Received: 4 December 2019 | Accepted: 15 August 2020

DOI: 10.1111/evj.13340

GENERAL ARTICLE

Equine Veterinary Journal WILEY

Fetal morphological features and abnormalities associated with equine early pregnancy loss

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Funding information
The current study was funded by the Thoroughbred Breeders Association (VET/RS/250 and TBA/EPL/2018) with support by the Horserace Betting Levy Board.

Abstract

Background: Early pregnancy loss (EPL) occurs in approximately 8% of equine pregnancies, although the aetiology is mostly unknown and embryonic/fetal morphological abnormalities associated with EPL are not defined.

Objectives: To compare the morphology of EPL to clinically normal embryos/fetuses and previously described embryonic/fetal developmental milestones. To identify morphological abnormalities associated with equine EPL.

Study design: Observational case-control study.

Methods: Embryos/fetuses were obtained from clinically normal Thoroughbred and pony pregnancies (n = 11) and following EPL from Thoroughbred mares (n = 27). The crown-rump length (CRL) of embryos/fetuses was measured and macroscopic morphology and developmental age were determined independently by three blinded examiners. Sagittal sections of EPL (n = 13) and control (n = 6) embryos/fetuses were assessed microscopically. Fisher's exact test was used to determine significance ($P < .05$) and correlations were expressed by Pearson coefficient.

Results: Age and CRL were strongly positively correlated in clinically normal Thoroughbred and reference (n = 15, $R = .9$ (95% CI: 0.8-1.0), $R^2 = .9$, $P < .0001$) but not EPL embryos/fetuses (n = 19, $R = .1$ (95% CI: −0.4 to 0.5), $R^2 = .01$, $P = .75$). Relative to controls, the CRL of EPL embryos/fetuses was smaller, with evidence of intrauterine growth retardation (IUGR) in 3/8 fetuses assessed. In 9/13 EPL embryos/fetuses, nonspecific neural tissue alterations were identified including disruption of developing pros-, mes- and rhombencephalon and the presence of haemosiderin, indicating premortem haemorrhage. Failed neural tube closure was identified in 1/13 EPL embryos/fetuses. Subcutaneous haemorrhage was present in 14/27 EPL embryos/fetuses.

Main limitations: Autolysis significantly affected 15/27 EPL embryos/fetuses, excluding them from complete assessment. The IUGR reference cut-off values were based on a small number of controls.

Conclusions: Morphological features associated with equine EPL were a mismatch between embryonic/fetal size and age, and alterations of the developing neural tissue and localised subcutaneous haemorrhage. Failed neural tube closure was confirmed as a rare specific abnormality.

KEYWORDS
horse, equine reproduction, pregnancy outcome, fetus, developmental abnormalities

1 | INTRODUCTION

Pregnancy loss is of significant importance in equine reproductive medicine and is associated with animal welfare[1] and economic implications.[2] In mares, the majority of pregnancy failure occurs between the initial diagnosis and day 65 of gestation and is commonly referred to as 'early pregnancy loss' (EPL). In the United Kingdom, EPL was recently described to have an incidence of 6.4% between days 14 and 42 and of 1.6% between days 43 and 65 of pregnancy,[3] comparable to observations in distinct broodmare populations worldwide over the last 20 years.[4-8] Multivariable and epidemiological studies have identified several risk factors for EPL,[4-6,9] although the aetiology remains unknown in >80% of mares suffering this condition.[10,11]

Anatomical and morphological abnormalities of the fetus are recognised in miscarriages in women. Poland et al[12] describe abnormalities of the external morphology in more than half of spontaneously aborted human conceptuses. They define both specific and general defects, the latter categorised as 'growth disorganisation' and 'developmental inconsistency'. Specific abnormalities have been reported in 16.4% human fetuses,[13] with the central nervous (CNS), cardiovascular (CVS) and urinary system presenting as the most commonly affected anatomical structures.[12,14]

Limited macroscopic and histological descriptions exist for normal equine fetuses of different gestational ages,[15-17] although no data are available on the morphological features of fetuses obtained from mares suffering EPL. Further, whether developmental abnormalities, described in early human fetuses, are present in the horse is unknown. A unique placentation and prolonged establishment of the microvillous feto-maternal attachment[18,19] suggest that aetiologies of early pregnancy failure in mares may be specific and cannot be assumed to mirror observations in women. Thus, the aim of the current study is to describe the morphology of equine embryos/fetuses obtained from mares suffering EPL (EPL embryos/fetuses). We hypothesised that morphological abnormalities more commonly present in embryos/fetuses from EPL but not embryos/fetuses from clinically normal and terminated pregnancies (clinically normal embryos/fetuses). The objectives were (a) to compare the macroscopic and histological morphology of EPL to clinically normal embryos/fetuses and developmental milestones described in the literature and (b) to identify morphological abnormalities associated with equine EPL.

2 | MATERIALS AND METHODS

2.1 | Clinically normal embryos/fetuses

Clinically normal conceptuses were obtained from Thoroughbred (n = 6) and pony (n = 5) broodmares between days 29 and 40 of gestation. All mares were housed at Royal Veterinary College Biological Services Unit. Mares in oestrus were gynaecologically examined at 24 hours intervals and pregnancies were established by artificial insemination, using a commercial dose of chilled semen of a Thoroughbred/pony stallion of proven fertility. Pregnancies were first diagnosed on day 14 post-ovulation and the development of conceptuses was monitored ultrasonographically twice weekly. Clinically normal pregnancies (those with heartbeat and of expected size and the presence of anatomical features for the gestational age) were isolated using sterile uterine lavage after detaching the allantochorion from the endometrium via mild transrectal manipulation and processed as previously described.[20] Conceptus recoveries required 10-30 minutes.

2.2 | EPL embryos/fetuses

Conceptuses from Thoroughbred broodmares suffering EPL were obtained by the attending veterinary surgeon, shipped to the laboratory and dissected as previously described.[20] Age inappropriate size and/or appearance of a conceptus, collapsing embryonic/fetal membranes, absence of embryonic/fetal cardiac activity and failure to advance between repeated examinations were interpreted as ultrasonographic signs of nonviability of a pregnancy. Participating mares were owned by commercial stud farms, who consented for further analyses of the pregnancy. Conceptuses obtained from mares suffering EPL and submitted from 2013 to mid-May 2019 were eligible for the current study by the inclusion criteria of a gestational age of days 22 to 46 and the presence of a mostly intact embryo/fetus prior to fixation (27/89 submissions, Table S1). The definition of a 'fetus' was applied to specimens with a gestational age of 35 days and beyond, based on the observation, that by this time the embryo has completed essential organogenesis and has prominent fore- and hindlimb buds. Those specimens with a gestational age of 34 days and younger are referred to as an embryo.[21]

2.3 | Macroscopic morphology

2.3.1 | Clinical gestational age

For clinically normal embryos/fetuses, the gestational age was defined as the day post-ovulation when the pregnancy was terminated. Gestational ages of EPL embryos/fetuses were deemed as the day post-ovulation when a pathology of the developing conceptus was noted by ultrasonography (n = 26) or for specimens, where this information was not available, as the day of the uterine lavage (n = 1). According to general stud medicine protocols, participating mares were examined gynaecologically at 24-48 hours intervals during oestrus. Therefore, for those experimental outcomes where a more accurate gestational age was required, subpopulations of embryos/fetuses with consistent clinical and morphological developmental ages (defined by the presence of external morphological features, see below) were included in the analysis and then provided in the results.

2.3.2 | Macroscopic imaging

All embryos/fetuses were fixed and subsequently stored in 70% ethanol (4°C). For morphological imaging, a stereoscopic microscope (SMZ-1500, Nikon) and camera attachment (LH-M100C-1, Nikon) were used and contrast and illumination were provided by an additional gooseneck light source. All embryos/fetuses were photographed partly at X 0.75 magnification and images were assembled to complete left and right lateral views using Photoshop (Photoshop PS, Adobe Inc.). Additional photographs of single anatomic structures or abnormalities of each sample were taken at a higher magnification.

2.3.3 | Embryonic/fetal size

Measurement of the crown-rump length (CRL) was performed on left lateral photographs of all Thoroughbred entire and intact embryos/fetuses (using ImageJ (Fiji software, National Institutes of Health, Laboratory for Optical and Computational Instrumentation, University of Wisconsin)). Pony embryos/fetuses were excluded due to the unknown impact of breed on early fetal growth. Seven EPL embryos/fetuses and one control embryo/fetus were excluded from measurements due to damage sustained in collection and/or processing. The CRL was defined as the greatest length of a c-shaped embryo/fetus, encompassing its cranial ('crown', ie embryonic/fetal head or cervical curvature) to caudal curvature ('rump'). Results published by Jenner et al[17] served as reference. The mean mare age for the control and EPL embryos/fetuses was 11 (range 2-20) and 10.9 (range 3-19) years respectively. Intrauterine growth retardation (IUGR) was defined as previously described[22] using the 10th percentile of the CRL of all reference and clinically normal embryos/fetuses available for two developmental age categories: (a) >28/30 to <35 days and (b) >37/40 days (Table S2). The remaining developmental age categories were represented by a small number of reference and clinically normal embryos/fetuses and not analysed.

2.3.4 | Macroscopic assessment

The macroscopic morphology of clinically normal and EPL embryos/fetuses was assessed independently by two of three different and blinded examiners, using left and right lateral photographs at ×0.75 magnification of each sample. A standardised protocol, including the assessment of 21 individual parameters assigned to seven categories (outer appearance, surface ectoderm, appendices, musculoskeletal, sensory, digestive and CNS), allowed for a conclusion on the presence of autolysis, the developmental age and a morphological diagnosis. All embryos/fetuses were assessed by at least two examiners and their agreement served for verification of the results, and was 84%, 92% and 96% for the presence of autolysis, developmental age and morphological diagnosis respectively.

2.3.5 | Presence of autolysis

Macroscopic autolysis was defined by a board-certified pathologist as an impairment or the loss of integrity of the embryonic/fetal surface tissue and diffuse discolouration of an embryo/fetus consistent with post-mortem autolysis previously described.[23]

2.3.6 | Developmental age

The presence of external anatomical features of embryos/fetuses was used to assign embryos/fetuses to one of five developmental age categories (Table S2). Based on previous studies, these features included morphological changes of the body shape, limbs and pontine flexure, pigmentation of the retina primordium and translucency of the skin.[15,16]

2.3.7 | Morphological diagnosis

A morphological diagnosis was achieved by each examiner based on the observations on the standardised assessment parameters, compared with the morphology of healthy equine embryos/fetuses described in the literature.[15,16]

2.4 | Histological morphology

2.4.1 | Sample processing

The inclusion criterion for further histologic assessment of whole embryos/fetuses was an agreed macroscopic diagnosis (EPL n = 13; control n = 6). Whole embryos/fetuses were dehydrated and paraffin embedded using a Vacuum Infiltration Processor (Tissue-Tek VIP5, Sakura

Finetek UK Ltd). Sagittal serial sections (6 μm) of the entire embryo/fetus were created (HM 325 Rotary Microtome, Leica Biosystems) and subsequently transferred onto slides (Superfrost Plus Micro Slides, VWR International). The entire embryo/fetus was sectioned and a representative subset of slides, for example every 20th slide, beginning with slide 10 of each embryo/fetus, was stained with haematoxylin and eosin (H&E, VWR International) using a standard protocol. Additionally, two slides of each sample were assigned for Gram staining (Gram stain, VWR International), following the standard methodology.

2.4.2 | Histologic assessment and imaging

The histological morphology of clinically normal and EPL embryos/fetuses was assessed by one examiner, using all H&E- and Gram-stained slides of an embryo/fetus (median of 7.5 (3-11) slides per embryo/fetus depending on the embryonic/fetal size) and a microscope (Olympus CX31, Olympus) at ×4 to ×100 magnification. A standardised protocol, which assessed the surface ectoderm, musculoskeletal, CNS, sensory, CVS, respiratory, gastrointestinal and urogenital systems as well as the umbilical cord and attached embryonic/fetal membranes, was applied for the H&E-stained tissues. Anatomical formation (present/not present), presence of extracellular oedema (fluid accumulation/empty spaces) and haemorrhage (present/not present), defined as extravascular erythrocytes and/or haemosiderin, were recorded for each tissue. Pronounced cellularity of embryonic/fetal tissues was graded as none, <10 and >10 cells per 40 × field, averaged for five fields. Microscopic autolysis was interpreted as a dissociation of epithelial and endothelial cells, progressive cellular shrinkage and poor staining in the absence of a host response. Gram-stained slides were assessed for the presence (yes/no) and type of bacteria (Gram-positive/negative) and their anatomical location within a sample. A selection of histology slides was additionally imaged at different magnifications using a Leica DM4000B Upright Microscope with camera application.

2.5 | Data analysis

The descriptive data were entered and summarised in Excel (Office 365 Personal, Microsoft). *P* values and agreement between examiners in the macroscopic assessment were determined using Fisher's exact test and the correlation between embryonic/fetal age and size was tested by Pearson correlation in GraphPad (Prism 8, GraphPad Software, Inc.). A $P < .05$ was deemed to be significant.

3 | RESULTS

3.1 | Sample quality and integrity

Macroscopic evidence of autolysis more commonly affected embryos/fetuses from EPL (15/27) compared with those from clinically normal pregnancies (0/11, $P = .0003$, Figure 1A left, middle and right panel). Autolysis was additionally detected by one examiner only, suggestive of mild autolysis, in 5/27 EPL and 1/11 clinically normal specimens. Within the EPL samples, the presence of macroscopic autolysis was associated with examiners unable to assign or agree a morphological diagnosis (11/15, Table S3). In contrast, a diagnosis was assigned and agreed in 10/11 normal embryos/fetuses. Only Thoroughbred embryos/fetuses with an agreed macroscopic diagnosis (n = 13 EPL; n = 6 control) were additionally analysed histologically (Tables S1 and S3). This subpopulation of embryos/fetuses included six EPL and five controls with no gross autolysis, four EPL and one control with mild autolysis and three EPL with autolysis (Table S4). Histological evidence of mild autolysis was found in all EPL (13/13) compared with no clinically normal (0/6) embryos/fetuses ($P < .0001$, Figure 1B, left and right panels).

3.2 | Embryonic/fetal age

The clinical age reported by the veterinary surgeon ranged between 29 and 40 days for clinically normal and 28 and 46 days for EPL pregnancies. As the clinical age of the specimens may be affected by uterine retention, we additionally calculated a developmental age using morphological features, previously described as indicators of embryonic/fetal age.[15,16] An agreed developmental age was reached by both examiners in significantly more embryos/fetuses from clinically normal (11/11) than EPL pregnancies (13/27, $P = .011$, Table S4). A similar proportion of EPL embryos/fetuses were agreed by both examiners to be not ageable (11/27) due to damage or autolysis. A further 3/27 EPL embryos/fetuses had no agreement of developmental age between examiners. Both clinical age and developmental age were available for 11 clinically normal and 12 EPL embryos/fetuses (Table S4). These two ages aligned in 10/11 clinically normal and 10/12 EPL samples. One clinically normal embryo had a 1 day age discrepancy consistent with the 24 hours interval between gynaecological examinations. An EPL fetus aged 35-37 days developmentally and 42 days clinically presented with nontranslucent and multi-layered surface ectoderm and a CRL of 9.2 mm, in alignment with the other features contributing to this developmental age category. A second EPL fetus with a mismatch of a developmental age of >28/30 to 34 days and clinical age of 44 days also showed features and a CRL, aligning with the developmental age. Clinically, this conceptus appeared normal on ultrasonography with a heartbeat present on days 28 and 31, but with a clinically reported maternal progesterone level of 0 ng/mL.

3.3 | Uterine retention time post-demise

Although the clinical and developmental age aligned in the majority of the EPL embryos/fetuses assessed (10/12), due to the day range of the developmental age categories it was still possible that

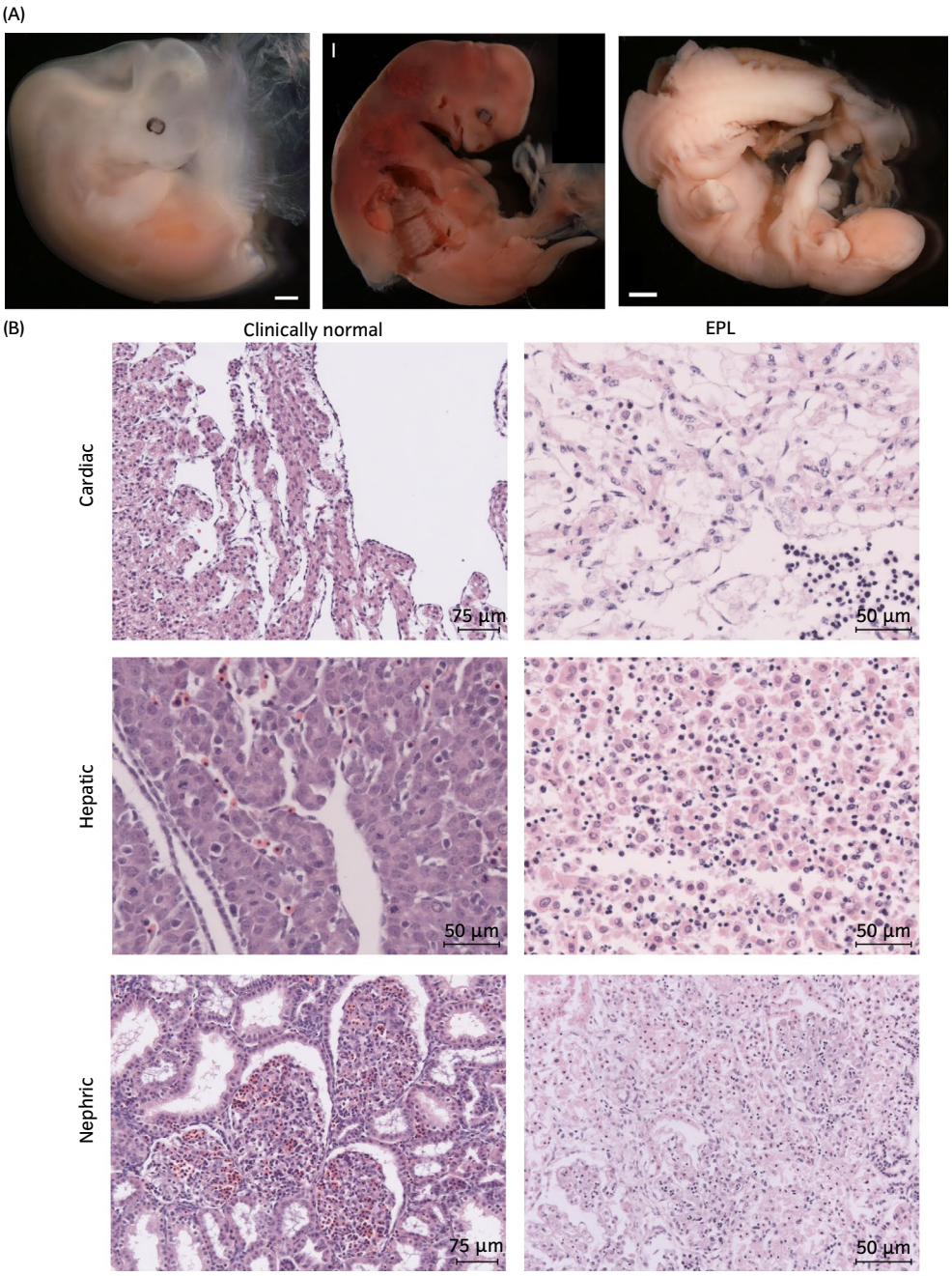

FIGURE 1 Sample quality and tissue integrity. A, Comparative macroscopy of a clinically normal (left panel) and two EPL fetuses (middle and right panel) of different sample quality. Autolysis was not present in the clinically normal but was in the EPL fetuses, as indicated by a diffuse discolouration and the partial (middle panel) to complete (right panel) loss of tissue integrity. The white scale bar indicates 1 mm. B, Comparative histology of cardiac (top), hepatic (middle) and nephric (bottom) H&E-stained tissue of a representative clinically normal (left panel) and EPL (right panel) fetuses. Alterations of the tissue architecture, including the separation of cells and nuclear changes, were observed and interpreted as microscopic evidence of autolysis

the 10 specimens with aligned ages may have been retained for a time period prior to diagnosis of pregnancy failure. Next, for those conceptuses, we calculated the maximum uterine retention time. We compared the youngest age of the applicable developmental age category (which could result in an overestimation but not underestimation) with the reported clinical age of these 10 embryos/fetuses. The median maximum uterine retention time post-demise was 4 days (range 2-8 days). The two fetuses with a mismatch between development and clinical age had a maximum uterine retention time of 7 and 15 days.

3.4 | Growth pattern

CRL could be accurately measured in only a subset of specimens as embryos/fetuses were not always fully intact (eg tearing occurred

during uterine lavage or processing, Table S4). Gestational age and CRL of EPL embryos/fetuses were compared with reference values published by Jenner et al[17] and the subpopulation of clinically normal Thoroughbred embryos/fetuses with a CRL recorded (n = 5) (Figure 2). A strong positive correlation of embryonic/fetal age and CRL occurred in the control embryos/fetuses (n = 15, $R = .9$ [95% CI: 0.8-1.0], $R^2 = .9$, $P < .0001$) but not the EPL samples (n = 19, $R = .1$ [95% CI: −0.4 to 0.5], $R^2 = .01$, $P = .75$). As this lack of correlation might be explained by prolonged uterine retention, the analysis was repeated for the subpopulation of EPL embryos/fetuses with a uterine retention time of 4 days or less (n = 6). There remained no correlation between clinical gestational age and CRL in this subset of EPL embryo/fetuses ($R = -.21$, $R^2 = .05$). This absence of a correlation appeared to be due to the relatively smaller EPL conceptuses, even if one considered a maximum uterine retention time of 4 days and their developmental age. Next, we calculated the proportion of EPL embryos/fetuses that had IUGR defined as those in the 10th percentile for size for the developmental period. There were eight embryos/fetuses that had CRLs recorded and an agreed developmental age that fell into the age categories where we had enough reference values to calculate IUGR (Table S2). IUGR was identified in 3/8 embryos/fetuses all developmentally aged to be a minimum of 38 days with clear regional subdividing (fore)limbs, bending towards the midline and a disappearing pontine flexure but with CRLs below the calculated threshold (Table S4). Two fetuses were markedly (CRL of 9 and 10.5 mm) and one was mildly undersized (16.4 mm).

3.5 | Morphological abnormalities

A morphological diagnosis was reached and agreed by both examiners in 13/27 embryos/fetuses obtained from EPL pregnancies by macroscopic and in 12/13 samples by histological assessment. All eligible clinically normal embryos/fetuses allowed for a diagnosis in macroscopy (n = 11/11) and histology (n = 6/6, Table S5).

3.6 | CNS

Abnormalities of the developing CNS were identified in more EPL than clinically normal embryos/fetuses in the macroscopic (clinically normal: 2/11; EPL: 10/13, $P = .01$) and histological assessment (clinically normal: 0/6; EPL: 12/12, $P < .0001$). The gross abnormalities noted in two control embryos/fetuses were: abnormal development of the forebrain and fourth ventricle (n = 1) and abnormal shape of midbrain (n = 1). The CNS of these two control embryo/fetuses had a normal histological structure. Two types of abnormalities were noted macroscopically in EPL samples: (a) failure of closure of the neural tube (1/13) (Figure 3A) and (b) cystic disruption of the developing neural tissue primarily involving the mesencephalon and sometimes accompanied by an irregular pattern of the CNS surface (9/13, Figure 4A, left, middle and right panel). All clinically normal and EPL embryos/fetuses (7/7) aged developmentally between 26 and 37 days (and thus younger than the fetus with failure of neural tube closure) presented macroscopically and histologically with a closed neural tube. Further, histological assessment of the CNS of the fetus with failed neural tube closure revealed a clear epithelial lining of the developing neural tissue surrounding the neural tube (Figure 3B), thus secondary tissue disruption (eg uterine lavage or processing) can be excluded.

In the remaining embryos/fetuses with CNS abnormalities (9/13), the anatomy of the developing pros-, mes- and rhombencephalon was not clearly appreciable (Figure 4A) and accompanied by a partly cellular and dense vs acellular to cavernous appearance of the CNS (Figure 4B lower panels) but no other organs. Frequently, extracellular oedema and haemorrhage were associated with these nonspecific abnormalities. The presence of haemosiderin, indicating premortem haemorrhage, was observed in the CNS tissues of 3/13 EPL embryos/fetuses. This was in contrast to the developing CNS of 6/6 clinically normal embryos/fetuses histologically assessed which appeared intact and well-organised (Figure 4B upper panels). To determine whether the morphological CNS changes were associated with bacterial infection, Gram staining of a representative

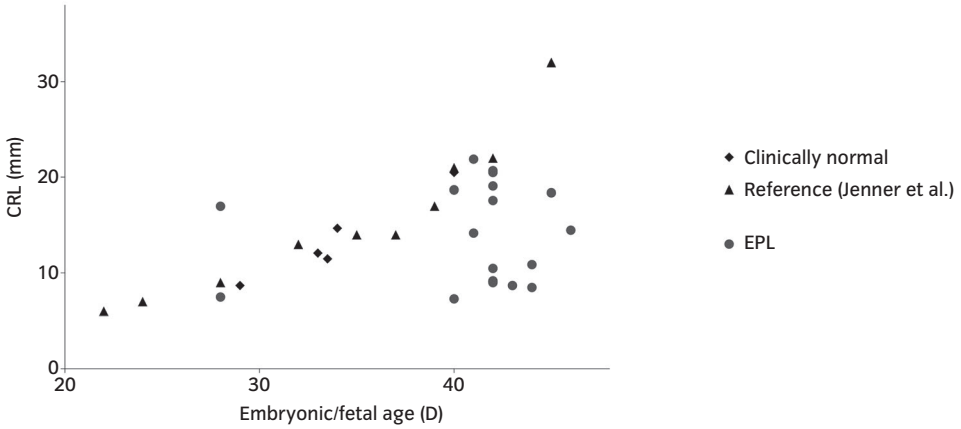

FIGURE 2 Embryonic/Fetal growth pattern. Comparison between the gestational age and CRL of reference (black triangles) (n = 10, 17) together with clinically normal Thoroughbred (black diamonds) (n = 5) and EPL embryos/fetuses (grey circles) (n = 19). A strong positive correlation of fetal age and CRL occurred in the reference and clinically normal Thoroughbred ($R = .9$ (95% CI: 0.8-1.0), $R^2 = .9$, $P < .0001$) but not the EPL embryos/fetuses (n = 19, $R = .1$ (95% CI: −0.4 to 0.5), $R^2 = .01$, $P = .75$)

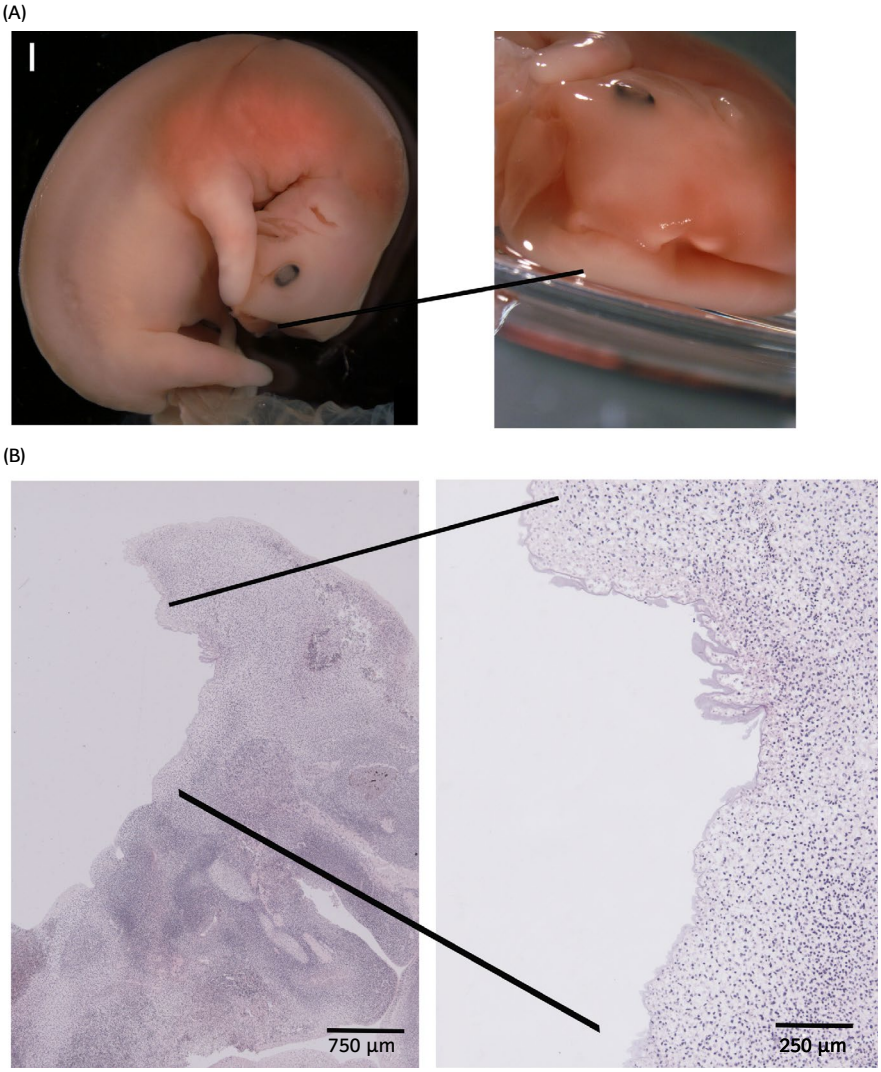

FIGURE 3 Specific morphological abnormalities of the CNS. A, Macroscopic appearance of a failed closure of the neural tube in a fetus obtained from an EPL pregnancy on gestational day 46. The black line highlights the defect at a low- (left panel) and a high-power view (right panel). The white scale bar indicates 1 mm. B, Corresponding histological presentation of the defect, shown at low (left panel, scale bar 750 µm) and high magnifications (right panel, scale bar 250 µm; both H&E stain)

histological section from the embryos/fetuses with evidence of neural tissue disruption was performed. Gram-positive microorganisms were seen within the neural, but no other embryonic/fetal tissues or the associated membranes, tissue of 1/10 EPL samples with CNS abnormalities. Gram-negative bacteria were not observed.

3.7 | Subcutaneous haemorrhage

A distinct bilateral dark-red discolouration located predominately in the area of the embryonic/fetal neck, shoulder and cranial thorax was observed in EPL embryos/fetuses and referred to as 'red patching' in the current study (Figure 5A). 'Red patching' was more common in EPL (14/27) compared with clinically normal embryos/fetuses (0/11, $P = .010$), and corresponded to the histological finding of localised severe subcutaneous haemorrhage in 8/13 EPL samples also assessed histologically (Figure 5B left and right panel). A further 4/13 embryos/fetuses obtained from EPL pregnancies and with no macroscopic evidence of 'red patching' presented either with the same histological changes (n = 3 embryos/fetuses) or a generalised to disseminated haemorrhage (n = 1).

4 | DISCUSSION

This is the first study to describe the macroscopic and histological features of embryos/fetuses obtained from mares suffering EPL. Similarly to descriptions of spontaneously aborted human conceptuses,[12,24] accuracy of the morphological diagnosis and determination of the developmental age were impaired due to autolysis in EPL embryos/fetuses. Although, eligibility of conceptuses obtained from EPL pregnancies required a mostly intact sample on submission to the laboratory, the post-fixation macroscopic assessment revealed that only 13/27 embryos/fetuses allowed for a morphological diagnosis, thus reducing the number of specimens available for a complete assessment. Nevertheless, a developmental age and morphological diagnosis were achieved with over 90% agreement between examiners in 11 clinically normal and 13 EPL embryos/fetuses. Microscopically, all embryos/fetuses obtained from clinically normal Thoroughbred pregnancies and 12/13 EPL embryos/fetuses allowed for a diagnosis. Specimens obtained following early pregnancy failure were characterised by a mismatch of embryonic/fetal size and age, irregularities of the CNS structure and subcutaneous haemorrhage, features that were absent in control and reference embryos/fetuses of similar

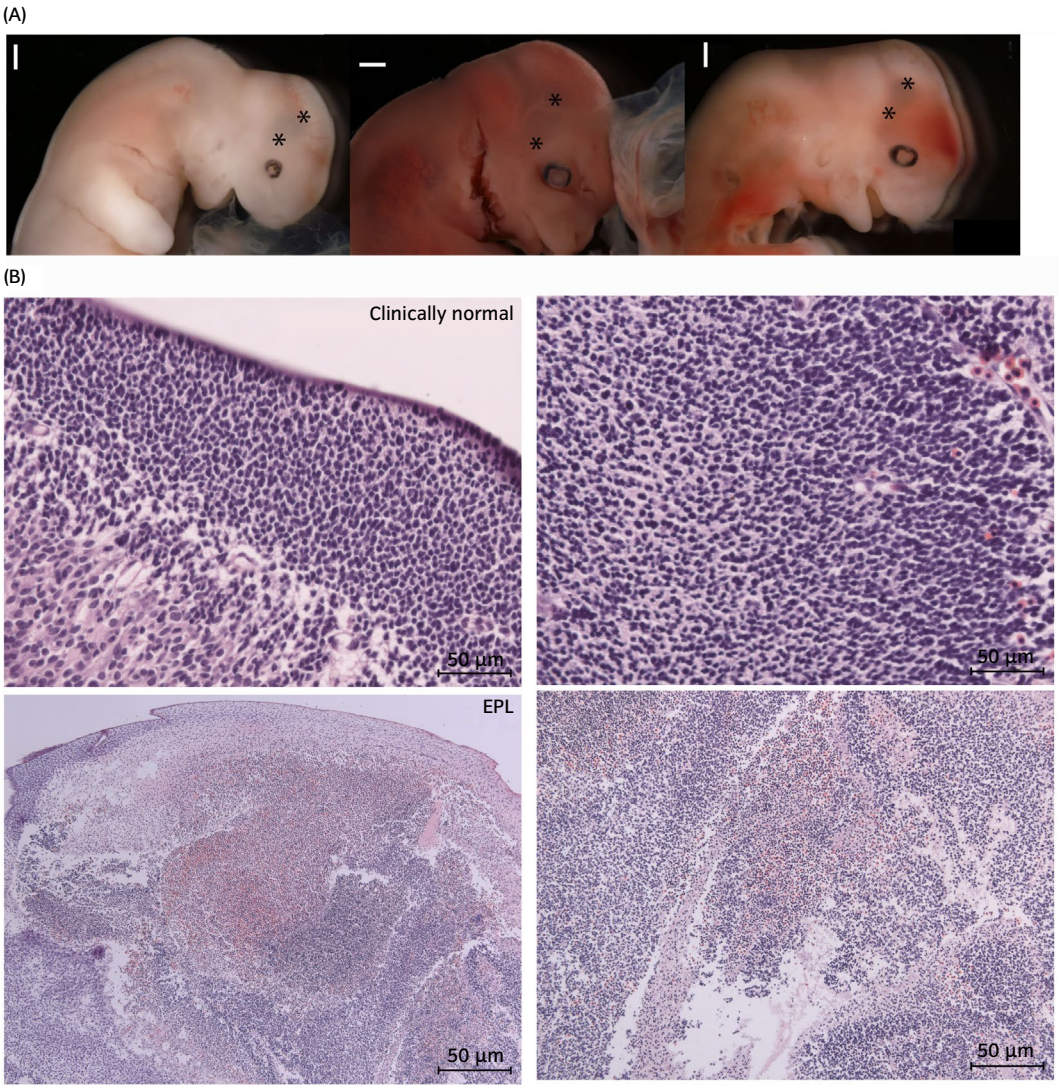

FIGURE 4 Nonspecific morphological abnormalities of the CNS. A, Representative images of EPL embryos/fetuses, presenting with a macroscopic abnormal, cystic (indicated by *) to disrupted appearance of the mesencephalon. The white scale bar indicates 1 mm. B, Clinically normal (upper panels) and abnormal (lower panels) appearance of the developing neural tissue (H&E stain). Abnormalities associated with EPL imply alternations of cellular and dense areas vs acellular to cavernous regions as well as extracellular oedema and haemorrhage. Contrary, the CNS presents intact and well-organised in clinically normal embryos/fetuses

gestational ages and that could not always be explained by uterine retention.

Although the clinically reported gestational age of EPL pregnancies may contain a 24 hours ovulation inaccuracy and be confounded by uterine retention, this potential error was acknowledged by comparison of the gestational and developmental age. Gestational and developmental age coincided for the majority of the ageable EPL embryos/fetuses in the present study, thus in alignment with the median maximum uterine retention time post-demise of 4 days. Gestational and developmental age did not align in 2/27 embryos/fetuses obtained from mares suffering pregnancy failure.

Despite the age-appropriate developmental morphology of embryos/fetuses being confirmed above, a correlation between gestational age and CRL, as described by others,[17] was not observed among the EPL specimens, a finding that persisted following exclusion of conceptuses with prolonged uterine retention. This suggests a general mismatch of embryonic/fetal size and age. In comparison to reference and clinically normal embryos/fetuses, their size was significantly smaller and 3/8 embryos/fetuses eligible for assessment were found to have IUGR. In human pregnancy, growth retardation is commonly defined as a birth weight below the 10th percentile of a reference population[24-26] and this is also described in the horse.[22] In this study, the 10th percentile of the CRL of reference and clinically normal embryos/fetuses was calculated to define IUGR for early equine pregnancy. A small number of control embryos/fetuses, available for the calculation of 10th percentile reference values for embryonic/fetal size, is a limitation to the current study and subsequent research is encouraged to determine the relevance of IUGR and potential early initiators of this condition, well-described for advanced gestational stages in

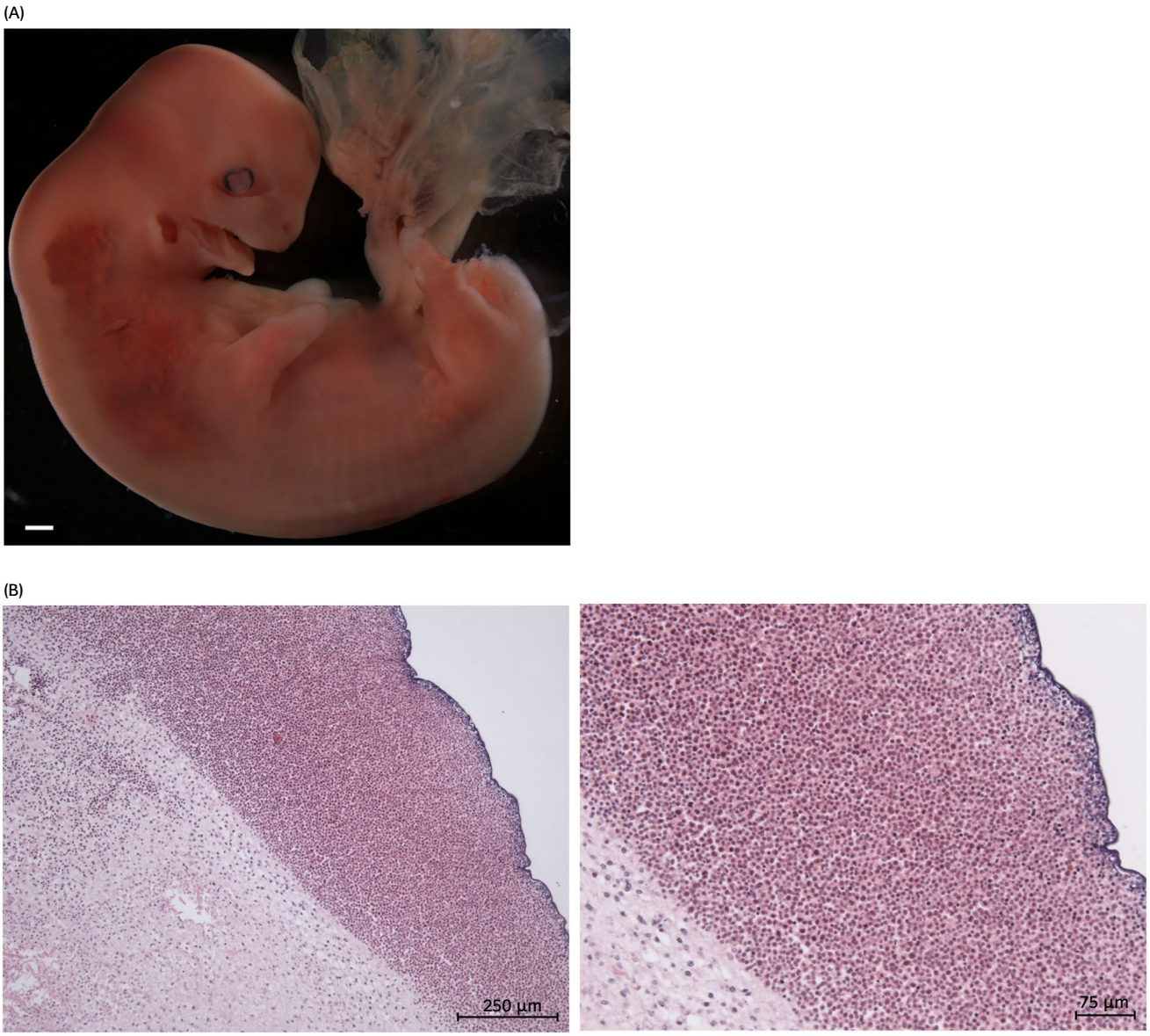

FIGURE 5 Subcutaneous haemorrhage. A, Representative image of a distinct bilateral dark-red discolouration located in the area of the neck and shoulder of a fetus obtained from an EPL pregnancy on gestational day 42. The white scale bar indicates 1 mm. B, Corresponding histological appearance illustrated in the subcutaneous accumulation of nucleated fetal erythrocytes at low (left panel) and high magnification (right panel; both H&E stain)

the mare.[27-29] Nevertheless, of the 3/8 embryos/fetuses identified with IUGR, two showed marked growth restriction consistent with embryos/fetuses of a much earlier gestational age and their diagnosis would be unlikely to be impacted by changes in the reference values.

An association of IUGR with congenital malformations is described for humans and mice.[26,30] A similar correlation may be suspected for equine conceptuses and one fetus with a confirmed failure of neural tube closure also was small for gestational age in our study. Development of the equine conceptus depends on the endometrial supply and embryonic/fetal absorption of histotroph until approximately day 40 of gestation.[18,19,31] The importance of histotrophic allantochorion cells in promoting optimal growth in late pregnancy has also recently been highlighted.[29] Therefore, it is plausible endometrial pathologies that impact on histotroph production may be involved in the pathogenesis of equine EPL, further strengthened by the association of early pregnancy failure with the presence of uterine cysts.[9] Whether endometrial impairments cause the IUGR observed here is not known, although worthy of further investigation. Pregnancy loss in women is frequently associated with general defects of the embryo or early fetus.[12] A disturbed progression of developmental landmarks existed among failed equine embryos/fetuses and may make determination of the developmental age challenging and account for some of the disagreement between examiners on developmental age in the current study.

Observations in mice show a correlation between placental defects and abnormalities of the embryonic/fetal CNS and CVS,[30] both alterations also recognised in the present study. A definite and specific morphological anomaly was confirmed in one EPL fetus as failed closure of the neural tube. As the sample size was relatively small, and only one case identified, it is likely that this is a rare cause of pregnancy loss. Nevertheless, to our best knowledge, it represents the first described case of a major developmental pathology in an equine fetus from a clinical case of early pregnancy failure. Closure of the neural tube is a time-dependent process, expected to be completed by gestational day 28 in human fetuses[32] and based on our observations here, it closes at a time prior to 28 days in the horse. A developmental age based on limb features and surface ectoderm of the confirmed case of failed neural tube closure was >37/40 days, aligning with the gestational age of 46 days corrected by the median maximum uterine retention time following demise of 4 days. Therefore, this gestational age combined with the histological finding of a clear epithelial lining of the developing tissue surrounding the neural tube strongly supports the diagnosis.

Abnormalities of the CNS, a common feature of human EPL,[12,14] were also observed in the majority of EPL specimens with a macroscopic diagnosis. Histology was a more sensitive method to detect alterations of the neural tissue in EPL embryos/fetuses, thus macroscopic changes may represent advanced stages of an impaired CNS integrity. Due to the presence of autolysis in some of the EPL embryos/fetuses, it is possible that a proportion of the neural tissue pathologies identified here reflect autolysis post-mortem. Equally, it is unlikely to explain all the changes observed. Gross anatomical alterations to the CNS, such as cystic disruption of the developing neural tissue, along with the presence of haemosiderin within the CNS indicating premortem haemorrhage suggest that these pathologies were present prior to demise of the conceptus. Although infection was previously described as an underlying cause of equine EPL,[3,11] we propose it to be a very minor contribution to the morphological abnormalities in the cases of pregnancy failure studied here, since microorganisms were observed within the neural tissue of only one EPL fetus.

A localised and severe subcutaneous haemorrhage presented in EPL embryos/fetuses and was interpreted as a specific morphological feature associated with equine pregnancy failure. This may be related to ante-mortem haemorrhage and a degree of hypostatic congestion, although an underlying primary or secondary embryonic/fetal cardiovascular disorder cannot be excluded. Cardiovascular anomalies, together with CNS and urinary system anomalies, are the most frequently observed malformations in human conceptuses,[12,14] suggesting in-depth research on embryonic/fetal cardiovascular failure in the horse is warranted. Clinically normal embryos/fetuses presented with variable degrees of subcutaneous disseminated to diffuse erythrocytes, presumably originating from manual pregnancy termination, although the patterns of these changes were distinct from the EPL embryos/fetuses.

In summary, a failed closure of the neural tube was confirmed as a definite and specific abnormality in a fetus obtained from a mare suffering EPL and should be considered as a possible although rare cause of pregnancy failure. Additionally, morphological features of equine embryos/fetuses associated with early pregnancy failure were a mismatch of embryonic/fetal age and size, alterations in the macroscopic and histological structure of the neural tissue and distinct subcutaneous haemorrhage. Subsequent research is encouraged to reveal the aetiology of equine EPL. Genetic causes are suspected, based on well-described associations between chromosomal and morphological anomalies in humans and mice, including specific disorders of the neural tube and CVS.[13,33-35] Further, a relationship of a small embryonic/fetal size and structural anomalies with subchromosomal pathologies was recently found[36] and chromosomal[13,33-36] and teratogenic causes, such as maternal diabetes,[37] are known to be meaningful for pregnancy outcome in various species.

ACKNOWLEDGEMENTS

We thank the participating stud farms and clinicians at Newmarket Equine Hospital, Rossdales Veterinary Surgeons and Equine Reproductive Services (UK) and Dr Jennie Henderson for providing clinical material. The authors are grateful to the staff of the Biological Services Unit of the Royal Veterinary College London for care and management of mares and to Daniel Hampshire, Elaine Shervill, Andrew Hibbert and Ruby Chang for their valuable technical support.

CONFLICT OF INTEREST

No competing interests have been declared.

AUTHOR CONTRIBUTIONS

A. de Mestre designed the present study, which was executed by A. Kahler, A. de Mestre, I. McGonnell, H. Smart and A. Kowalski. Data analysis was performed by A. Kahler with K. Smith and D.C. Wathes supporting data analysis and interpretation. The manuscript was prepared by A. Kahler and A. de Mestre with input and final approval by all authors.

ETHICAL ANIMAL RESEARCH

Animal care and procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 guidelines set by the Home Office and Ethics Committee of the Royal Veterinary College London (PPL 70/6944 and 70/8577).

OWNER INFORMED CONSENT

Owners gave consent for their animals' inclusion in the study using an owner consent form approved by the Royal Veterinary College Ethics Committee (URN 2012/1169 and URN 2017–1660–3).

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/evj.13340.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are openly available in the Royal Veterinary College repository at https://doi.org/10.34840/S712-PX78.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Kahler A, McGonnell IM, Smart H, et al. Fetal morphological features and abnormalities associated with equine early pregnancy loss. *Equine Vet J*. 2020;00:1–12. https://doi.org/10.1111/evj.13340