 mm in diameter is imaged within the uterine lumen (Ginther, 1998). At this stage, the embryo is not visible, and the anechoic fluid fills the yolk sac. The best time to identify twins and reduce one vesicle is approximately 14–16 days (Figure 1). Mares are usually rechecked around 25 days to ensure a live embryo remains. A small hyperechoic structure with a beating spot (beating heart) can be seen at the junction of the allantois and yolk sac that occupy 25% and 75% of the vesicle, respectively (Ginther, 1998). At 40 days, the embryo reaches the dorsal part of the vesicle, the umbilical cord is formed, and it can now be referred to as a fetus. Pregnancy diagnosis can continue to be performed at any stage of gestation using the transrectal ultrasound approach (Renaudin, 2018). The transabdominal ultrasound approach is another way to image fetal parts or fluids by simply placing the transducer on the mare's ventral abdomen cranial to the mammary glands (Renaudin, 2018). It is the safest way to diagnose pregnancy with small or nervous horses and the only way to identify twins after 90 days of gestation.

2.2 | Endocrine approach

The first significant deviation in hormone secretion from normal cyclicity that is directly related to pregnancy and detectable in maternal blood is the extension of luteal function and progesterone secretion associated with the maternal recognition of pregnancy (Klein & Troedsson, 2011; Swegen, 2021). Although not a reliable diagnostic indicator (Allen, 1978), it is an essential event in establishing pregnancy, occurring by day 15 post-ovulation (Hershman & Douglas, 1979). Yet, progesterone concentrations are commonly assessed in early pregnancy, and may be higher in mares that maintain their pregnancies to day 14 (Hollinshead et al., 2022) or 15 (Ginther, 1985) than those that do not. Progesterone concentrations are often determined in embryo recipient mares to verify the adequacy of luteal function (Ball, 2011; Kelleman, 2013), since the associated manipulation of the reproductive tract may disrupt luteal function and decrease progesterone concentrations (Foss & Crane, 2004). Based on the outcome of pregnancies established by embryo transfer into ovariectomized mares given supplemental progesterone (Ginther, 1985; McKinnon et al., 1988; Shideler et al., 1982), many practitioners use 2-4 ng/mL as a critical concentration range for judging the adequacy of luteal function and mares with lower progesterone concentrations may be placed on supplemental progestin (Ball, 2011; Kelleman, 2013). Yet, different immuno-assays can yield significantly different concentration estimates (Wynn et al., 2018) and interpretations of results are best evaluated using established reference ranges for that laboratory. Furthermore, there is no objective evidence that supplemental progestin therapy actually increases foaling rates in mares with low progesterone, and embryonic loss is unlikely to result from luteal insufficiency (Conley, 2023) based on the relatively few clinical cases reported to date (Betteridge et al., 2018; Canisso et al., 2013; Irvine et al., 1990; Newcombe, 2000). Even if more frequent, as some have suggested (Betteridge et al., 2018), the effectiveness of altrenogest to rescue pregnancies remains unproven. For these and other reasons relating to the androgenic effects of altrenogest on fetal fillies, the use of this supplemental progestin, although common, remains controversial (Conley, 2023).

One of the first clinically reliable changes in the endocrine profile distinguishing pregnant from non-pregnant mares is the secretion of pregnant mare serum gonadotropin (Allen, 1969c; Cole & Hart, 1930), or equine chorionic gonadotropin (eCG; Figure 2), from the chorionic girdle (Allen & Moor, 1972; Moor et al., 1975) of the developing chorio-allantois (Catchpole & Lyons, 1934). The detection of eCG by bioactivity (Cole & Hart, 1930) or immunoassay (Allen, 1969b, 1969c; McCaughey et al., 1973; Richards, 1967; Walker, 1977; Wide & Wide, 1963) can be used as an indicator of pregnancy (Cole & Hart, 1930) if blood samples are drawn from mares approximately 40–120 days of gestation (Figure 2). Yet, there is considerable variability in the size and extent of endometrial cup formation in individual mares (Antczak et al., 2013) and in secretion of eCG (Allen, 1969b; Antczak et al., 2013; Day & Rowlands, 1947; Hoffmann et al., 1996; Holtan et al., 1975; Manning et al., 1987; Murphy & Martinuk, 1991; Wilsher & Allen, 2011). Besides being dynamic, the secretion of eCG is also relatively short-lived, peaking approximately 50–70 days before declining again in most mares (Allen, 1969b; Cole & Hart, 1930; Hoffmann et al., 1996), and is as variable among mares as is the development and viable lifespan of the cup tissue (Antczak et al., 2013). Cup viability is seemingly independent of the pregnancy itself, surviving after pregnancies

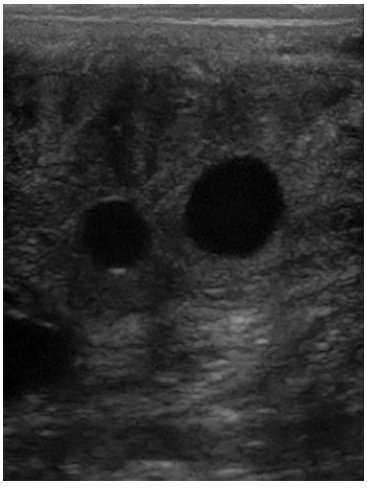

FIGURE 1 Early detection of twin pregnancy: transrectal ultrasound image showing two vesicles side by side at 14 days post ovulation. One vesicle measures 16 mm and the other one 14 mm.

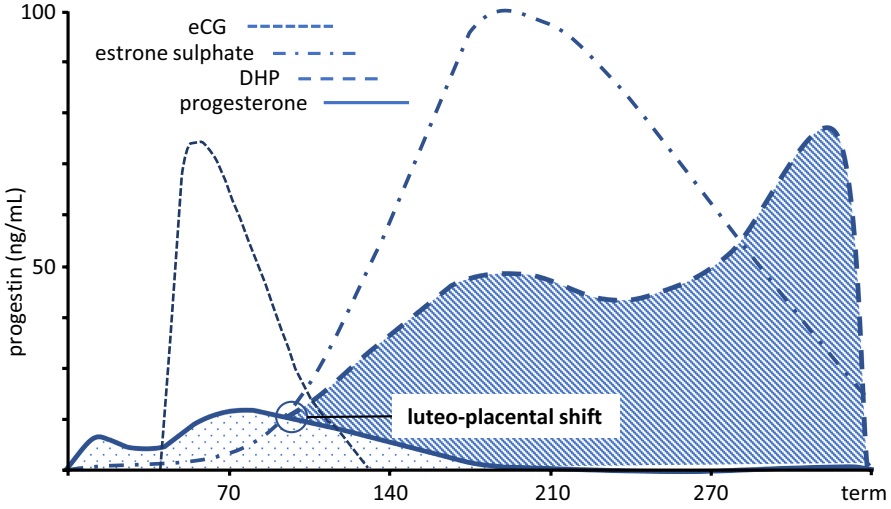

FIGURE 2 Schematic representation of steroid hormone and equine chorionic gonadotropin (eCG) profiles during equine gestation (days). Peak eCG at 60–70 days revives progesterone secretion by the primary corpus luteum but luteal progesterone secretion declines with eCG thereafter as endometrial cups regress. Development of the chorio-allantois coincides with the initial increase in estrone sulphate and with increasingly more 5α- DHP which brings about the luteo-placental shift (as indicated) approximately 110–120 days of gestation. Thereafter, DHP concentrations exceed those of progesterone more and more as gestation proceeds, along with a second substantial increase in estrone sulphate secretion. This is fueled by androgens from the developing fetal gonads that peak approximately 210 days of gestation but declines as the gonads regress towards parturition. Adapted from (Hoffmann et al., 1996; Legacki et al., 2016a, 2016b).

are lost (Allen, 1969a; Jeffcott et al., 1987; Mitchell, 1971; Squires et al., 1980), which increases false positive diagnostic rates (Mitchell, 1971). Cup tissue can sometimes fail to regress, secreting eCG for prolonged periods of time (Allen & Wilsher, 2012; Hoffmann et al., 1996), sometimes past foaling (Crabtree et al., 2012; Hoffmann et al., 1996). Resumption of normal cyclicity can be delayed by eCG (Penzhorn et al., 1986) and so too can fertility (Rathwell et al., 1987) in mares that lose their pregnancies after cups have formed, prompting both medical (Crabtree et al., 2012) and surgical (Huber et al., 1993) approaches to remove or hasten the regression of cup tissue, with questionable success (Podico et al., 2020). The combination of inherent variability of eCG, the necessity for well-timed blood collection and the possibility of false positives limits the practical value of eCG as a pregnancy diagnostic aid.

Chorio-allantoic development, endometrial cup formation and eCG secretion are also associated with increases in the secretion of several steroids in maternal blood (Legacki et al., 2016a, 2016b), including progesterone, estrone sulphate (Figure 2) and even androgens (Figure 3a) among others (Daels et al., 1996; Kindahl et al., 1982; Legacki et al., 2016a, 2019; Terqui & Palmer, 1979). The increase in estrone sulphate in blood (Kindahl et al., 1982; Terqui & Palmer, 1979) and urine (Daels et al., 1991a, 1991b; Evans et al., 1984) that begins around day 35–37 of pregnancy has been used in pregnancy diagnosis (Evans et al., 1984; Hyland et al., 1984), more reliably after 40–50 days of gestation. The concomitant rise in progesterone around day 35 (Figure 2) marks a functional revival of the primary CL that occurs before any accessory luteal tissue develops (Bergfelt et al., 1989; Daels et al., 1998; Hoffmann et al., 1996; Holtan et al., 1975; Squires & Ginther, 1975). This initial luteal response is consistent with the molecular nature of eCG as a more heavily glycosylated equine LH (Bousfield et al., 1987; Sherman et al., 1992; Sugino et al., 1987) exhibiting LH bioactivity in horses (Stewart & Allen, 1981). Whether or how much eCG contributes to the increase in estrone sulphate is unclear, perhaps because conceptus tissues are also involved (Figure 3b). For instance, although eCG stimulated progesterone and oestrogen secretion in pregnant mares, and did so only when luteal tissue was present (Daels et al., 1998), eCG only stimulated luteal progesterone but not oestrogen secretion in vitro (Daels et al., 1995). Luteal tissue expresses the steroidogenic enzymes necessary for oestrogen synthesis but luteal aromatase (CYP19A) expression decreases coincident with the initial increase in estrone sulphate (Albrecht et al., 1997, 2001; Albrecht & Daels, 1997). Furthermore, there is little evidence that luteal tissue by itself can actually sulphate estrone (Brown et al., 2006) to the degree conceptus and endometrium can (Heap et al., 1982). Still, ovariectomy (Kasman et al., 1987; Terqui & Palmer, 1979) and prostaglandin-induced luteolysis (Kasman et al., 1987) delay the estrone sulphate rise, even in pregnant mares maintained on exogenous progesterone (Daels et al., 1990; Daels, DeMoraes, et al., 1991a). This led the authors to conclude that the ovaries were a major contributor to the rapid increase between days 33 and 39 of gestation, even though urinary estrogens plummeted immediately after fetal death at 55 days of pregnancy in one mare (Daels et al., 1990), implicating significant conceptus involvement.

As much evidence as there is indicating that the ovaries are involved in estrone sulphate secretion in early equine pregnancy, there is additional convincing evidence that the conceptus itself contributes in a major way. Oestrogen concentrations increase markedly in fetal fluids after day 20 and fetal membranes themselves express considerable oestrogen synthetic capacity (Heap et al., 1982).

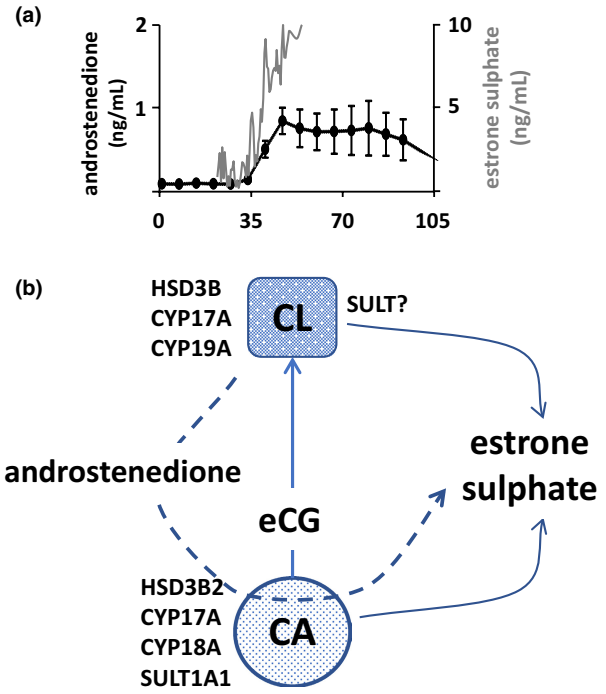

FIGURE 3 Representation of events contributing to the initial rise in estrone sulphate in early equine pregnancy. (a) Increase in androstenedione concentrations in maternal blood coincident with the initial rise in maternal estrone sulphate in early equine pregnancy. (b) Schematic depicting steroid secretion and steroidogenic enzyme expression of the primary corpus luteum (CL) and the chorio-allantois (CA) and their potential interaction contributing to estrone sulphate secretion coincident with endometrial cup formation and equine chorionic gonadotropin (eCG) stimulation of luteal androstenedione secretion. Enzymes critical in androgen (HSD3B; CYP17A1), oestrogen (CYP19A) and steroid sulphation (SULT1) are expressed in the CL or CA and specific isozymes are designated where known.

Estrone sulphate concentrations drop rapidly after the loss of pregnancy, either as a result of prostaglandin administration or surgical removal of the fetus (Kasman et al., 1988), even though progesterone concentrations may not change for several days (Kasman et al., 1987). Estrone sulphate drops after induction of fetal death at day 45 of pregnancy (Hyland & Langstrom, 1990; Jeffcott et al., 1987) without affecting either eCG or progesterone secretion (Jeffcott et al., 1987). As noted earlier, fetal tissues have considerable capacity for oestrogen synthesis, and equine endometrium is very active in sulphation (Heap et al., 1982). Chorio-allantoic steroidogenic enzyme expression at day 45 of gestation is also consistent with the synthesis of estrone sulphate (Loux et al., 2020), and there were strong positive correlations between chorio-allantoic expression of CYP17A and CYP19A with maternal serum dehydroepiandrosterone (DHEA) and estrone sulphate, respectively, in maternal blood (Loux et al., 2022). This suggests that eCG stimulation of luteal androgen secretion (Albrecht et al., 1997; Daels et al., 1998; Daels et al., 1996; Legacki et al., 2016a, 2016b) may provide substrate for aromatization in the chorio-allantois and sulphation in the fetal membranes or endometrium (Heap et al., 1982, 1991; Loux et al., 2020) as illustrated (Figure 3b). This would represent one of the earliest (and most novel) examples of a functioning maternal-fetal unit.

This model of cooperative steroidogenic exchange and interaction between the eCG-stimulated ovary and the early-developing chorio-allantois has not been previously proposed and has yet to be adequately explored. The increase in chorioallantoic expression of CYP17A at day 45 of gestation bears a notable resemblance to the early increase in expression of CYP17A in the bovine placenta that is associated temporally with early fetal adrenocortical organization (Conley et al., 1992) and corticoid synthesis (Lund et al., 1989). There is a similar activation of adrenal corticoid synthesis in the ovine fetus (Tangalakis et al., 1994) that is associated with a dramatic increase in maternal concentrations of DHEA (Conley & Reynolds, unpublished observations) which almost certainly reflects an increase in CYP17A in the early ovine chorio-allantois. Lastly, the increase in estrone sulphate secretion between days 24 and 30 of gestation in the pig (Robertson & King, 1974), as seen in fetal fluids of both bovine and ovine foetuses (Robertson et al., 1985), is likely also fueled by an increase in chorio-allantoic androgen synthesis. The temporal coordination of steroidogenic enzyme expression during early development of the chorio-allantois and the fetal adrenal gland in ungulates, and the increase in CYP17A and CYP11A in the developing equine chorio-allantois, suggests that the mechanisms are involved are well-conserved across species.

3 | FETO-PLACENTAL WELL-BEING EVALUATION

3.1 | Ultrasonographic approach

Transrectal and transabdominal ultrasonography are combined to assess the health of the placenta, fetal fluids and fetus.

3.1.1 | Placental evaluation

Even though the placenta is large, much can be imaged. The most caudal area of the uterus cranial to the cervix, and parts of the uterine body and horns, can be evaluated via transrectal and transabdominal ultrasonography, respectively (Renaudin et al., 1997, 2003). A normal healthy placenta should not be distinguishable from the uterus except after 280 days of gestation where the chorio-allantois looks edematous in the cervical area (Renaudin, 2018; Renaudin et al., 1997). The combined thickness of the uterus and placenta (CTUP) is measurable by ultrasound. Transrectally, the 5.0 to 7.5-MHz linear transducer is placed at the placenta-cervical junction, 2.5–5 cm cranial to the cervical internal os, to obtain an image where the fetal fluids, the dorsal and ventral parts of the uterine body and a vascular space (branch of the uterine artery) are well visualized. CTUP is measured from the ventral aspect of the uterine body, one cursor placed on the border of the uterus in contact with the vessel margin and the other cursor on the border of the chorio-allantois

in contact with the allantoic fluid. It is important that neither the amniotic membrane nor allantoic vessels are included in the measurements and that no fetal parts put pressure on the placenta; the axis of the measurement should be perpendicular to the axis of the ventral part of the uterine body. Three measurements from two to three different images should be taken and the mean CTUP calculated. Transabdominally, the same probe is used. CTUP is measured placing one cursor at the border of the uterus and the other one on the border of the chorio-allantois in contact with the allantoic fluid, keeping the axis of the measurement perpendicular to the axis of the uterus. Measurements should be taken in all four quadrants of the placenta (right cranial, right caudal, left cranial, left caudal) in areas where there is no fetal contact, and preferably where the placenta is not folded (Bucca et al., 2005). In light breed horses, the mean CTUP plateaus approximately 4 mm between 4 and 9 months of gestation, and then increases 1.5–2 mm each month. There appears to be small variations in normal CTUP between most breeds (QH/light breed) (Renaudin et al., 1997), Thoroughbred (Colon, 2008; Troedssen et al., 1997), Warmblood (Coutinho da Silva et al., 2013; Hendriks et al., 2009), Crioulo (Souza et al., 2010) and Standardbred (Bucca et al., 2005). In draft horses, CTUP values are significantly higher than in any other breed reaching 15 mm late in pregnancy (Kimura et al., 2018). Increased CTUP >7 mm between days 90 and 270, >8 mm between days 271 and 300, >10 mm between days 301 and 330 and >2 mm after day 330, with or without placental separation, are suggestive of placentitis in most breeds (Renaudin, 1999; Troedssen et al., 1997; Troedssen & Macpherson, 2011). In draft horses, placentitis is more frequently diagnosed when CTUP is greater than 15 mm. The transrectal ultrasound approach is best to assess ascending placentitis which most commonly affects the cervical star region, while the transabdominal ultrasound approach is particularly useful in suspected nocardioform placentitis or diffuse hematogenous placentitis due to Leptospirosis. Ultrasonography is the most useful tool for monitoring placental changes in mares affected by placentitis, whether clinical (associated with premature udder development with or without streaming of milk and vulvar discharge) or subclinical (thickening of the placenta). Treatment should be initiated when placentitis is first diagnosed via ultrasound whether clinical signs are present or not, because attempts to treat mares with clinical signs are often disappointing. Standard treatment includes the use of large spectrum antibiotics, anti-inflammatory drugs, altrenogest and pentoxifylline (LeBlanc, 2010). Response to treatment is evaluated weekly or biweekly by ultrasound. Decreased CTUP, or a return to normal values, are indicative of positive response to treatment, while increased CTUP prompts a change in treatment strategy.

3.1.2 | Fetal fluid evaluation

Both allantoic and amniotic fluids can be imaged on ultrasound and assessed for their echogenicity and maximum depth. Fetal fluid echogenicity is best evaluated using the transrectal approach (Renaudin et al., 1997). The amniotic fluid generally contains more (and smaller) free-floating particles than the allantoic fluid after 4 months of gestation, likely due to fetal parts in motion that are actively stirring sedimented particles within the amniotic fluid. Both fluids should look dark grey (hypoechoic with few hyperechoic foci suspended in anechoic medium), except for the amniotic fluid after 10 months of gestation that is light grey (semi-echogenic) (Renaudin et al., 1997). Increased echogenicity with hyperechoic fluid is abnormal and may be associated with passage of meconium in utero, haemorrhage, or inflammatory debris, which may reflect fetal hypoxia, placental detachment, or placental infection, respectively (Sertich, 1993; Vaala & Sertich, 1994). It has also been reported in mares with reproductive loss syndrome at 60 days gestation (Powel, 2011) and with fetal death. The normal amniotic membrane should be thin and undulating. Thickened or turgid amniotic membranes have been associated with some cases of placentitis (Troedssen & Macpherson, 2011) and umbilical cord torsion, and hydrops amnion (Slovis et al., 2014), respectively. Excessive amount of fetal fluid or hydrops allantois/amnion, often suspected late in pregnancy when mares have an enlarged abdomen for gestational age, can be confirmed by measuring the maximum allantoic and amniotic depth using the transabdominal ultrasound approach (Reef et al., 1995). Allantoic maximum depth (AllMD) is measured by placing one cursor on the edge of the placenta in contact with the allantoic fluid and the other cursor on the amniotic membrane once it reaches its lowest point on the ultrasound image. Amniotic maximum depth (AmnMD) is measured by placing one cursor on the amniotic membrane (at its highest point on the ultrasound image) and the other cursor on the fetal skin with the connecting line perpendicular to the placental surface. Reef reported excessive AllMD and AmnMD as being superior to 22.1 and 14.9 cm, respectively (Reef et al., 1996).

3.1.3 | Fetal well-being evaluation

Signs of fetal well-being are associated with normal fetal heart rate (HR), activity, presentation, and size for gestational age.

Fetal HR can be obtained either after viewing the cardiac cavity on B-mode by using a stopwatch or M-mode. In late gestation when foetuses are in anterior presentation, peripheral pulses from the external carotid artery can be determined by transrectal ultrasonography (Bucca, 2022). In normal pregnancies, fetal heart rhythm is regular (Reef et al., 1995) and HR decreases as gestation advances (Adams-Brendemuehl & Pipers, 1987; Bucca, 2006; Reef et al., 1995). Fetal HR accelerations of 15–40 beats per minute in rate, and of 20–40 s in duration, generally occur in response to fetal activity (Adams-Brendemuehl & Pipers, 1987; Renaudin, 2018). Yet, fetal movement without fetal HR accelerations are observed routinely, while accelerations in the absence of detectable stimuli occurs only 5% of the time (Adams-Brendemuehl & Pipers, 1987). Normal mean fetal HR values, at rest (HRR) and after movement (HRM), have been reported for different gestational ages (Adams-Brendemuehl & Pipers, 1987; Bucca et al., 2005; Hendriks et al., 2009; Reef et al., 1995; Renaudin, 1998).

In summary, HRR is above 100 bpm between 100 and 250 days gestation, above 80 bpm between 250 and 300 days gestation and above 60 bpm after 300 days gestation (Renaudin, 2018). Normal HRM value is represented by adding 15–40 bpm to the HRR. Occasionally low HR, below 50 bpm, may be observed in term foetuses and may be considered normal if they represent only short-lived episodes (Bucca, 2011). Transient elevations of HRR above 120 beats/min, during the last weeks of pregnancy, is not of concern either, unless it fails to return to baseline (Bucca, 2011). Persistent tachycardia or bradycardia are concerning as the former has been observed preceding abortion (Reef et al., 1996) and stillbirth (Adams-Brendemuehl & Pipers, 1987; Bucca, 2011), and the latter is considered the most reliable indicator of impending fetal demise (Colles et al., 1978; Rantanen & Kincaid, 1998) but is often preceded by fetal tachycardia. Sustained tachycardia may also be brought on by painful maternal conditions or systemic disease (Bucca, 2022). Bradycardia or lack of HR variation suggests central nervous system (CNS) depression likely attributable to hypoxia (Bucca, 2006). Fetal asystole ultimately identifies fetal demise and can be confirmed with colour Doppler (absence of blood flow) (Rantanen & Kincaid, 1998; Reef et al., 1996). Cardiac arrhythmias are commonly associated with a negative outcome (Bucca, 2022).

Fetal activity includes any fetal movements (extension and flexion of the limbs, neck and head, whole body rotations around the fetal long axis, spinning and translation). It is graded on a scale of 0 to 3 for the entire examination period (approximately 20–30 mn). It is grade 0 if no movement is detected throughout the entire examination period, 1 if only a small amount of movement is detected (<33% of the examination time), 2 if the fetus is fairly active (33%–66% of the examination time), and 3 if the fetus is very active and few or no quiet periods are detected (>66% of the examination time) (Reef et al., 1995). Fetal activity declines as gestation advances and is reduced to rotation of the abdomen and thorax, and extension and flexion of the head, neck, and limbs when, after 9 months of gestation, the hindlimbs are trapped in the umbilical cord horn (Ginther, 1998). Between 100- and 250-day gestation, fetal activity varies from 2 to 3; between 250- and 320-day gestation from 1 to 3; and after 320-day gestation from 1 to 2 (rarely grade 3 except from 5 to 72 h before parturition) (Reef et al., 1995, 1996; Renaudin, 2016). Inactivity late in gestation is common with normal foetuses having dormant resting activity for up to 60 mn or longer on occasion (Fraser et al., 1973). Therefore, fetal inactivity late in gestation should be interpreted with caution and should warrant re-evaluation within 24 h. Fetal activity is very important to ensure satisfactory muscular development and skeletal joint function that ultimately promotes successful postnatal adaptation. It reflects CNS function and development, and depressed CNS function, most often due to hypoxia, results in decreased activity. Abnormal activity such as prolonged hyperactivity or inactivity, or sudden bouts of excessive activity followed by abrupt cessation, have been associated with poor fetal outcomes (Fraser et al., 1973).

Fetal presentation can easily be evaluated (Figure 4). Fetuses can be found in anterior, posterior or transverse presentation up to

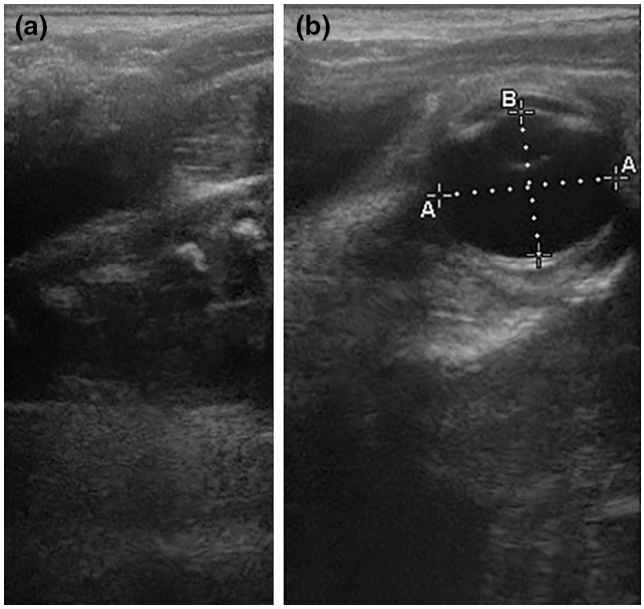

FIGURE 4 Fetal presentation: (a) transrectal ultrasound image showing the fetal tail consistent with posterior presentation at 242 days of gestation; (b) transrectal ultrasound image showing a fetal eye consistent with anterior presentation at 229 days of gestation. Eye length (eyL) between A and eye depth (eyD) between B were measured to calculate eye approximated volume (eyV = eyL × eyL × eyD). The head of the mare is to the right and tail of the mare to the left of both images.

240–260 days of gestation (Bucca et al., 2005; Renaudin, 2018). After that time, all fetuses should be in anterior presentation. It is a sign that the fetus reaches a neurological age, which allows the internal ear to respond to a maternal directional signal (Ginther, 1998). When fetuses are not in anterior presentation after passing 260 days, the fetus should be reexamined any time prior to parturition to ensure it has rotated. This is very valuable information. If the fetus is not appropriately presented, there is a high risk of dystocia. Therefore, the mare should foal in a veterinary hospital where assisted vaginal delivery and C-section can be performed.

Assessment of *fetal size* is a significant part of feto-placental well-being evaluations. Fetal optimal development and well-being are suggestive of a highly functional placenta, efficient feto-maternal exchange pathways, and genetic normalcy (Bucca, 2022). In case of placental insufficiency and/or fetal abnormality, intrauterine growth retardation may occur. Fetal growth can be assessed, via ultrasound, using multiple biometric parameters: biparietal diameter (BPD), head circumference (HC), eye length, eye width, eye length + width, eye surface area, eye approximated volume (eyV) (Figure 4b), aortic diameter (aortD), abdominal diameter (AD), abdominal circumference (AC), intercostal distance, femur length (FL) (Adams-Brendemuehl & Pipers, 1987; Bucca et al., 2005; Kahn & Leidl, 1987; Reef et al., 1995; Renaudin et al., 2000) and recently the first phalanx length (P1L) (Renaudin, 2021). In QH, among those variables, BPD, eyV, aortD and FL are mainly used because they are highly correlated with days of gestation (Renaudin et al., 2000). The table established in light breed horses serves as a reference for predicted measurements of

the main biometric parameters for each day of gestation from 100 to 342 days of gestation (Renaudin et al., 2022). Even though fetal growth will likely vary with breed, the table established using mainly quarterhorse (QH) can be used in warmblood (WB), standardbred (STB), thoroughbred (TB) up to 200 to 250 days of gestation. Later on, P1L can be measured in addition to BPD and eyV. Interestingly, when P1L reaches or exceeds the width of the ultrasound image (on most ultrasound machines = 52.5 mm), most pregnant QH mares are at 300 days of gestation or more, and therefore within 4–6 weeks of parturition (Renaudin, 2021). P1 proximal and distal epiphyses appear and eventually close at defined times in gestation. A normal sized QH fetus at 10 months of age should have both proximal and distal epiphyses present on transrectal ultrasound with P1L inferior to the length of the ultrasound image (52.5 mm). And by 11 months of age, the distal epiphysis should be closed and P1L superior to the length of the ultrasound image (Renaudin, 2021).

3.1.4 | Biophysical profile

Reef et al. (Reef et al., 1996) modified the biophysical profile (BPP) previously developed by Adams-Brendemuehl and Pipers (Adams-Brendemuehl & Pipers, 1987) so that it would be predictive of perinatal events in the late gestation. Adapted from the human BPP (Manning, 1990), it is a composite of six parameters: fetal HR (HRR and HRM), fetal aortic diameter, maximum fluid depth, uteroplacental contact, uteroplacental thickness and fetal activity (Reef et al., 1996). Breathing movement has been removed from the BPP parameter likely due to difficulty to consistently image the excursions of the diaphragm between the thorax and abdomen and expansion of the ribcage. For each parameter, fetuses are assigned a 0 score if their condition is abnormal and a 2 if it is normal. To calculate the BPP, these scores are summed, resulting in a total score between 0 and 12. A low score of 8 or less was a definite indication of an impending negative outcome. Yet, a high score is not reliably predictive of a positive outcome (Reef et al., 1996). The equine BPP's predictive value of normalcy is not as uniformly accurate as it is in human medicine. Palmer suggested that the lack of sensitivity and specificity may have to do with the selection of parameters measured (Palmer, 2000). To reduce the time of examination, Vincze et al. (Vincze et al., 2019) introduced a rapid examination protocol (REP) including three parameters: FHR (acute hypoxia marker), aorticD (chronc hypoxia marker) and CTUP (placentitis marker). Both protocols have similar sensitivity, specificity and accuracy. Even though they show promise, they need refinement.

3.1.5 | New emerging use of Doppler technology

Doppler technology has recently been applied to assessments of the uterine and umbilical arteries of pregnant mares (Klewitz et al., 2015; McGladdery & Ousey, 2016), although neither are very informative. Specifically, the former has poor predictive value for pregnancy outcomes and the latter suffers from inconsistent visualization of the cord past 250 days at the time when most gestational disorders in mares tend to be more clinical. Further studies in this area should focus on new ultrasound techniques able to consistently visualize the equine umbilical cord for doppler interrogation throughout gestation. In contrast to umbilical cord vessels, the fetal carotid artery can be readily visualized by transrectal ultrasonography from mid-gestation to term, in the equine fetus in anterior presentation (Bucca, 2006) allowing establishment of normal Doppler indices in healthy uncomplicated pregnancies (Bucca et al., 2020). Recording of abnormal carotid waveforms in the equine fetus, suggesting systemic blood volume redistribution, could become a critical parameter to predict the risk of adverse pregnancy outcomes (Bucca et al., 2020). Blood flow pattern alterations generally occur in response to poor placental function, intrauterine growth restriction, fetal anaemia, hypoxia and acidosis. Yet, in the author's experience, it is not an easy task to obtain carotid waveforms due to fetal and maternal movements.

3.2 | Endocrine approach

Steroid concentrations, primarily progesterone, 5α-dihydroprogesterone (DHP) and estrone sulphate can provide valuable information on the viability of the fetus and of the pregnancy in general. Development and maturation of the equine chorio-allantois enable it to synthesize and secrete enough progestin in the form of DHP to sustain pregnancy without ovarian progesterone, an event known as luteo-placenta shift that coincides with a secondary, more dramatic increase in the secretion of estrone sulphate (Figure 2). The luteo-placental shift may begin as early as day 50 in some mares and is complete in most by day 120 (Holtan et al., 1979) when pregnancies can survive without ovarian support. The chorio-allantoic placenta in horses does not secrete progesterone. In fact, when measured using mass spectrometry, progesterone is undetectable in most mares after 200 days of gestation. This is because the equine chorio-allantoic placenta expresses a different spectrum of steroidogenic enzymes than luteal tissue, one that includes 5α-reductase in particular, that metabolizes virtually all the progesterone it synthesizes to 5α- DHP an equally bioactive progestin in horses (Scholtz et al., 2014). DHP binds tissues like the endometrium (Jewgenow & Meyer, 1998) and mammary gland (Chavatte-Palmer et al., 2000) that are rich in progesterone receptors. DHP concentrations remain below those of progesterone until the endometrial cups regress and ovarian progesterone secretion declines from approximately gestation day 70–80 (Holtan et al., 1991; Legacki et al., 2016a, 2016b). As pregnancy progresses, the decrease in progesterone and the increase in DHP from the developing chorio-allantois eventually results in an inversion of the ratio of the two pregnanes (Figure 2), so that DHP exceeds progesterone concentrations, at approximately days 110–120 of gestation. Mass spectrometry, typically liquid chromatography tandem mass spectrometry (LC–MS/MS), can distinguish progesterone from DHP in a single analysis and provides quantitative estimates

of concentrations that immuno-assays cannot (Wynn et al., 2018). The inversion of the ratio of progesterone:DHP provides objective evidence that placental viability and development are sufficient for pregnancy maintenance and may be valuable in confirming that exogenous progestin therapies like altrenogest are no longer needed. Altrenogest is androgenic (Naden et al., 1990), more so than testosterone in human assays (McRobb et al., 2008), with possible effects on long-term fertility (Conley, 2023). Confirming the luteo-placental shift can support rational decisions to cease altrenogest treatments, potentially benefiting the health of filly foals.

The luteo-placental shift coincides with the start of the unusual growth (Wesson & Ginther, 1980) and steroid secretion of the fetal gonads (Pashen et al., 1982) that ultimately fuels the secondary rise in estrone sulphate (Raeside, 2017), peaking approximately 7 months of gestation (Hoffmann et al., 1996; Legacki et al., 2019; Raeside, 2017). This secondary increase in estrone sulphate comes by way of metabolism of dehydoepiandrosterone (DHEA) from the fetal gonads (Legacki et al., 2017) that is ultimately aromatized to estrone in the placenta and then sulphated, most likely in the placenta and endometrium (Loux et al., 2020). Oestrogen synthesis, therefore, occurs by way of a feto-placental unit (Raeside, 2017) and so remains a biomarker of fetal viability throughout gestation. Fetal death precludes gonadal steroid secretion, resulting in a rapid decline in estrone sulphate. Based on fetal gonadal and adrenal tissue steroid concentrations (Legacki et al., 2017), the gonads likely contribute significantly to the high concentrations of pregnenolone in the fetus (Ousey et al., 2003) that some believe fuels placental progesterone and ultimately DHP synthesis. Concentrations of other progesterone and DHP metabolites also increase during this period, particularly in the last months of pregnancy, to exceedingly high levels in some mares (Holtan et al., 1991; Legacki et al., 2016a, 2016b). These are weak progestins (Legacki et al., 2016a, 2016b) and concentrations of them are extremely variable among mares. Therefore, although they increase in some mares with placentitis (El-Sheikh Ali et al., 2020), determining concentrations of pregnane metabolites is of limited diagnostic value (Ousey et al., 2005), even if multiple samples are examined to determine trends. Sometime in the weeks before foaling, the chorio-allantois loses 5α-reductase expression (Legacki et al., 2018) which likely contributes to the rapid decline in DHP and other pregnanes preceding parturition. But neither expression nor enzymatic activity of HSD3B and CYP19A changes, suggesting that the loss of steroidogenic function is isolated to SRD5A1. This remains a unique observation with respect to placental steroidogenesis at parturition, among livestock species certainly, and the mechanisms involved in differential regulation of placental gene expression are unknown.

4 | FETAL AGE EVALUATION

Fetal age is not always known with certainty, as when mares are left intentionally pastured with stallions or bred accidentally, or when pregnant mares are sold or rescued without any information on the pregnancy. It is, therefore, important to estimate fetal age and thereby ascertain an approximate time of foaling. Yet, because gestation length is so variable in the mare (310–374 days), estimates are unreliable.

4.1 | Ultrasonographic approach

Fetal age is predicted by measuring the main biometric parameters (BPD, eyV, FL, AortD) (Adams-Brendemuehl & Pipers, 1987; Bucca et al., 2005; Kahn & Leidl, 1987; Reef et al., 1995; Renaudin et al., 2000) as well as P1 (Renaudin, 2021). A table established for light-breed horses can be used to obtain fetal age associated with the measurement of each biometric parameter evaluated (Renaudin et al., 2022). In a recent study involving 23 healthy QH pregnancies, fetal age was predicted within 2 weeks from 100 to 200 days gestation (with FL, EyV or BPD) and within 3 weeks from 201 to 300 days (with BPD, EyV) and after 301 days (with EyV) (Renaudin et al., 2022). FL was the most reliable biometric parameter from 100 to 150 days, predicting age within 1 week with a very small margin of error (+2 days) (Renaudin et al., 2022). The same table can be used in WB, STB, TB with similar accuracy, as in QH up to 200–250- days gestation (Renaudin unpublished data). Some data are available in Shetland ponies, Crioulo, TB, STB and Dutch WB mares. In Shetland types of ponies (100–250 kg), the predicted day before parturition can be calculated from the regression equation DBP = 265.16–0.21* (eyL in mm) (Turner et al., 2006). In Crioulo mares, gestational age can be estimated using the formula: $y = 8.3756x + 11.90$, where y represents the gestational age in days, and x represents the eyL in millimetres (Hartwig et al., 2013). Based on our study in QH (Renaudin et al., 2022), fetal age is best predicted during mid-gestation. After 250 days gestation, when accuracy based on BPD or EyV decreases, P1L and the presence or absence of its epiphyses can help narrow down fetal age, assuming that the fetus and placenta are normal and it is not a twin pregnancy. In QH, if only P1 diaphysis is present, then the mare is likely less than 300 days gestation (Renaudin, 2021). As described above, if both P1 proximal and distal epiphyses are visible on ultrasound, mares are likely in their tenth month of pregnancy, and if the proximal epiphysis is present and the distal absent, more likely in their eleventh month of pregnancy (Renaudin, 2021).

4.2 | Endocrine approach

Considered in toto, steroid secretion during equine gestation is as complicated as it gets during gestation in any mammal, with the possible exception of human pregnancy (Conley, 2016). This is because it includes the secretion of a suite of pregnane metabolites (Holtan et al., 1991) as well as androgens and estrogens (Legacki et al., 2016a, 2016b), some of which are of the B-ring unsaturated variety. As described above, increased androgen and oestrogen secretion is initiated coincident with eCG secretion, which is followed by the luteo-placenta shift to DHP and then peak secretion of

estrone sulphate (feto-placental unit) (Legacki et al., 2016a, 2016b). As estrone sulphate concentrations decline from their peak at 7 months (Figure 2), there is an increase in DHP metabolites, more steeply in the final few months (Legacki et al., 2016a, 2016b; Wynn et al., 2018). Many of these steroids, including pregnanes, androgens and estrogens, can be monitored by LC–MS/MS in a single analytical run. The secretory patterns so defined over time within the same mare allowed these longitudinal, time-resolved data to be mathematically modelled for the first time, generating algorithms predictive of the stage of pregnancy and perhaps even time of foaling (Shorten et al., 2021) as a proof of concept requiring refinement.

Recent studies on steroidogenesis in the fetal adrenal glands and gonads have provided insights into the sources of steroid precursors that are required for the synthesis of both pregnanes and estrogens during equine gestation. It has long been known that the equine fetus, unlike the ovine fetus, has extremely high concentrations of unconjugated pregnenolone (Holtan et al., 1991; Ousey et al., 2003), and some have suggested that this represents fetal adrenal synthesis (Fowden et al., 2008). But there are reasons to doubt this (Conley, 2016). Pregnenolone concentrations are no higher in the fetal adrenal glands than they are in the fetal gonads at any point from 4 months of gestation (Legacki et al., 2017), and the fetal gonads are many times larger in size (Wesson & Ginther, 1980). This suggests that the gonads may contribute more than the adrenal glands to fetal pregnenolone concentrations, which provides substrate for placental pregnane sysnthesis (Ousey et al., 2003). In addition, administration of adreno-corticotropic hormone (ACTH) to the equine fetus does not acutely increase pregnane concentrations in maternal blood, as would be expected but only after days (Rossdale et al., 1992). Fetal betamethasone administration which would have been expected to suppress the fetal hypothalamic–adrenal axis also increased maternal pregnane levels (Rossdale et al., 1992) as did maternal dexamethasone (Ousey et al., 2011). The increase in maternal pregnane concentrations in response to fetal or maternal glucocorticoid seems more likely to result from effects directly on chorio-allantoic steroidogenesis than it does fetal stress (Allen et al., 2002) but warrants further investigation. Finally, the well-known secretion of B-ring unsaturated androgens (Tait et al., 1983, 1985) by the equine fetal gonads that are metabolized to the equine-specific estrogens (Bhavnani, 1988) by the equine placenta, remains an enduring steroidogenic mystery. Human patients suffering Smith-Lemli-Opitz syndrome, with a deficiency in the final enzyme involved in cholesterol synthesis, 7-dehydrocholesterol Δ7-reductase, also secrete B-ring unsaturated estrogens (Shackleton et al., 1999). Yet, transcript analysis of equine fetal gonads and adrenal glands (Legacki et al., 2017) found robust expression of the gene encoding 7-dehydrocholesterol Δ7-reductase and no difference between these tissues as was expected (Conley & Ball, unpublished observations). This unusual steroidogenic pathway awaits a more comprehensive transcriptomics investigation of the complex pathways involved in cholesterol synthesis, and perhaps the related pathways for Δ7-desaturated steroids that are intermediates in vitamin D synthesis that is so unusual in horses (Uhl, 2018).

5 | PREDICTION OF PARTURITION

Mares are said to be 'at term' when they reach 340 days gestation. Yet, the length of gestation is so variable among mares that predicting the day of parturition remains problematic. There are many other factors influencing gestation length including fetal gender, daylight, parity, and age. Male-product pregnancies are slightly longer, by 2–3 days on average (Satué et al., 2011). Mares have longer gestations when daylight period is shorter (Astudillo et al., 1960) and subjecting mares to 16 hours of daily light from the beginning of December advanced the date of parturition (Hodge et al., 1982). Aged and multiparous mares have longer gestation (Satué et al., 2011) that may be attributable to degenerative changes in the endometrium, poor fetal nutrition and delayed development. Primiparous mares, which are usually younger, also have longer gestations perhaps because they are simply not fully mature, anatomically and/or physiologically (Satué et al., 2011).

5.1 | Ultrasonographic approach

Several parameters such as cervical relaxation, presence of stomach rugae and gastro-intestinal peristalsis, and distal limb ossification are useful to determine fetal readiness for birth. Ultrasound studies using the transrectal approach have shown that cervical remodelling in the mare is a slow and gradual process, initiating as early as 9 months of gestation, and taking several weeks to achieve completion (Bucca, 2022). Cervical relaxation occurs close to parturition and is apparent when cranial and caudal diameters of the cervix are comparable, and when mucosal engorgement, thinning of the muscular layer, associated with a marked distinction in echolucency between mucosa and muscularis is observed by ultrasound (Bucca & Fogarty, 2011). Ultrasonographic changes in the fetal gastro-intestinal tract that occur with progressive fetal maturity may indicate fetal readiness for birth (Agnew et al., 2019). Stomach rugae are absent prior to 12 days of parturition but are present thereafter. Peristalsis appears, and then becomes progressively continuous, approximately 18 and 5 days before parturition, respectively. Peristalsis, in combination with tail head relaxation of the mare and day of gestation, can predict the day of parturition with a 95% CI ± 4 days. In addition to fetal GI tract changes, signs of bone maturation of the distal limb can be detected via transrectal ultrasonography (Renaudin, 2021). Based on a study in QH, distal limb ossification can be considered complete when the metacarpal 3 distal epiphysis and P1 and P2 proximal epiphysis are present and reach the palmar aspect of their respective diaphysis, and all sesamoid bones are present (Renaudin et al., 2023). A regression model based on eye length and width (distance from the cornea to the optic disc), in addition to parity and age as covariates, has been established in STB mares to predict parturition. At best, depending on the node, prediction is within 10 days (Lanci et al., 2019).

5.2 | Endocrine approach

The prediction of foaling dates based on endocrine algorithms was far less accurate than was prediction of the gestational stage (Shorten et al., 2021). Yet, the data used to generate those models made use of sera collected from relatively few mares with a sampling frequency that was far less as the pregnancy progressed. Therefore, there was a paucity of the data needed to develop models with the goal of predicting foaling. Foaling may also be preceded by a limited number of changes in steroidogenic enzyme expression in the chorio-allantois (Figure 2). There was a complete disappearance of 5α-reductase (SRD5A1) expression in the chorio-allantois between 300 days of gestation and the day of delivery but no significant change in expression of other enzymes (HSD3B1 or CYP19A) (Legacki et al., 2018). Levels of DHP and other pregnane metabolites decline sharply, but only over the last 2–3 days before foaling (Legacki et al., 2016a, 2016b). Therefore, whether or not algorithms developed from maternal serum metabolomics data collected prior to the final days of gestation will be predictive of foaling dates remains to be determined. The data collection in the experiments described above was not designed for the purpose of algorithm development in general, let alone for predicting foaling dates. Although reasonably predictive of gestation stage, algorithm development served only as a proof of concept (Shorten et al., 2021). Future studies should incorporate frequent sampling during the last month of gestation at least to better explore the potential for steroid metabolomic analysis and algorithm development to predict foaling dates.

6 | CONCLUSION

Ultrasonography remains the most effective tool for pregnancy diagnosis from as early as 9 days post ovulation and can be performed at any stage of gestation using transrectal and/or transabdominal approaches. Twins can be identified and reduced early in gestation and placental abnormalities such as placentitis can also be diagnosed later in gestation, sometimes before the appearance of clinical signs, thereby facilitating early treatment that can salvage pregnancies. Ultrasonography is the best tool to diagnose hydrops, and fetal well-being can be monitored by assessing fetal heart rate, activity, presentation, and size for gestational age. Any abnormalities such as persistent tachycardia or bradycardia often precede fetal demise and should prompt re-examination. Assessment of pregnancy viability based on BPP and REP is of limited predictive value. The use of doppler to evaluate fetal intracranial blood flow impedance, and to detect abnormal blood flow redistribution to the brain, could become a critical parameter to predict the risk of an adverse pregnancy outcome. Fetal age evaluation is well predicted within 2–3 weeks with ultrasound until 200–250 days gestation in most light breed mares using common biometric parameters such as FL, BPD, and eye volume. Later, distal limb ultrasonographic evaluation is more useful at determining whether gestation is at 10 or 11 months. Ultrasonography is not very good at predicting parturition. Yet, it can identify signs of fetal maturation that can be used as an indication of fetal readiness for birth. Endocrinology can add a more functional element to the morphological information provided by ultrasound. The secretion of estrone sulphate requires both maternal and fetal viability and input, even at the initial rise early in gestation, and fetal demise results in a rapid drop in estrone sulphate and progesterone metabolites later in gestation. Endocrine testing is especially useful when rectal palpation is difficult or impossible, as with mini breeds. Although only a proof of concept, there is considerable potential for the development of algorithms to estimate the stage of gestation, and perhaps even foaling dates, using mass spectrometry that allows multiple steroids to be assessed in a single analysis.

AUTHOR CONTRIBUTIONS

Catherine Renaudin wrote the ultrasonographic approach sections, and Alan Conley wrote the endocrine-related sections of the manuscript.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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