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THE USE OF TRANSRECTAL ULTRASOUND TO INVESTIGATE THE EFFECT OF PROGESTERONE SUPPLEMENTATION ON EARLY EMBRYO LOSS IN SHEEP

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Abstract

Reproductive wastage in the form of early embryo loss represents a significant economic forfeiture for sheep producers. There is substantial ambiguity surrounding the causes and preventative strategies for early embryo loss, primarily due to the limited capacity to detect early pregnancy (<day 30 gestation). Experimentation with progesterone (P_4) supplementation as a mitigation measure for loss has yielded inconclusive results, warranting further investigation into its efficacy. As such, the objective of this study was to determine the earliest point of gestation transrectal ultrasound (TRUS) can diagnose pregnancy (including multiples), as well as investigate whether the addition of exogenous P_4 can reduce embryo loss. Merino ewes (n=62) were synchronised and inseminated artificially (AI) via laparoscopy 60 hours post sponge removal (day 0) and were randomly allocated into either a control (no CIDR), CIDR on day 0 (CIDR @D0) or CIDR on day 3 (CIDR @D3) treatment groups. CIDRs were removed from both groups on day 17 post AI. Each ewe was subjected to TRUS on days 10, 12, 14, 17, 19 and 28. Blood was also collected from all ewes on day 19 of gestation for a P_4 blood assay, against

which TRUS diagnoses could be compared. All ewes were also subject to transabdominal ultrasound on day 54 of pregnancy which was the standard against which all TRUS diagnoses were compared to analyse the accuracy of pregnancy detection via TRUS. The accuracy of TRUS for correctly diagnosing ewes as pregnant on days 12, 14, 17, 19, and 28, was 62.2%, 50.0%, 64.9%, 86.5% and 100%, respectively, while the percentage of ewes correctly diagnosed as having multiples on the same days, was 40.6%, 22.6%, 38.2%, 50% and 97.2% respectively. The accuracy of detecting pregnancy (and multiples) was similar ($P>0.05$) on days 12, 14 and 17, though differed significantly ($P<0.05$) from days 19, 28 and 54, between which, there was no difference ($P>0.05$). Embryo loss was found to occur predominantly prior to implantation since embryo loss (%) varied significantly ($P<0.05$) between ovulation (D0), pre- (D12 and 14) and peri-implantation (D17 and 19). There was no major change in percent embryo loss following peri-implantation ($P>0.05$). Exogenous P_4 from D0 was found to increase embryo loss ($P<0.05$), while P_4 from D3 was of no advantage ($P>0.05$; similar to the control). In summary, TRUS is capable of detecting early embryos and has reaffirmed earlier conclusions of loss occurring prior to implantation (<day 19 gestation). It can also be concluded that progesterone supplementation in early pregnancy is of little benefit, if provided later in pregnancy it is also expected to have little effect, due to majority of losses occurring prior to implantation.

Key Words: early embryo loss, sheep, transrectal ultrasound, progesterone supplementation

Introduction

It has been estimated that between 20-30% of all fertilised ova are lost within the first month of gestation (Edey, 1969, Quinlivan et al., 1966). This substantial rate of embryo loss in early pregnancy (<day 30 of gestation) is further supported by the fact that sheep possess high fertilisation rates (>90-95%) (Diskin and Morris, 2008, Mitchell et al., 1999, Restall et al., 1976) and experience few late-gestational losses (Dixon et al., 2007). Consequently, early embryo loss represents a significant economic forfeiture for sheep producers. Moreover, considerable uncertainty surrounds the exact cause, timeline and prevention of early embryo loss. Numerous factors have been linked to early losses, including maternal age (Mulvaney, 2011, Shorten et al., 2013), nutrition (Abecia et al., 1997, Parr et al., 1982, Viñoles et al., 2012), genetics (Bodin et al., 1992), endocrine factors (Ashworth et al., 1989, Diskin and Niswender, 1989, Wilmut et al., 1986) and environmental conditions (Dutt, 1963). The interaction between these factors, further complicates the comprehension of embryo loss.

Ambiguity surrounding early embryo loss can be partially attributed to the limited capacity to detect early pregnancy (<day 30 of pregnancy). There are various methods of pregnancy detection currently used for sheep (Fthenakis et al., 2012, Ganaie et al., 2009, Garcia et al., 1993, Jones et al., 2016), varying in accuracy, expense and stage of pregnancy which they can be reliably utilised. Transabdominal ultrasonography is widely used within industry, yet can only be used between days 40-70 of gestation (Fthenakis et al., 2012, Ganaie et al., 2009, Jones et al., 2016), rendering it incongruous for early pregnancy detection. Alternatively, blood hormone analysis, particularly for progesterone (P_4) or pregnancy associated proteins, can be employed relatively accurately (>90%) by days 15-19 of gestation (Karen et al., 2003, McPhee and Tiberghien, 1987). Yet, this method is labour intensive, expensive and incurs a lag period between sampling and result, compromising the viability of this option for large scale sheep

production. Furthermore, this method fails to identify multiple conceptuses, a crucial element for investigating early embryo loss (Boscos et al., 2003).

Transrectal ultrasound (TRUS) has been shown to detect positive signs of pregnancy as early as day 16 and accurately (100%) diagnose gestation by day 20 (Romano and Christians, 2008). Few investigations into the efficacy of TRUS for identifying very early pregnancy in sheep exist. Those that do have non-uniform conclusions, likely a consequence of deviation in transducer frequency, operator experience, pregnancy stage and technique (González de Bulnes et al., 1998, Karen et al., 2004). Despite this, studies have established that with time and stronger transducer frequency (5-7.5mHz), accuracy increases (Dinc et al., 2001, Garcia et al., 1993, Romano and Christians, 2008). A logical progression of an even higher frequency transducer (10MHz) increasing the likelihood of accurate pregnancy diagnosis prior to day 20, is feasible. Furthermore, since individual conceptuses have been observed at day 19 via TRUS (Garcia et al., 1993, González de Bulnes et al., 1998), multiple embryo identification could be achieved. If an accurate TRUS early pregnancy diagnosis protocol could be established, the exploration of possible preventative methods for early embryo loss could ensue.

Current preventative studies, including management (Kenyon et al., 2013), nutrition (Robinson et al., 2002), and hormone supplementation (Cam et al., 2002, Kleemann et al., 1994) have returned contradictory results. Amongst these hormonal additives, exogenous progesterone supplementation has been explored, owing to its pivotal role in establishing and maintaining pregnancy (Ashworth et al., 1989, Cam et al., 2002), yielding inconclusive results (Ashworth et al., 1989, Diskin and Niswender, 1989, Kleemann et al., 1991, Kleemann et al., 1994). At present, sources of exogenous progesterone are readily available and utilised extensively within industry, in the form of pessaries for assisted reproduction programs (Bartlewski et al., 2015). Should P_4 supplementation prove capable of preventing embryo loss, this would be an affordable and simple solution to a national source of reproductive wastage within the industry. As such, the present study was conducted to establish the earliest stage of pregnancy that

transrectal ultrasound can accurately detect pregnancy in ewes with a 10 MHz transducer. Simultaneously, the study aimed to observe the effect of exogenous progesterone supplementation during the very early stages of pregnancy as a possible mitigation measure for early embryo loss.

Materials and Methods

Procedures conducted during the trial were approved by the University of Sydney Animal Ethics Committee (protocol number 965).

Oestrous Synchronisation and Artificial Insemination

Mature ewes of merino and merino cross breeds (n=62, aged 2-6, body condition score 2-3) were housed at the University of Sydney, Camden campus, NSW, Australia, for the duration of the trial. Ewes remained on a pasture based diet, supplemented with lucerne hay during limited pasture availability. The investigation took place during the 2016 breeding season. Ewes were synchronised for oestrus with intravaginal flugestone sponges (30mg, Ova-Gest®, Vetiquinol, Brisbane, QLD, Australia) for 14 days. At sponge removal, ewes were injected with pregnant mare serum gonadotropin (PMSG; 400iu, Pregnecol, Vetoquinol, France). 60-hours post sponge removal, ewes were artificially inseminated via intrauterine laparoscopy with fresh semen collected via artificial vagina, from one Coopworth ram to prevent any differences in embryo survival to be caused by inter-male variation. The day of insemination was designated day 0 (D0) of pregnancy. At insemination, ewes were randomised into three treatment groups; control (n=21) receiving no exogenous hormone treatment, CIDR @D0 (n=20), at insemination ewes received intravaginal controlled internal drug release device (CIDR; 300mg, Zoetis, Silverwater, NSW, Australia) and CIDR @D3 (n=21), ewe received a CIDR on day 3 post insemination. All CIDRs were removed on day 17 post insemination.

Pregnancy Diagnosis

Transrectal Ultrasound

Each ewe was subjected to transrectal ultrasound on day 10, 12, 14, 17, 19 and 28, between 7:00 and 13:00. Ewes were not withheld from food or water beforehand. Prior to the procedure, an enema was performed to remove faeces from the rectum. The same experienced operator conducted transrectal ultrasound using a Esaote Germany, MyLab™One VET, equipped with electronic linear array 10-5 MHz transducer (SV3513 Vet), modified to retain a rigid position and facilitate internal contact with the reproductive tract. Ewes were restrained in dorsal recumbency and had the lubricated probe, inserted approximately 20cm into the rectum where landmarks including the bladder, ovaries, uterine horns and endometrium were identified. Using these landmarks, the transducer was rotated 180° to identify indicators of pregnancy. Pregnancy was defined as the presence of a trophoblastic expansion on days 12 and 14, an embryonic vesicle on days 17 and 19 and an embryo on day 28. Typical observations for each stage are included in Figure 1. Embryo number was established by identifying completely separate entities within the reproductive tract. Ovulation rate was established on the first and second scanning day (Day 10 and 12) by observing the number of corpus lutea (CL) present on both ovaries (Figure 1). Progressive scans involved the observation of uterine endometrium height, fluid-filled endometrium lumen, trophoblastic expansion, embryonic vesicle, presence of an embryo, embryo length, fetal heartbeat and crown rump length, as embryos developed (Figure 1).

Real-time Transabdominal Ultrasound

On day 54 of pregnancy, pregnancy status and litter size of all ewes was determined via transabdominal ultrasonography. The examination was completed using an Ovi-Scan 6; Axial 3.5MHz transducer (BCF Ultrasound Australasia; Mitcham, Australia) where the probe was placed on the ventral abdominal wall, adjacent to the udder. Accuracy was considered to be

100% on this day and as such was used as the standard to compare the accuracy of TRUS results.

Progesterone Assay

Blood samples (5-10ml) were collected in heparinized vacutainers (Edwards Group Pty. Ltd., Narrellan, Sydney, NSW, Australia) via jugular venipuncture from each ewe on day 19 post-insemination. The samples were immediately centrifuged at 1500g for 15 minutes, then stored at -20°C until needed for analysis. Samples were analysed with ImmunoChem Coated Tube Progesterone 125 RIA Kits [ICN Pharmaceuticals, Inc, Costa Mesa, CA, United States of America; intra-assay CV < 10%, sensitivity; 0.02ng/ml, cross reactivity: progesterone (100%), oestradiol (<0.01%)], as per manufacturer's instructions. Concentrations above 1.0ng/mL were considered pregnant.

Statistical Analysis

The sensitivity of TRUS diagnosis for each scan day was defined as the ability of TRUS to correctly diagnose pregnancy in those ewes detected as pregnant on day 54 (true positives). Sensitivity is indicative of the accuracy of TRUS to correctly diagnose pregnancy. Whilst specificity was defined as the ability of TRUS to correctly detect the non-pregnant ewes on day 54 as non-pregnant on the TRUS scan day (true negatives). An incorrect pregnancy diagnosis was thus defined as an ewe detected as non-pregnant via TRUS, subsequently diagnosed as pregnant by transabdominal ultrasound on day 54 (false negative). The sensitivity and specificity of TRUS were calculated for each TRUS scan day, as follows:

$$Sensitivity\ (\%) = \frac{number\ of\ true\ positives}{number\ of\ true\ positives + false\ negatives}$$

$$Specificity\ (\%) = \frac{number\ of\ true\ negatives}{number\ of\ true\ negative + false\ positives}$$

The sensitivity and specify of using TRUS to detect multiples was calculated in the same way.

Sensitivity of P_4 assay pregnancy diagnosis results were compared to day 54 diagnoses, to determine the sensitivity (defined as the ability of P_4 assay to correctly diagnose pregnancy in ewes that there were subsequently detected as pregnant on day 54), and specificity (the ability of P_4 assay to correctly diagnose non-pregnant ewes, successively diagnosed as pregnant on day 54). The proportion of ewes diagnosed as pregnant on day 19 by both P_4 assay and TRUS was also compared.

All statistical analyses were completed using GENSTAT (Version 18.1, VSN International, Hemel Hempstead, UK). An ordinal regression was used to determine whether there was a significant difference in the number of animals correctly identified as pregnant (as well as multiples) between TRUS days, where scan day was the model to be fitted. Residual maximum likelihood models (REML) were used to analyse the percentage of ewes detected as pregnant on each scanning day (fixed effects; scan day, random effect; ewe tag), cumulative embryo loss per developmental period (fixed effects; developmental period (ovulation; D10, pre-implantation; D12-14, peri-implantation; D17-19, post-implantation; D28; established pregnancy; D54), random effect; ewe tag), treatment effect on embryo loss (fixed effects: treatment (CIDR @D0, CIDR @D3, control), random effects; ewe tag), and size of structures over time (fixed effects; structure size, random effects; ewe tag). For all analyses, a result of $P<0.05$ was considered significant.

Results

Detection of Pregnancy and Accuracy of Diagnosis

Pregnancy Detection

The percentage of ewes detected as pregnant was similar (P>0.05; Figure 2) on days 12 (38/54; 70.4%), 14 (34/54; 63.0%), 17 (30/54; 55.6%), 19 (34/54; 63.0%), 28 (36;54; 66.7%) and 54 (37/54; 68.5%) of gestation.

Accuracy of Detecting Pregnancy

The percentage of ewes correctly diagnosed as pregnant on days 12, 14, 17, 19, 28 and 54 was 62.2%, 50.0%, 64.9%, 86.5%, 100% and 100%, respectively. Ewes pregnant on day 12 was similar (P>0.05) to days 14 and 17, though differed significantly (P<0.001) from the remaining days. Ewes pregnant on day 14 varied from day 17 (P<0.05) and the remaining days (P<0.001). Day 17 was significantly different from days 19, 28 and 54 (P=0.001) whilst days, 19, 28 and 54 were all similar (P>0.05) to one and other. The proportion of ewes diagnosed as pregnant overtime can be observed in Figure 3.

The percentage of ewes correctly diagnosed as having multiples on days 12, 14, 17, 19, 28 and 54 was 40.6%, 22.6%, 38.2%, 50%, 97.2% and 100%, respectively. Multiple diagnoses on day 12 was similar to days 14 and 17 (P>0.05), though varied significantly from day 19 (P=0.018) and the remaining days (P<0.001). Day 14 different significantly (P<0.05) from day 17 and days 19, 28 and 54 (P<0.001). Day 17 was similar (P>0.05) to 19, while both days 17 and 19 differed significantly (P<0.001) from days 28 and 54. Day 28 and 54 did not vary significantly (P>0.05) from one and other. The variation in proportion of ewes detected as pregnant each TRUS scan day is highlighted in Figure 4.

There was no difference (P>0.05) between TRUS and P_4 assay pregnancy diagnosis methods on day 19 of gestation, in terms of the percentage of ewes correctly diagnosed as pregnant

(classified as pregnant by day 19 TRUS and P_4 assay and subsequently as pregnant on day 54; Figure 5).

Embryo Loss

The average ovulation rate per ewe was 2.05±0.09 and the average number of embryos lost/ewe was 1.17 ± 0.12 (Figure 6). Fetilisation rate was assumed to be 100%, thus the lack of embryo loss at the ovulation stage of pregnancy (Figure 7). Cumulative embryo loss was significantly different ($P<0.001$) between pre-implantation and post implantation periods. There was no further embryo loss after peri-implantation (60.4%), with percent embryo loss being similar ($P>0.05$) for post implantation (64.8%) and established gestation (60.5%; Figure 7). There was no significant difference ($P>0.05$) between embryo loss of treatments over time, therefore, total embryo loss per treatment was compared. Ewes which were given CIDRs at Day 0 recorded significantly ($P<0.001$) more embryo loss (5/22; 77.2%; Figure 8) than ewes which did not receive any P_4 (16/32; 51.6%) or received P_4 from Day 3 (11/25; 56.0%). There was no difference ($P>0.05$) between the control ewes and those that received P_4 on Day 3 (Figure 8).

Size of Embryo and Structures

Embryonic vesicle size was similar ($P>0.05$; Figure 9. A) between days 12 (7.5±0.12mm) and 14 (8.4±mm), while all other days varied significantly ($P<0.001$) from one and other. Additionally, over time a similar trend was observed for the average embryo size ($P<0.001$; Figure 9. B).

Discussion

To our knowledge, the present study is the first to employ a 10MHz transducer for transrectal ultrasound of ewes in early pregnancy (<day 30 of gestation). Consequently, this method was capable of identifying individual embryos as early as day 12 of gestation, and diagnose pregnancy relatively accurately, earlier than previously reported (Garcia et al., 1993, Gearhart et al., 1988, Romano and Christians, 2008, Schrick and Inskeep, 1993). Additionally, TRUS confirmed the majority of embryo loss occurred prior to implantation, as concluded by others (Quinlivan et al., 1966, Wilmut et al., 1986). Furthermore, the present investigation demonstrated that progesterone failed to mitigate early loss, rather heightened loss when provided from the first day of gestation. Such a result could be attributed to an asynchronous relationship between the uterine environment and embryo, possibly due to hormonal imbalances hampering embryonic implantation and development (Ashworth et al., 1989).

Since the introduction of transrectal ultrasound as a method of detecting early pregnancy, the accuracy of its detection has proved pivotal to its viability as a pregnancy diagnostic and exploratory tool. Here, TRUS was capable of detecting pregnancy (and multiples) from day 12, with the accuracy of diagnosis on this day (pregnant; 62.2%, multiples; 40.6%) equivalent ($P>0.05$) to days 14 (pregnant; 50.0%, multiples; 22.6%) and 17 (pregnant; 64.9%, multiples; 38.3%), meaning similar results can be achieved on these days. Accuracy was maximised from day 19 (pregnant; 86.5%, multiples; 50.0%) and onwards. The current study identified pregnancy earlier than the previously reported days 15-20 of gestation (Gearhart et al., 1988, Romano and Christians, 2008, Schrick and Inskeep, 1993). The reported days from which TRUS accuracy of diagnosis was maximised, ranged from 20 (Romano and Christians, 2008) to 25 and beyond (Gearhart et al., 1988, Schrick and Inskeep, 1993). Transducer strength could account for differences in pregnancy detection and accuracy of diagnosis as a 7.5MHz

transducer was utilised by Romano and Christians (2008) and Schrick and Inskeep (1993) while Gearhart et al. (1988) utilised a 5MHz probe, unlike the present study that employed a 10MHz transducer. Furthermore, differences between the day of TRUS scan, variation in technique, pregnancy definition and operator experience, could contribute to the varied results (González de Bulnes et al., 1998). A noteworthy contribution to TRUS inaccuracy for all investigations is the loss of embryos prior to the standard against which results are compared (day 54 here). A false positive result (pregnant on TRUS scan day but subsequently non-pregnant on day 54/standard day), on any day could be attributed to embryo loss rather than an inaccuracy in detection. This phenomenon could explain the slight reduction in accuracy (increase in false negative results) for day 14 of gestation compared to days 12 and 17 (Figure 3; Figure 4). Furthermore, around the 14th day scan, those ewes that had failed to conceive or that may have experienced embryo loss, undergo corpus luteum regression (luteolysis), in line with the oestrous cycle (Roberts, 2007, Silvia et al., 1984). The changes in the uterine environment around this time could impede pregnancy detection, further compromising accuracy of pregnancy diagnosis. Despite compromising TRUS accuracy, identification of ewes that have lost embryos earlier in pregnancy can allow for prompt management such as culling or re-joining, rather than waiting for the traditional 6-8 week transabdominal ultrasound. This benefit of TRUS is further exacerbated by similar ($P>0.05$) percentages of ewes detected as pregnant across days 12 to 54 (Figure 2). TRUS can still identify the same percentage of ewes as pregnant compared to a day 54 transabdominal scan, yet is also capable of identifying multiple embryos, facilitating a more thorough exploration of embryo loss. A comparison between TRUS and blood hormone assay on day 19 of pregnancy was also possible in the current investigation, finding no difference ($P>0.05$) in the accuracy of detection of the two methods (Figure 5). It is important to note that exogenous progesterone supplementation did not interfere with P_4 assay results as CIDRs were removed on day 17 and blood collected on day 19, allowing sufficient time for excess progesterone to be metabolically degraded (Bedford et al.,

296 1972). TRUS provides a diagnosis in real time and an indication of embryo number in early
297 pregnancy. On the other hand, P_4 assay requires blood sample collection and analysis, incurring
298 a lag period between sample and diagnosis, consequently generating time and monetary costs,
299 while also failing to identify embryo number. These factors render TRUS as a preferable option
300 compared to P_4 assay, particularly for large-scale sheep producers seeking early pregnancy
301 diagnosis. Additionally, TRUS can provide more opportunities for sheep producers to better
302 match ewe needs, particularly multiple-bearing ewes, with resources, such as feed, from an
303 earlier point of gestation. In the current study, the use of TRUS with a 10mHz transducer
304 enabled an earlier detection of pregnancy. Furthermore, it facilitated the confirmation of loss
305 occurring prior to implantation (days 14-19 of gestation), in sheep.
306
307 Early embryo losses represent considerable reproductive potential, warranting further research
308 attention to decipher the underlying causes and potential preventative measures. In the present
309 study, TRUS detected no further embryo loss post-implantation (Figure 7), indicating that the
310 critical period for embryo loss and resultant study is before implantation. This conclusion agrees
311 with a study in Romney Marsh ewes, that experienced the majority of losses prior to day 30 of
312 gestation, with most losses (~50%) concentrated to the period immediately preceding day 18
313 (Quinlivan et al., 1966). Incidentally, implantation in sheep occurs between days 14 and 18
314 (Guillomot et al., 1981), coinciding with this period of high embryo loss. This involves
315 implantation or the adhesion of the conceptus to the uterine endometrium (Spencer et al.,
316 2004). Concurrently, the maternal recognition of pregnancy occurs, where Interferon-τ (oIFN-τ),
317 secreted from the conceptus, suppresses the secretion of the luteolytic factor, prostaglandin-
318 F2α (PGF2α; (Bindon, 1971, Roberts, 2007) , conserving corpus luteum function that is
319 essential for pregnancy establishment and maintenance (Ott et al., 1993, Roberts, 2007).
320 Various factors have been found to influence both implantation and the maternal recognition of

pregnancy compromising embryo survival, namely maternal nutrition (Abecia et al., 1997, Parr et al., 1982, Viñoles et al., 2012), age (Mulvaney, 2011, Shorten et al., 2013), management (Dutt, 1963), genetics (Bodin et al., 1992) and inadequate or imbalanced progesterone concentrations (Ashworth et al., 1989, Diskin and Niswender, 1989, Wilmut et al., 1986). One of the most prominent factors is maternal nutrition. Undernourished ewes fed diets below maintenance requirements, prior to and following mating, have been shown to display an increased ova wastage rate compared to their adequately fed counterparts (Abecia et al., 2015, Edey, 1966, Rhind et al., 1989). Furthermore, malnutrition has been linked to reduced embryonic secretion of oIFN-τ in-vitro, suggesting luteolysis is induced due to elevated endometrial secretions of PGF2α (Abecia et al., 1999). Moreover, ewes fed above-maintenance diets (2x maintenance ration) post-insemination, displayed lower pregnancy rates (48%) compared to undernourished ewes (67%; 0.25 x maintenance ration)(Parr et al., 1987). Such a finding has been associated with an increased metabolic clearance rate of progesterone with elevated food intake, since following feeding, blood-flow is directed to the splanchnic region, that is largely responsible for progesterone metabolism (Bedford et al., 1972, Parr, 1992). These conclusions emphasise the pivotal role nutrition plays during peri-implantation. Another highly influential factor on embryo survival peri-implantation is progesterone concentration (Diskin and Niswender, 1989). Progesterone is crucial for early embryo development and establishing pregnancy (Ashworth et al., 1989). It has been previously suggested that inadequate progesterone in sheep could initiate luteolysis or interrupt the relationship between embryo and uterus, thus initiating embryo loss (Ashworth et al., 1987). Such conclusions of elevated embryo mortality due to hormonal imbalances around insemination have also been drawn in cattle (Larson, 2009, Lonergan et al., 2007, Morris and Diskin, 2008), pigs (Almeida et al., 2000, Bouwman et al., 2012) and horses (Wilsher et al., 2012). Ashworth et al. (1989) concluded that in sheep the higher the P_4 concentration on days 0-1 of pregnancy, the higher embryo survival.

As such, it was postulated that progesterone supplementation around the time of mating could potentially boost embryo survival rates (Ashworth et al., 1989). It is the complexity of interactions between the numerous factors such as nutrition, progesterone, management, maternal age and genetics, known to influence embryo survival around the time of implantation, that warrants further investigation into the effect of P_4 supplementation during early pregnancy and how it could influence embryo loss or possible survival.

Despite considerable studies, exogenous progesterone supplementation as a means for preventing embryo loss is still inconclusive. In the present study, provision of exogenous P_4 from the time of insemination proved counterproductive to embryo survival, indeed it increased loss (Figure 8). Additionally, in the current study ewes supplemented with P_4 from day 3 of pregnancy showed no improvement in embryo survival rates compared to the control ($P>0.05$; Figure 8). Earlier findings of Kleemann et al. (1994), concluded that ewes provided with P_4 (CIDRs, 300mg progesterone) from day 1 of gestation, for either 3 or 6 days, had lower ($P<0.05$) pregnancy rates (1.65 and 1.72, fetuses per pregnancy, respectively), compared to progesterone provided from days 3-6 (2.00 fetuses per pregnancy) and a control (1.98 fetuses per pregnancy). These findings are parallel to the current findings in terms of P_4 failing to increase embryo survival from insemination, yet are contradictory with respect to the ability of P_4 to prevent embryo mortality when provided from day 3 of gestation. Such a difference could be attributed to variation in length of P_4 supplementation and time of CIDR insertion between the studies. Another earlier investigation found that P_4 failed to increase the portion of ewes that lambed or gave birth to multiples when provided 3 days post-mating (Kenyon et al., 2005), complementing the findings reported here. Interestingly, in other studies P_4 supplementation has been found capable of increasing litter size (Kleemann et al., 1991) and promoting fetal growth of surviving singletons and multiples (Kleemann et al., 1994). The current study did not explore different P_4 supplementation regimes, such as providing P_4 later in pregnancy, though the

372 conclusion of embryo loss occurring prior to implantation questions the efficacy of this method,
373 since majority of losses would have already occurred. The contradictory findings on the success
374 of P_4 supplementation as an embryo loss prevention method could be explained due to
375 differences in the concentration and length of P_4 supplementation, the number of ewes in study,
376 alongside ewe nutritional status and the interaction between such factors. Despite this, it has
377 been suggested that reduced embryo survival rates under P_4 supplementation post-mating
378 could be attributed to asynchrony between the uterine environment and embryo (Ashworth et
379 al., 1989). Following ovulation, the ewe's progesterone profile follows three distinct phases that
380 are essential for embryo establishment (Ashworth et al., 1989, Wilmut et al., 1985). Initial P_4
381 concentrations remain low, before days 3 to 7 where maternal serum levels increase to 3-
382 6ng/ml (Ashworth et al., 1989), and then remain at a concentration of approximately 5ng/ml, that
383 is typical of the luteal phase and crucial for pregnancy retention (Ashworth et al., 1989, Manalu
384 and Sumaryadi, 1998). Therefore, irregularly high serum progesterone concentrations due to P_4
385 supplementation during the preliminary days of pregnancy could account for elevated embryo
386 loss. Such a conclusion challenges the value of exogenous P_4 supplementation as a
387 preventative means for early embryo loss. Furthermore, high progesterone concentrations are
388 known to cause the downregulation of progesterone receptors (Spencer et al., 1995, Spencer
389 and Bazer, 1995). It has been postulated that such an event can result in a downregulation of
390 endogenous P_4 production in cattle (Mann and Lamming, 2001). If this were also the case for
391 sheep, an appropriate concentration of exogenous P_4 would be fundamental for improving
392 embryo survival, suggesting the amount provided in this investigation may have been outside
393 this critical threshold. The results of the current study showed that P_4 had no effect on mitigating
394 embryo loss and if given immediately around time of fertilisation, it can even increase loss.
395 While future work on the effect of P_4 could be helpful in further confirming our results, it could be
396 worth focusing on other strategies, such as administration of GnRH from day 12 of pregnancy

(Cam et al., 2002) or maternal diet composition, in conjunction with TRUS to try and improve early embryo survival in sheep.

The advent of specialised and more powerful transducers facilitates the observation and differentiation of different structures and characteristics of individual embryos, not previously seen. As observed here, overtime the size of the embryonic vesicle and embryo itself could be measured and as expected there was a significant increase in size over time ($P<0.001$; Figure 9). The mean embryonic vesicle size (mm) was 7.92±0.13, 8.10±0.24, 2.80±0.51 and 14.1±0.68 for days 12, 14, 17 and 19, respectively. In comparison, an early investigation measuring embryos of Colombia x Lincoln ewes mated with Suffolk rams, found the embryonic disc to measure 1.8, 4.5 and 6.0mm for days 14, 17 and 19, respectively (Bryden et al., 1972). Furthermore, the mean size (mm) of the embryo on days 17, 19 and 28 in the present study, was found to be 1.47±0.38, 4.10±0.14 and 14.18±0.02, respectively. Whilst, Bryden et al. (1972) estimated the embryo to measure approximately 4, 7 and 17mm on the same days. Differences between measurements could be attributed to sheep breed, definitions of structures being measured and ultrasound technology versus physical measurement, particularly since the orientation of the conceptus in relation to the transducer determines what angle the measurement is taken from.

Conclusion

The present study identified pregnancy and diagnosed multiple pregnancies via transrectal ultrasound earlier and with a higher degree of accuracy than previously reported. Embryo loss was confirmed to occur predominately prior to implantation, as such future investigations into prevention can focus on a smaller window during gestation. Additionally, progesterone

supplementation as a preventative measure for embryo loss has proved to have little effect during early pregnancy, yet is likely to have a more pronounced impact in later gestation for surviving embryos and their subsequent development. In conclusion, this study has developed an accurate, safe and welfare friendly transrectal ultrasound regime for continued research and exploration of embryo development in an effort to improve early embryo survival and ultimately improve the reproductive efficiency for the industry.

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Figures and Tables

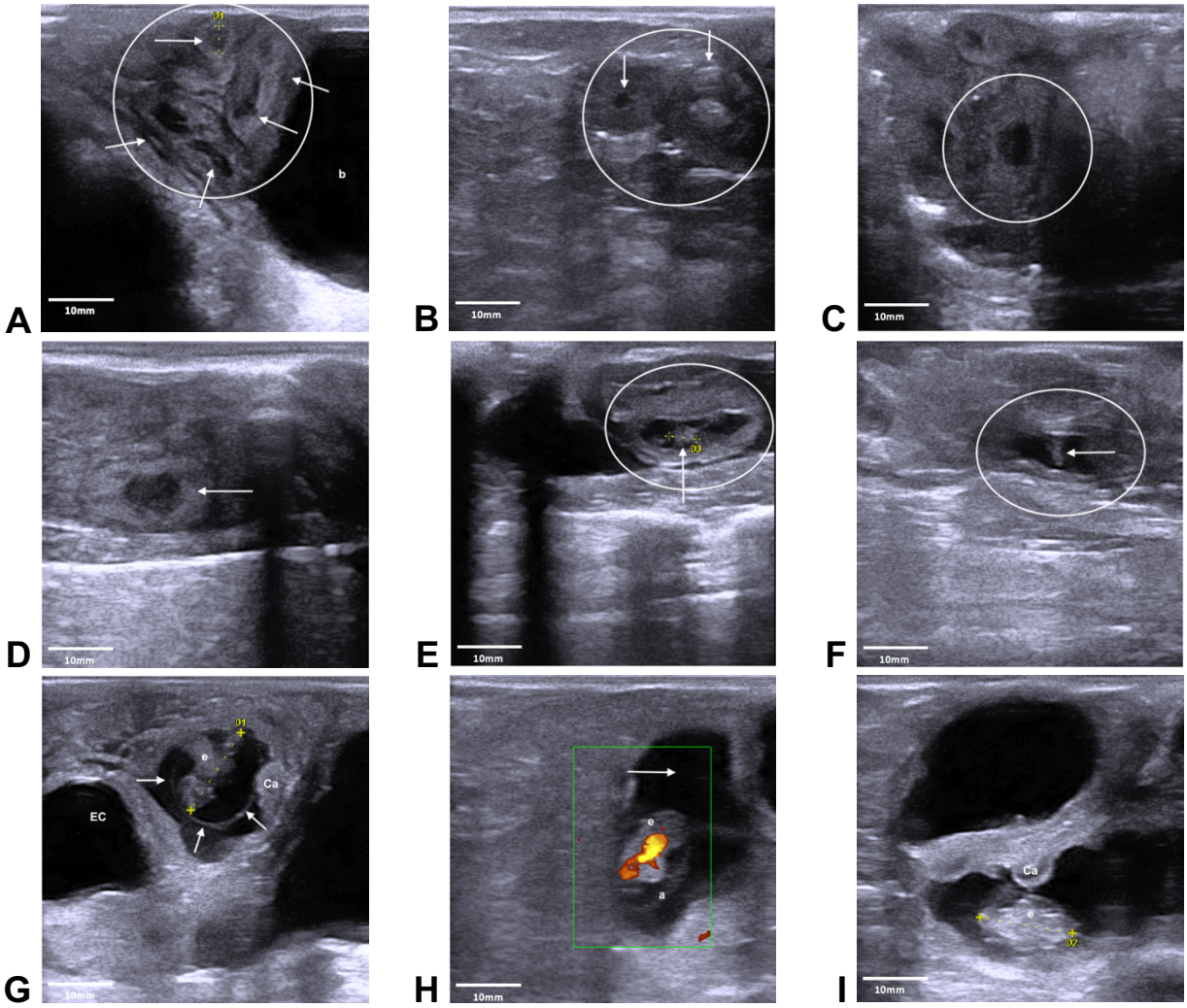


Figure 1. Transrectal ultrasound (Esaote Germany, MyLab™One VET, equipped with electronic linear array 10-5 MHz transducer (SV3513 Vet)), images of the ovary, uterus and conceptus during early pregnancy in sheep (oestrus = day 0). **A** Day 10: The coiled uterus (circle) is located cranial to the bladder (b). Within the coiled uterine horns, the lower echogenic endometrium (arrows) is well distinguished. **B** Day 10: Two corpora lutea (arrows) are present on the ovary (circle), documenting recent ovulation. **C** Day 12: Trophoblastic expansion (within the circle): At the site of trophoblastic expansion the endometrium appeared enlarged. A dark oval shaped structure expanded within the uterine lumen. Boundaries between endometrium and trophoblast could not be distinguished at this stage. Yet the trophoblast expanded the uterine lumen exceeding the regular endometrial height by 2-fold. **D** Day 14:

Trophoblastic expansion (arrow): Low echogenic oval-shaped trophoblast within the uterine lumen, further expands in length but not in height. **E** Day 17: Embryonic vesicle (circle) with 4 mm embryo (arrow) within. **F** Day 19: 4 mm embryo (arrow), distinctly present inside the embryonic vesicle (circle). **G** Day 28: Embryo (e) surrounded by the amniotic membrane (arrows). A caruncle is in close proximity of the embryo (Ca). Crown rump length of the embryo measures 1.4 cm. Fluids of the embryonic cavity (EC) expand further into the adjacent uterine horn. Caudal to the bladder that is bordering the pregnant uterus. **H** Day 28: Power Doppler of the embryo heart. Embryo (e) is surrounded by the amnion (a) and the fluid filled embryonic cavity (arrow). **I** Day 28: 1.4 cm embryo (e), situated below a placental caruncle (Ca). Above this the fluid filled embryonic cavity expands into the uterine horn.

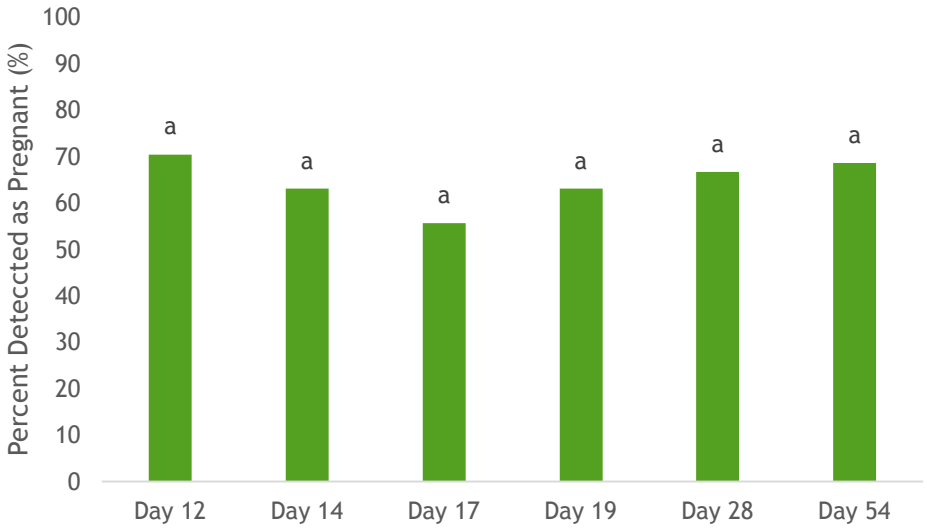
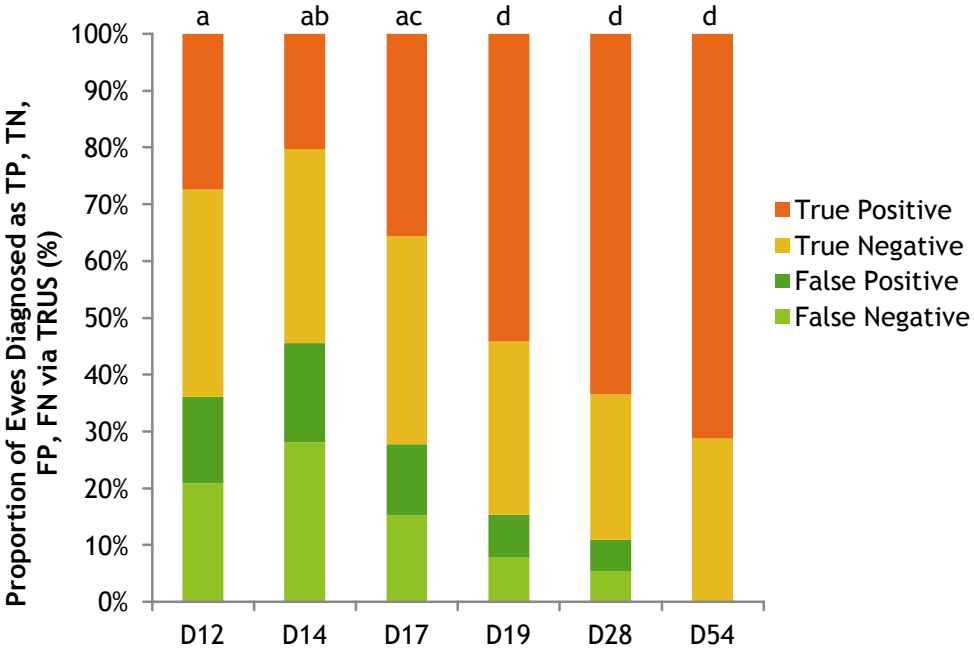


Figure 2. Percentage of ewes detected as pregnant via transrectal ultrasound (P<0.05) on days 12, 14, 17, 19, 28 and 54 of pregnancy. Columns with common superscripts are similar (P=0.667).



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646 **Figure 3**. Proportion (%) of ewes detected via transrectal ultrasound (TRUS) as either a true positive (TP;
647 ewe classified as pregnant on TRUS scan day and day 54), true negative (TN; ewe classified as non-
648 pregnant on TRUS scan day and day 54), false positive (FP; ewe defined as pregnant via TRUS and non-
649 pregnant on day 54), false negative (FN; ewe classified as non-pregnant on TRUS scan day and
650 pregnant day 54). Note day 54, is the standard criteria against which TRUS diagnoses were compared.
651 Columns without common superscripts differ significant ($P<0.05$).

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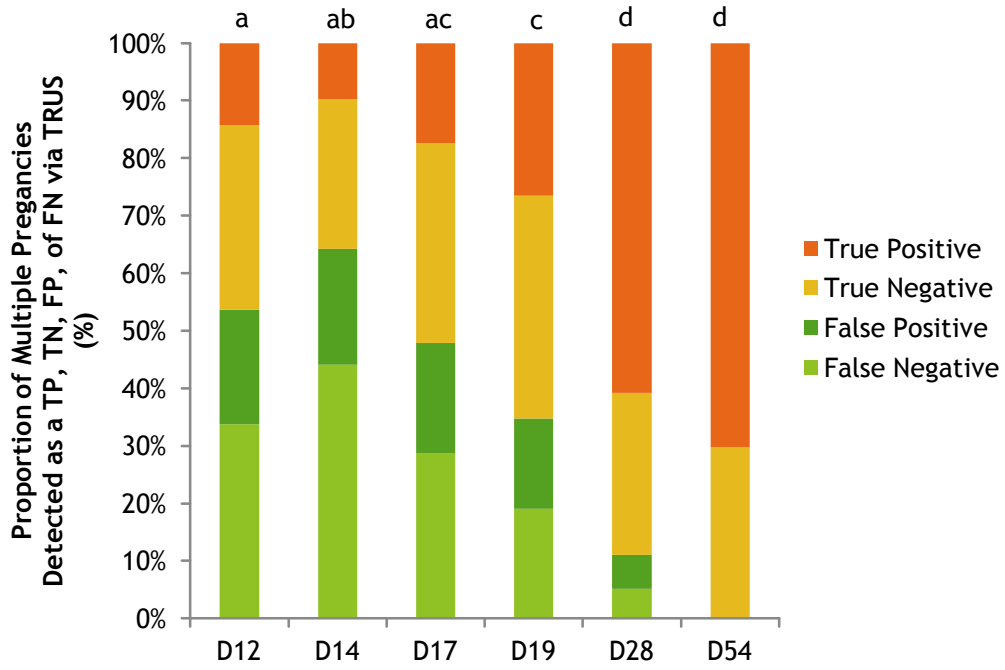

653

654 **Figure 4**. Proportion (%) of multiple pregnancies in ewes detected via transrectal ultrasound (TRUS) as a
655 true positive (TP; ewe classified as having multiples on TRUS scan day and day 54), true negative (TN;
656 ewe classified as not having multiples on TRUS scan day and day 54), false positive (FP; ewe defined as
657 having multiples on TRUS scan day and not on day 54), false negative (FN; ewe classified as not having
658 multiples on TRUS scan day and having multiples on day 54). Note day 54 is the standard criteria against
659 which TRUS diagnoses were compared. Columns without common subscripts differ ($P<0.05$).

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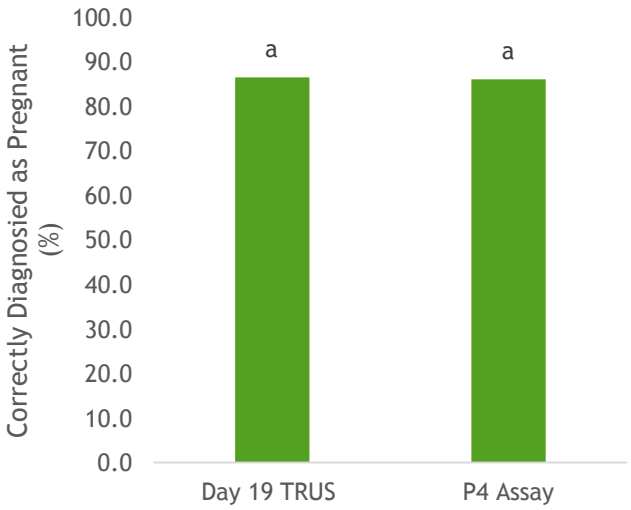


Figure 5. Percentage of ewes correctly diagnosed as pregnant (classified as pregnant on day 19 and subsequently as pregnant on day 54) via trnsrectal ultrasound (TRUS) and progesterone blood hormone assay (P_4 assay) on day 19 of gestation. Standard criteria for pregnant/non-pregnant diagnosis for both TRUS and P_4 Assay was day 54 transabdominal ultrasound. Columns without common superscript differ significantly ($P<0.05$).

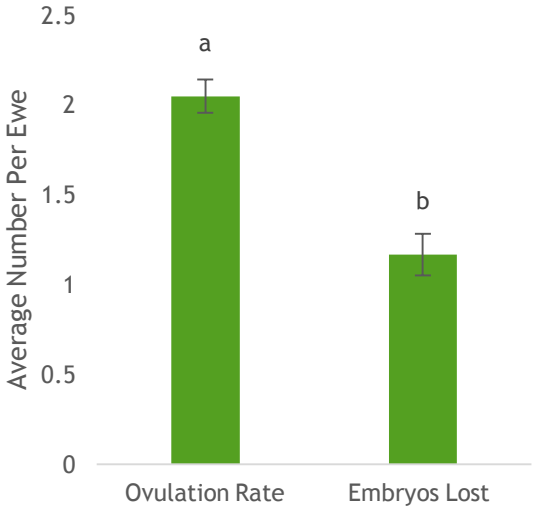
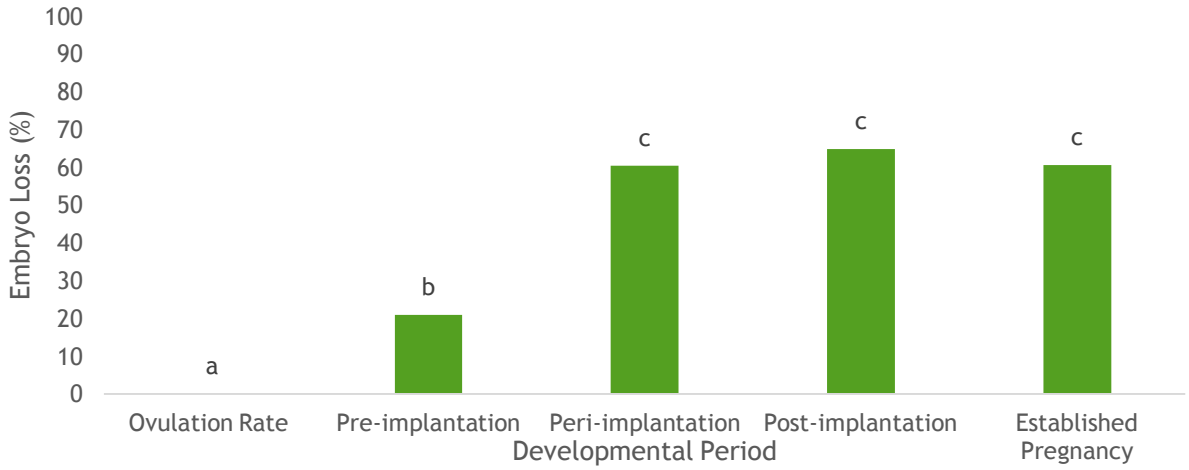


Figure 6. Mean (±SEM) ovulation rate and number of embryos lost per ewe. Ovulation rate was determined via transrectal ultrasound on days 10 and 12 if gestation (oestrus = day 0), and final embryo number determined on day day 54 via transabdominal ultrasound. Columns with dissimilar superscripts are different ($P<0.05$).



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675 **Figure 7**. Cumulative embryo loss (%) for each developmental period, assuming 100% fertilisation,

676 therefore no loss is assumed at ovulation. Columns without common letters differ significantly (P<0.001).

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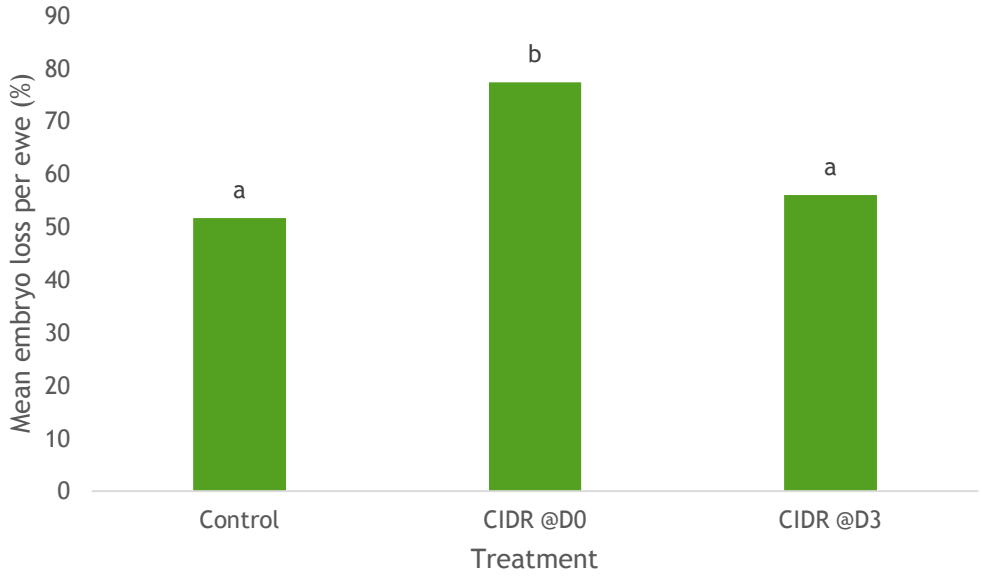

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679 **Figure 8**. Mean embryo loss (%) per ewe per treatment (CIDR @D0, CIDR @D3, control) during

680 gestation, assuming 0% loss at ovulation and 100% fertilisation. Columns without common letters differ

681 significantly (P<0.001).

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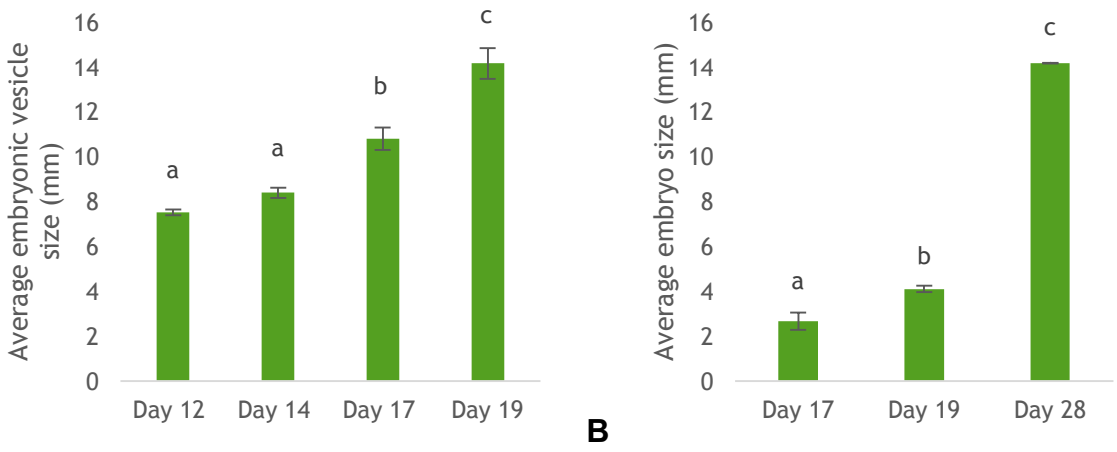

Figure 9. Mean (±SEM) size of structures as gestation progresses. **A** Mean (±SEM) embryonic vesicle size (mm), measured when possible during transrectal ultrasound procedure. **B** Mean (±SEM) embryo size (mm), measured when possible during transrectal ultrasound procedure. Columns without common superscripts differ significantly (P<0.001).

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- Use double line spacing throughout (including reference list and figure legends).
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Protein: SOX2

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Gene symbols should be in italics with all letters capitalised; protein designations should be the same as the gene symbols but not italicised. For example:

Gene/RNA/DNA: *SOX2*

Protein: SOX2

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Protein: Sox2

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- Justify the omission of any observations from the analysis.

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- Your article may be sent to the Statistical Advisor for comments.

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