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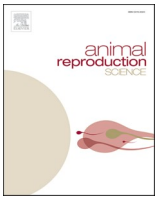

Equine fetal-sex determination from mid-gestation to term using serum conjugated steroid profiling by liquid chromatography-tandem mass spectrometry

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ABSTRACT

Refined profiling of conjugated estrogens and androgens during equine pregnancy using liquid chromatography-tandem mass spectrometry (LC-MS/MS) could provide accurate fetal-sex determination. Current methods for fetal-sex prediction remain limited by timing, accuracy, and operator expertise. This study investigated sex-specific differences in maternal conjugated steroid profiles to develop a reliable, non-invasive predictive method. Samples were collected from 141 mares of various breeds starting at sixteen weeks of pregnancy. The samples were pooled according to gestational stage, divided into 28-day blocks, and analysed using a validated LC-MS/MS method. Quantified analytes included estrone, estradiol, equilin sulfates, glucuronides, and dehydroepiandrosterone sulfate. Method validation encompassed linearity, trueness, precision, accuracy, uncertainty, quantification limits, recovery, matrix effects, carryover, sensitivity, and stability. The effects of fetal sex, breed, parity, and maternal age on steroid concentrations were investigated. Data were split into a Test group for model development and a Validation group for performance assessment. Fetal sex was the principal factor influencing conjugated estrogen and dehydroepiandrosterone profiles ($p = 0.01–0.0001$). Female pregnancies exhibited higher classical conjugated steroid concentrations at block 5, whereas male pregnancies showed delayed elevations persisting until term. Equilin derivatives were consistently lower in

Abbreviations: ACN, Acetonitrile; AUC, area under the curve; BSA, bovine serum albumin; CffDNA, circulating cell-free fetal DNA; CI, 95 % confidence intervals; CLSI, Clinical and Laboratory Standards Institute guidelines; CTUP, combined thickness of the uterus and placenta; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone 3-sulfate; DS, showjumping-type; ECG, equine chorionic gonadotropin; E1, estrone; E1G, estrone β-D-Glucuronide; E1S, estrone 3-Sulfate; E2, 17β-estradiol; E2G, 17β-Estradiol 3-β-D-Glucuronide; E2S, 17β-Estradiol 3-O-Sulfate; EQG, equilin 3-O-β-D-Glucuronide; EQS, 17β-Dihydro-Equilin 3-Sulfate; F, female; GC-MS, gas chromatography-mass spectrometry; GLMM, generalized linear mixed models; ICE, Icelandic; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOQ, lower limits of quantification; M, male; Min-max, minimum-maximum values; MP, Miniature Shetland pony; OR, odds ratios; PBS, phosphate-buffered saline; P4, progesterone; PRE, Spanish Purebred; QC, quality control; RB, relative bias; RSD, relative standard deviation; SD, standard deviation; STB, Standardbred; Se, sensitivity; Sp, specificity; T, gestational time in months; T^2, quadratic term of gestational time; TA US, transabdominal ultrasonography; TR US, transrectal ultrasonography; US, ultrasonography.

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males. Estrone sulfate (E1S) showed reliable univariate performance for fetal-sex prediction (area under the curve (AUC) 0.710–0.865, blocks 5–10). Multivariate models using all conjugated steroids achieved superior accuracy (AUC 0.747–0.912, blocks 5–11), providing an alternative method to univariate model and ultrasonography. The validated LC-MS/MS method improves understanding of equine feto-placental endocrinology and offers a non-invasive tool for practical fetal sexing.

1. Introduction

Estrogens, alongside progestogens and androgens, are steroid hormones derived from cholesterol metabolism and synthesized in horses by the conceptus, gonads, and adrenal glands, while the allantochorion later contributes to their metabolic transformation. In equine embryos, the onset of estrogen production has been reported to occur at 12 days of gestation (Heap et al., 1982; Zavy et al., 1979 and Zavy et al., 1984; Flood et al., 1979; Raeside et al., 2004). As gestation progresses, maternal ovaries produce progesterone (P4) and estrogens until placental steroidogenesis predominates around day 100 (Terqui and Palmer, 1979; Holtan et al., 1979; Daels et al.,1991; Ginther, 1992). The equine allantochorion metabolizes maternal C21 steroid precursors into progestogens, while fetal androgens serve as substrate for estrogen synthesis (Raeside et al., 1979; Bhavnani et al., 1969; Hamon et al., 1991; Legacki et al., 2017). This androgen-to-estrogen conversion is primarily mediated by placental aromatase, a cytochrome P450 enzyme, whose activity peaks at approximately 5 months of gestation, coinciding with maximal maternal serum estrogen levels (Loux et al., 2020). Prior to excretion, estrogens are conjugated by sulfotransferases or UDP-glucuronyltransferases, yielding sulfonated or glucuronidated metabolites that enter the maternal circulation (Loux et al., 2020). Concentrations of conjugated estrogens in maternal serum subsequently decline gradually until parturition (Nett et al., 1975; Ousey, 2004; Conley, 2016; Ledeck et al., 2022).

Despite detailed characterization of temporal estrogen profiles in mares, the influence of fetal sex and maternal factors remains incompletely described. Most equine steroid profiling studies have relied on immunoassays (Canisso et al., 2017; Esteller-Vico et al., 2017; Rance and Park, 1978; Satué et al., 2011), which, although previously considered the gold standard, are limited by cross-reactivity, restricted analytical sensitivity (Se), and specificity (Sp) (Dufour et al., 2021), potentially obscuring subtle steroid variations. As an alternative, gas chromatography-tandem mass spectrometry (GC-MS/MS) has been used in equine, providing accurate and specific measurements of progestogens (Holtan et al., 1991) androgens, and estrogens in plasma (Marshall et al., 1999). Long sample preparation, including concentration and derivatization of non-volatile compounds such as steroids, as well as extended analysis time, makes GC-MS/MS time-consuming and unsuitable for large sample sets. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) overcomes the limitations of both immunoassays and GC-MS/MS by decreasing sample preparation and analysis times, while providing highly sensitive and accurate quantification of multiple steroids. Recent LC-MS/MS reports have refined maternal estrogen patterns throughout equine gestation (Legacki et al., 2016; Genangeli et al., 2017; Dufour et al., 2021) and revealed breed-specific differences in unconjugated estrogen concentrations, while maternal age and parity appeared to exert minimal effects (Ledeck et al., 2022). No potential impact of fetal sex on steroids concentrations was reported in early studies (Raeside, 1976). Two studies have, however, reported associations between fetal sex and maternal plasma concentrations of P4 and testosterone, but these findings were based on immunoassays and limited sample sizes (Busato et al., 2021; de Lara et al., 2025). Investigating such differences in conjugated steroids across a large number of equine pregnancies using LC-MS/MS could provide an easy and reliable method for fetal-sex prediction.

Current methods for fetal sexing rely on techniques that are technically demanding or limited in accuracy. Since 1989, transrectal (TR) ultrasonography (US) has been considered the standard for early fetal sexing between 60 and 70 days of gestation (Curran and Ginther, 1989), whereas transabdominal (TA) US is employed at later gestational stages (120–210 days) due to fetal growth and positional changes (Van de Velde et al., 2018). Both approaches depend on identifying the genital tubercle, which migrates toward the tail in females (F) and the umbilical cord in males (M), accompanied by an increased anogenital distance in M fetuses (Curran and Ginther, 1989; Renaudin, 2000). Accuracy rates are reported at 99 % for TR and 90 % for TA examinations when performed by experienced operators (Holder, 2011), and Doppler or three-dimensional US may enhance the accuracy (Resende et al., 2014; Mebarki et al., 2019). Nevertheless, US-based sexing is operator-dependent. Circulating cell-free fetal DNA (cffDNA) analysis has emerged as a promising non-invasive alternative during late gestation (Aurich and Schneider, 2014). However, this approach may yield false negatives in F fetuses due to low cffDNA amount (Tonekaboni et al., 2020). Additionally, the mechanisms of cffDNA transfer across the equine placenta remain unclear, and further studies are needed to characterize its kinetics and assess the potential persistence of cffDNA from previous gestations (Aurich and Schneider, 2014; Kadivar et al., 2021). These limitations underscore the need for a new method that enable accurate, non-invasive fetal-sex determination, which has significant implications for breeding strategies and stud-farm management (Aurich and Schneider, 2014; Resende et al., 2014; Van de Velde et al., 2018).

Conjugated steroids in maternal circulation represent an unexplored field for fetal-sex determination. The present study used a validated LC-MS/MS assay to quantify maternal serum concentrations of sulfonated and glucuronidated steroid metabolites from sixteen weeks of gestation to term in mares carrying M or F fetuses. Analytes included estrone (E1) derivatives (Estrone 3-Sulfate (E1S) and Estrone β-D-Glucuronide (E1G)), estradiol (E2) derivatives (17β-Estradiol 3-O-Sulfate (E2S) and 17β-Estradiol 3-β-D-Glucuronide (E2G)), equilin derivatives (17β-Dihydro-equilin 3-Sulfate (EQS) and Equilin 3-O-β-D-Glucuronide (EQG)), and dehydroepiandrosterone 3-sulfate (DHEAS). The two objectives of this study were: (1) to characterize longitudinal changes in maternal conjugated steroid profiles according to fetal sex, accounting for maternal age, parity, and breed; and (2) to evaluate the potential of these steroid

profiles as non-invasive biomarkers for fetal-sex determination from mid-gestation to parturition.

2. Material and methods

2.1. Ethical approval

This study was conducted in accordance with the Ethics Committee for Animal Experimentation of the University of Liège (protocol no. 21–2392). All samples were collected with the informed consent of the animal owners.

2.2. Animals and study design

Between February 2020 and June 2024, a cohort of pregnant mares was enrolled from two private stud farms located in Wallonia, Belgium. The mares were housed and managed under standardized conditions in terms of feeding, exercise, and reproductive monitoring. A total of 104 different mares were sampled across years, for 199 investigated pregnancies. The study population included 116 Spanish Purebred (PRE), 68 showjumping-type (DS), 7 Standardbred (STB), 6 Icelandic (ICE), and 2 Miniature Shetland pony (MP) mares. Individual mare-specific data, including age, breed, and parity, were recorded, and fetal sex was confirmed by direct clinical examination at birth.

From the sixteen weeks of gestation until parturition, mares underwent monthly TR US to assess gestational health and detect signs of placentitis, which could interfere with circulating conjugated steroid concentrations. Examinations were performed using an EXAPad Mini® US device equipped with a 7.5–10 MHz linear transducer (IMV Imaging, Angoulême, France). The probe was positioned transrectally at the caudal pole of the placenta to measure the combined thickness of the uterus and placenta (CTUP), defined as the distance between a uterine vessel at the dorsal aspect of the uterine body and the allantoic fluid. Three CTUP measures were obtained at each examination, and their mean value was recorded. The echogenicity of the utero-placental unit, allantoic, and amniotic fluids were assessed by a single experienced operator to minimize inter-operator variability.

At each US session, blood samples were collected from the jugular vein into dry tubes (Vacuette® CAT serum sep clot activator tubes). Sampling was generally performed in the afternoon, and blood was centrifuged within two hours of collection (1500 x g for 15 min) (Hettich zentrifugen EBA 200™, Tuttlingen, Germany). Serum was divided into three 1-milliliter (mL) aliquots in sterile cryogenic tubes (LABSOLUTE®, Renningen, Germany) and stored at −80 °C until analysis.

For each sample, the interval between ovulation and sampling was calculated in days, and gestation length was defined as the period from ovulation to foaling. Gestational weeks were assigned sequentially (week 1 = days 0–7, week 2 = days 8–14, etc.), with week n corresponding to days [1 + (n - 1) x 7] to (n × 7). Samples were then grouped into 4-week (28-day) blocks to standardize the time unit despite variations in calendar month length (28–31 days). When multiple samples were available for the same gestation within a block, only the latest was used for analysis.

2.3. Inclusion and exclusion criteria

Mares were included when ovulation and foaling dates were documented, pregnancy was confirmed by US 14–21-days post-ovulation, and foal sex was reported at birth. Embryo-recipient mares were excluded. Exclusion criteria also comprised clinical or ultrasonographic signs of placental pathology (increased CTUP, heterogeneous cervical region, particles in fetal fluids, abnormal vulvar discharge, or premature udder development) and any adverse gestation outcomes, including twin pregnancy, abortion, prematurity, dysmaturity, weak-foal syndrome, or neonatal septicaemia. Placental abnormalities observed after foaling (swelling, congestion, or gross lesions) and euthanasia of the mare or foal for unrelated reasons during gestation or within the first postnatal week also led to exclusion.

2.4. Hormone assays in serum

Maternal serum concentrations of E1S, E1G, E2S, E2G, EQS, EQG, and DHEAS were quantified using an in-house LC-MS/MS method, validated according to Clinical and Laboratory Standards Institute guidelines (CLSI): CLSI C50 (Mass Spectrometry in the Clinical Laboratory: General Principles and Guidance), CLSI C57 (Mass Spectrometry for Androgen and Estrogen Measurements in Serum), and CLSI C62-A (Liquid Chromatography-Mass Spectrometry Methods).

Calibration and quality-control (QC) samples were prepared in phosphate-buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA) to minimize matrix interferences on analyte and internal-standard (IS) retention times. Certified reference standards were purchased from LGC Standards (Luckenwalde, Germany) and Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade acetonitrile (ACN) and water were obtained from Biosolve (Dieuze, France), while formic acid, PBS, and BSA were supplied by ThermoFisher Scientific (Kandel, Germany) and Merck KGaA (Darmstadt, Germany).

Calibration curves consisted of seven concentration points prepared by serial dilution of mixed spiking solutions, ranging from 2000 to 2.0 ng/mL for E1S, E2G, EQS, and EQG, and from 200 to 0.2 ng/mL for E1G, E2S, and DHEAS. Two independently prepared QC levels (low and high) were also prepared. Each batch comprised a blank with IS (to monitor carryover or contamination), and a double blank without IS or analytes (to confirm absence of background interference). Calibration and QC samples were analysed at both the beginning and end of each run to ensure accuracy and reproducibility.

For extraction, 400 μL of substitute matrix were aliquoted and spiked with 20 μL of the corresponding calibrator or QC solution and

20 μL of IS, except for the double blank. Equine serum samples (400 μL) were processed in parallel, spiked only with 20 μL of IS. Four deuterated IS were available (E1S-d_4, E2S-d_3, E2G-d_3, DHEAS-d_5), and each analyte was paired with the most structurally and physiochemically similar IS, and with the closest analyte retention time: sulfated analytes with sulfate IS, and glucuronide analytes with glucuronide IS. Proteins were precipitated by adding 1200 μL of ACN, followed by 20 s of vortexing and centrifugation (13600 rpm, 10 min, +4 °C; Ohaus, Nänikon, Switzerland). Supernatants were transferred to 96-well plates, evaporated to dryness under nitrogen (TurboVap® 96 Dual, Biotage, Uppsala, Sweden), and reconstituted in 100 μL of an organic-solvent reconstitution mix composed of water and ACN before injection.

Chromatographic separation was performed using an ExionLC Series UHPLC (AB Sciex, Framingham, Massachusetts, USA) equipped with a BEH C18 column (2.1 mm × 150 mm, 1.7 μm; Waters, Milford, USA). A binary gradient of water and ACN, each containing 0.1 % formic acid, was applied-starting at 25 % organic phase, gradually increasing to 95 %, and followed by re-equilibration. Detection was conducted on a QTrap 6500 + triple quadrupole mass spectrometer (AB Sciex, Framingham, Massachusetts, USA) operated in negative electrospray ionization and multiple reaction monitoring mode. Quantifier ions were used for analyte quantification and qualifier ions for identity confirmation. The Supplementary Data provide details of all MRM transitions used in this study. Data acquisition and processing were performed using Analyst and Sciex OS software (AB Sciex, Framingham, Massachusetts, USA). Quantification employed a weighted ($1/X^2$) quadratic regression model. Method validation followed CLSI recommendations using e.noval 4.1 software (Arlenda, Liège, Belgium). Linearity was evaluated over five independent runs, with correlation coefficients > 0.990. Trueness, precision, accuracy, and measurement uncertainty were determined from QC samples. Trueness was assessed by comparing the mean measured concentrations to reference values and expressed as absolute bias (ng/mL), relative bias (RB) (%), and recovery (%). Precision was assessed as intra-day repeatability and inter-day intermediate precision, expressed as standard deviation (SD) and relative SD (RSD). Accuracy was evaluated using β-expectation tolerance intervals (β-risk < 10 %), with acceptance limits of ± 20 % at the LOQ and ± 15 % above it. Measurement uncertainty, integrating bias and precision, was required to remain ≤ 15 %. The limit of quantification (LOQ) was defined as the lowest concentration that could be quantified with acceptable precision and accuracy (20 %). Limits of quantification were 0.2 ng/mL for E1G, E2S, and DHEAS, and 2.0 ng/mL for E1S, E2G, EQS, and EQG. These values were confirmed by daily analysis of four replicates over ten days. Extraction recovery was calculated as the ratio between the analyte response in extracted samples and that in non-extracted references. Matrix effects were evaluated by comparing analyte responses in ten equine serum samples to those in water standards. Carryover was evaluated by injecting the highest calibrator followed by blanks, with acceptance limits < 10 % of the LOQ for analytes and < 5 % for IS. Specificity was confirmed by the absence of interference from structurally related compounds or matrix constituents. Short-term stability during whole-blood storage at + 4 °C prior to centrifugation was also assessed to evaluate the impact of pre-analytical handling conditions. Analytes were considered stable when concentrations remained within ± 15 % of initial concentrations (± 20 % at the LOQ).

2.5. Statistical analysis

All analyses were performed using SAS software (version 9.4), and plots were generated with R software (version 4.4.1). The significance threshold was set at $p < 0.05$. Descriptive statistics were used to summarize the data. Categorical variables are presented as frequencies (%), and continuous variables as mean ± SD, median, first and third quartiles, and minimum (min) - maximum (max) values. For analytes where few measurements were below the LOQ, values were imputed using random values from a triangular distribution bounded between zero and the LOQ, to minimize bias associated with excluding undetectable values (Handelsman et al., 2020). Normality was tested using the Shapiro-Wilk test, and logarithmic or square-root transformations were applied when necessary. Pairwise associations among steroid concentrations were assessed using Pearson's correlation coefficients, with associated p-values reported. Generalized linear mixed models (GLMMs) were fitted for each hormone to evaluate the effects of gestational time (T, in months), its quadratic term (T^2), fetal sex, and their interactions (T × sex and T^2 × sex) on hormone concentrations. The dataset was divided into a Test group (119 pregnancies, 2020–2023) for model development, and a Validation group (22 pregnancies, 2024) to assess the performance of the developed models. Each fetus was an independent statistical unit, with one sample per block, as the analysis focused on fetal sex per gestation, although multiple foals could originate from the same mare.

In the Test group, univariate logistic regressions were used to assess associations between each hormone concentration and fetal sex, as well as mare-specific data (breed, age, and parity) for each block. Results were expressed as p-values, odds ratios (OR), and 95 % confidence intervals (CI). Multivariate logistic regressions with stepwise selection were then performed for each block. Predicted probabilities of M fetuses (p) were obtained from the logistic equation $L = \text{logit}(p) = \ln(p / (1 - p))$ with $p = 1 / (1 + e^{(-L)})$ and multiplied by 100 to express percentages. Model performance was assessed using the area under the curve (AUC) with 95 % CI for each block.

In the Validation group, probabilities derived from the multivariate test models were applied to estimate fetal sex for each sample based on continuous steroid concentrations. Foals were classified as M when $p \geq 50$ % and F when $p < 50$ %. Agreement between predicted and observed sex was evaluated using a 2 × 2 contingency tables, with Cohen's kappa statistic (95 % CI) calculated to quantify concordance (0 = none, 1 = perfect)). Discordance between classifications was compared using McNemar's test. The overall accuracy of predicted fetal sex was calculated for each block, across the pregnancy for each mare, and according to prior inclusion of mares in the Test group or not using Mann-Whitney tests. Differences in proportions of correctly classified samples were evaluated using Fisher's exact tests. A binomial logistic regression was also fitted to examine whether correct classification was associated with fetal sex or mare group.

3. Results

3.1. Population description

Over the five-year study period, 80 different mares met the inclusion criteria, contributing to a total of 141 pregnancies. Annual enrolment varied from 15.6 % to 27.7 %. In 2020, 23 mares gave birth to 11 F and 12 M foals. In subsequent years, first-time mare enrolment varied with 14/28 mares (17 F, 11 M) in 2021, 12/29 (13 F, 16 M) in 2022, 21/39 (17 F, 22 M) in 2023 and 10/22 (9 F, 13 M) in 2024. Across the study, one mare was enrolled every year, two mares were enrolled for four years, 17 mares for three years, 17 mares for two years, and 43 mares for a single year. Detailed annual inclusion and exclusion rates, together with foal-sex distribution are presented in Table 1.

The Test group comprised 119 pregnancies originating from 70 unique mares. Mares in this group had a mean age of 10.2 ± 5.2 years (range = 3–22) and a mean parity of 3.2 ± 3.1 (range = 0–11). Parity distribution included 26.9 % nulliparous, 13.4 % primiparous, and 59.7 % multiparous mares. The population included 52.9 % PRE mares, 41.2 % DS mares, 4.2 % STB mares, and 1.7 % ICE mares. Mean gestation length was 333.6 ± 18.3 days (range = 327–380). The Validation group in 2024 included 22 pregnancies, of which 10 were newly enrolled in the study, including 90.9 % PRE, 4.5 % ICE or MP mares. Mares in the Validation group had a mean age of 9.1 ± 5.3 years (range = 4–20) and a mean parity of 2.6 ± 3.3 (range = 0–11). Parity distribution was 31.8 % nulliparous, 22.7 % primiparous, and 45.5 % multiparous. Mean gestation length was 341.3 ± 10.3 days (range = 320–362).

3.2. Analytical method validation

The in-house validated LC-MS/MS method demonstrated excellent linearity, with weighted quadratic regression $R^2 > 0.995$ for all analytes. Trueness ranged from −7.7 to 5.1 % RB (mean = −1.8 %). Individual analyte RB (%) ranged from −2.7 to + 4.3 for E1S, −7.7 to −0.4 for E1G, −6.3 to −1.1 for E2S, −6.0 to + 0.1 for E2G, −2.2 to + 3.8 for EQS, −3.7 to + 5.1 for EQG, and −7.0 to −0.2 for DHEAS. Inter-day RSD remained < 7.0 %, while intra-day RSD was consistently < 5.0 %, all within validation acceptance limits (± 15 %, ± 20 % at LOQ). Relative uncertainties at the LOQ ranged from 8.9 % to 14.8 %, with E1S, E2G, and EQS showing the highest variability. Relative uncertainties were lower at mid-range concentrations (5.1–9.6 %) compared with those observed at the LOQ. The lowest mean uncertainties across all levels were observed for E1S (8.0 %), E2G (8.5 %), and DHEAS (8.6 %), while the highest were for E1G (9.2 %), E2S (9.5 %), EQS (9.9 %), and EQG (9.4 %). Extraction recovery was concentration-dependent, averaging 80.5 % at low concentrations (75.9 % for E1S to 83.9 % for E2S) and 94.4 % at high concentrations (89.8 % for EQS to 96.9 % for EQG). Matrix effects, normalized to IS, ranged from 89.6 % to 117.8 %, with E1G showing the highest value (117.8 %) and EQS the lowest (101.7 %). The IS ME were consistent across analytes, ranging narrowly between 93.0 % and 97.0 %. Coefficients of variation were calculated, ranging from 2.9 % to 8.4 % for IS ME and 3.6 to 10.0 % for corrected ME. All validation data are shown in Table 2. Carryover was negligible (< 0.004 % of injected solution). Whole blood stability at + 4 °C prior to centrifugation was generally acceptable for 24 h (± 15 % bias), although E1G exceeded the limit as early as 4 h (+21.0 %), EQG was unstable between 4 and 12 h, and E2S and DHEAS showed −18.7 % and −16.4 % at 8 h, respectively. In contrast, E1S and EQS remained within ± 10.0 %.

3.3. Hormone concentrations and gestational variation

A total of 588 blood samples from the Test group (119 pregnancies, 70 individual mares) were collected across gestational time points and used for model development, corresponding to a mean of 5.1 ± 1.6 samples per pregnancy. Most samples were collected in blocks 9 (15.6 %, 52 mares, 92 pregnancies), 10 (15.1 %, 51 mares, 89 pregnancies), and 8 (13.9 %, 51 mares, 82 pregnancies), followed by blocks 11 (13.6 %, 46 mares, 80 pregnancies), 7 (11.7 %, 48 mares, 69 pregnancies), and 12 (10.4 %, 41 mares, 61 pregnancies). All remaining blocks each contributed less than 9.0 %: block 6, 39 mares, 52 pregnancies; block 5, 35 mares, 41 pregnancies; block 4, 10 mares, 10 pregnancies; block 13, 8 mares, 9 pregnancies; and block 3, 3 mares, 3 pregnancies. Values below the LOQ were rare, ranging from 0 to 28 of 588 measurements per hormone, with 12 and 28 values for EQS and EQG, respectively. The only exception was E2G, which was consistently below the LOQ (2.0 ng/mL) in all enrolled mares and this steroid was therefore

Table 1
Annual enrolment, exclusions, final inclusion of mares, and foal-sex distribution.

Year	Mares enrolled (first-time enrolment)	Not pregnant	Placental pathology	Missing data	Euthanasia	Final mares included (first-time included)	Foal sex	Foal sex
2020	27 (27)	0	3	0	1	23 (23)	12 M	11 F
2021	32 (17)	0	3	1	0	28 (14)	11 M	17 F
2022	44 (18)	3	9	2	1	29 (12)	16 M	13 F
2023	58 (29)	3	11	4	1	39 (21)	22 M	17 F
2024	38 (10)	6	7	3	0	22 (10)	13 M	9 F
Total	199 (104)	12	33	10	3	141 (80)	74 M	67 F

This table presents the annual enrolment and final inclusion of mares from 2020 to 2024, including both first-time (in parentheses) and repeat participants. Mares not pregnant, or with placental pathologies, with missing data, or subjected to euthanasia were considered exclusion criteria. The final number of mares included in the study is reported, along with the sex distribution of foals born each year (M = male, F = female). The last row represents the total for each column over the five-year period.

Table 2
Analytical validation parameters for the LC-MS/MS quantification of seven conjugated steroids in maternal equine serum.

	E1S	E1G	E2S	E2G	EQS	EQG	DHEAS
LOQ (ng/mL)	2.0	0.2	0.2	2.0	2.0	2.0	0.2
Mean R^2	0.999	0.998	0.999	0.999	0.999	0.998	0.999
Mean relative bias (%)	-0.2	-3.9	-3.6	-2.6	0.9	0.5	-3.5
Mean risk (%)	0.5	2.3	2.7	1.0	1.5	1.6	2.0
Mean repeatability (RSD%)	3.0	3.1	3.0	3.3	3.6	3.3	2.6
Mean intermediate precision (RSD%)	3.8	4.3	4.5	4.0	4.7	4.5	4.1
Mean relative expanded uncertainty (%)	8.0	9.2	9.5	8.5	9.9	9.4	8.6
Mean recovery (%)	96.6	95.7	88.2	94.5	89.8	96.9	96.4
CV mean recovery (%)	2.8	4.2	4.0	4.5	4.7	3.5	3.6
Mean matrix effect (%)	86.8	111.0	96.9	99.3	95.5	100.2	98.6
CV mean matrix effect (%)	6.8	5.0	6.2	4.9	5.2	3.2	4.4

This table presents the analytical validation of an LC-MS/MS method for quantifying seven conjugated steroids: estrone 3-sulfate (E1S), estrone β-D-glucuronide (E1G), 17β-estradiol 3-O-sulfate (E2S), 17β-dihydro-equilin 3-sulfate (EQS), equilin 3-O-β-D-glucuronide (EQG), and dehydroepiandrosterone 3-sulfate (DHEAS) in maternal equine serum, following Clinical and Laboratory Standards Institute guidelines. Validation was performed over five independent runs, including seven calibration points and two quality control (QC) levels analysed in triplicate. Reported parameters include the limit of quantification (LOQ, ng/mL) determined from 40 replicates over 10 days, mean coefficient of determination (R^2), mean relative bias (%), mean risk (%), mean repeatability and intermediate precision (RSD%), mean relative expanded uncertainty (%), mean recovery (%) from five extracted samples compared to non-extracted references and its coefficient of variation (CV, %), as well as mean matrix effect (%) determined from 10 equine serum samples compared to standards in water and its CV (%).

excluded from analyses.

Pairwise Pearson correlations between hormones within gestational blocks were highly significant ($p < 0.0001$). Strong positive correlations were observed between E1S and E1G in blocks 5, 7, and 9–12 ($r = 0.707–0.826$, min-max across blocks), and similarly between E1G and E2S in blocks 5 ($r = 0.801$), 7 ($r = 0.727$), and 11 ($r = 0.706$). Between blocks 5–11 (excluding block 6; $r > 0.800$), E1S and E2S were also positively correlated. Additional positive correlations were noted between E1S and DHEAS and between E2S and DHEAS in block 5 ($r = 0.792$ and 0.790, respectively).

Temporal dynamics of conjugated steroids are illustrated in Fig. 1. To assess sex-specific temporal trajectories of conjugated steroids, GLMM revealed significant quadratic effects of gestational time for all hormones (T and T^2, $p < 0.0001$), reflecting peak and decline patterns. Linear and quadratic interactions with fetal sex were significant for E1S, E1G, and E2S ($p < 0.0001$), indicating

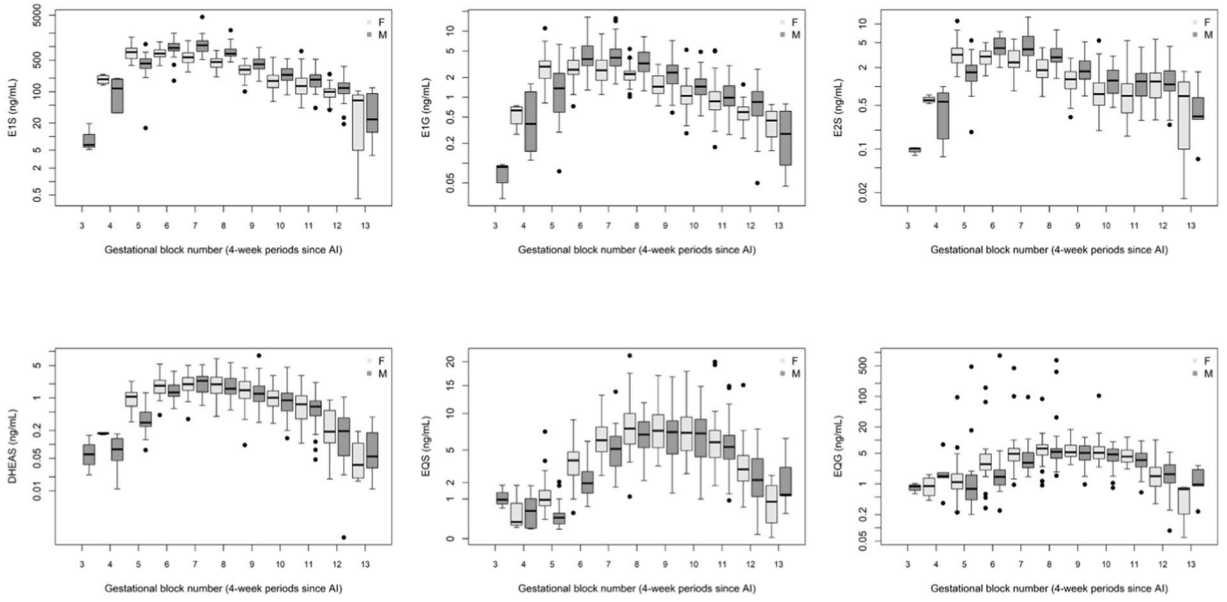

Fig. 1. Maternal serum concentrations of conjugated steroids across gestational blocks according to fetal sex in mare pregnancies. *Boxplots depict equine serum concentrations from 119 mares between 2020 and 2023 of estrone 3-Sulfate (E1S), Estrone β-D-Glucuronide (E1G), 17β-Estradiol 3-O-Sulfate (E2S), 17β-Dihydro-Equilin 3-Sulfate (EQS), Equilin 3-O-β-D-Glucuronide (EQG), and Dehydroepiandrosterone 3-Sulfate (DHEAS) across gestational blocks. Gestational weeks were assigned sequentially (week 1 = days 0–7, week 2 = days 8–14, etc.), and samples were grouped into 4-weeks (28-days) blocks for analysis. Dark gray boxes represent pregnancies carrying male fetuses (M) and light gray represent pregnancies carrying female fetuses (F). Horizontal lines within boxes indicate the median, boxes represent the interquartile range (Q1-Q3), and whiskers denote the minimum and maximum values within 1.5 × the interquartile range. Outliers beyond this range are shown as individual points.*

distinct gestational trajectories in M- versus F-bearing pregnancies. Peak E1S and E1G concentrations occurred at block 5 in F-bearing pregnancies (753.0 [535.0–963.0] ng/mL and 2.9 [1.9–3.5] ng/mL, respectively) and at block 7 in M-bearing pregnancies (1074.0 [782.0–1350.0] ng/mL and 4.0 [3.0–5.5] ng/mL, respectively). Peak E2S concentrations occurred at block 5 in F-bearing (3.2 [2.3–4.2] ng/mL) and block 6 in M-bearing pregnancies (4.1 [3.2–5.9] ng/mL). Significant effects of fetal sex ($p = 0.01$) and interactions with time ($T \times M$, $p = 0.004$; $T^2 \times M$, $p = 0.01$) were observed for EQS, peaking at block 8 in F-bearing (7.8 [5.6–10.3] ng/mL) and block 9 in M-bearing pregnancies (7.3 [4.6–8.9] ng/mL). Peak EQG concentrations occurred at block 8 for both sexes (6.4 [4.4–8.0] ng/mL for F, and 5.4 [3.9–6.6] ng/mL for M), with significant main effects of fetal sex ($p = 0.002$) and time interactions ($p < 0.005$). Finally, DHEAS concentrations were also sex dependent ($p = 0.02$), peaking at block 7 in both F- (2.0 [1.5–2.7] ng/mL) and M-bearing pregnancies (2.3 [1.3–2.9] ng/mL).

3.4. Fetal-sex prediction

Blocks 3, 4, and 13 were excluded from prediction analyses due to the limited sample sizes (3, 10, and 9 samples respectively). The remaining data from the Test group were included in the predictive analyses.

Mare-specific data (age, parity, and breed) did not significantly affect fetal sex. Univariate analyses revealed significant differences in hormone concentrations between M- and F-bearing pregnancies from block 5 onward. Table 3 presents the most predictive hormones for fetal sex at each gestational blocks, along with the robustness of the corresponding univariate model. During block 5, E1S, E1G, and E2S concentrations were significantly lower in M-bearing pregnancies compared to F ($p = 0.003$, 0.009, and 0.004, respectively). From block 6 through 10, E1S and E1G concentrations were significantly higher in M-bearing pregnancies ($p = 0.01$–0.0004 and 0.04–0.0001, respectively), whereas E2S remained significantly higher in M-bearing pregnancies through block 11 ($p = 0.01$–0.0001). Equilin conjugates showed different trend, with significantly lower concentrations in M fetuses for EQS from blocks 5–7 ($p = 0.004$, 0.004, and 0.01, respectively) and for EQG from blocks 10–11 ($p = 0.03$ and 0.04). Finally, DHEAS concentrations were significantly lower in M-bearing pregnancies only at block 5 ($p = 0.0001$).

Multivariate logistic regression demonstrated that combinations of conjugated steroids were significantly associated with fetal sex. At block 5, DHEAS predicted M fetuses (AUC of 0.858). At block 6, E2S (OR = 144.7) and EQS (OR = 0.02) were predictive of M (AUC = 0.912). Subsequent blocks showed varying predictive contributions: E1S at block 7 (OR = 66.6, AUC = 0.865), EQS and E1S at block 8 (OR = 0.2 and 517.9, AUC = 0.907), E1G and EQG at block 9–10 (AUC = 0.846 and 0.824), and finally, E2S and EQS at block 11 (OR = 5.6 and 0.3, AUC = 0.747).

Table 3
Performance of univariate models for prediction of fetal sex in mares across gestational blocks in the Test group.

Hormones	Block 5	Block 6	Block 7	Block 8	Block 9	Block 10	Block 11
E2S	***F>M***	**M>F**	**M>F**	**M>F**	**M>F**	**M>F**	**M>F**
OR	0.08	16.3	17.5	22.1	7.6	3.6	2.5
P-value	0.004	0.003	0.0002	< 0.0001	0.0004	0.003	0.01
AUC	0.821	0.759	0.813	0.804	0.721	0.710	0.683
EQG						***F>M***	***F>M***
OR						0.4	0.4
P-value						0.03	0.04
AUC						0.597	0.629
E1G	***F>M***	**M>F**	**M>F**	**M>F**	**M>F**	**M>F**	
OR	0.2	6.0	8.8	9.0	6.0	3.0	
P-value	0.009	0.01	0.0008	0.0004	0.0009	0.01	
AUC	0.778	0.726	0.764	0.741	0.723	0.660	
DHEAS	***F>M***						
OR	0.1						
P-value	0.0001						
AUC	0.862						
EQS	***F>M***	***F>M***	***F>M***				
OR	0.03	0.1	0.3				
P-value	0.004	0.004	0.01				
AUC	0.826	0.768	0.668				
E1S	***F>M***	**M>F**	**M>F**	**M>F**	**M>F**	**M>F**	
OR	0.03	6.1	66.6	161.1	23.5	5.6	
P-value	0.003	0.04	< 0.0001	< 0.0001	0.0001	0.002	
AUC	0.845	0.753	0.865	0.862	0.756	0.710	

This table summarizes the predictive performance of univariate models based on individual serum hormones for fetal-sex determination in mares across gestational blocks. Gestational weeks were assigned sequentially (week 1 = days 0–7, week 2 = days 8–14, etc.), and samples were grouped into 4-weeks (28-days) blocks for analysis. The test group included 119 mare pregnancies from 2020 to 2023. For each hormone and block, it reports whether concentrations are higher in female (F) or male (M) fetuses (F > M/M > F), the strength of the association with fetal sex (odds ratios, OR), the statistical significance of the association (p-values), and the discriminative ability of the model to correctly classify fetal sex (area under the curve, AUC; 0 = no discrimination, 1 = perfect discrimination). Hormones analysed include Estrone 3-Sulfate (E1S), Estrone β-D-Glucuronide (E1G), 17β-Estradiol 3-O-Sulfate (E2S), 17β-Dihydro-Equilin 3-Sulfate (EQS), Equilin 3-O-β-D-Glucuronide (EQG), and dehydroepiandrosterone 3-sulfate (DHEAS).

Multivariate models consistently achieved higher AUCs than univariate models, although univariate E1S models demonstrated robust predictive performance across blocks 5–10 (113–280 days of gestation), with AUC ranging from 0.710 to 0.865. A more discontinuous pattern was observed for E2S predictive ability, achieving significant performance from blocks 5–11 (113–308 days of gestation), with AUC ranging from 0.683 to 0.821. The remaining hormones displayed predictive capacity only at specific blocks, EQS and DHEAS at block 5 (AUC = 0.826 and 0.862, respectively), and EQG at block 11 (AUC = 0.629). All associated AUC values are provided in Table 4.

3.5. Model validation

In the Validation group (2024–2025), 171 blood samples were collected from 22 pregnancies, corresponding to a mean of 7.8 ± 1.3 samples per pregnancy. Sample distribution averaged 10 % per gestational block, except for blocks 2–4 (approximately 5 %). All corresponding sample sizes and M or F distributions for each gestational block are provided in Table 5. Values below the LOQ were imputed (2–38 per hormone). The proportion of values replaced per hormone ranged from 2 to 38 of 171 measurements, specifically: 3/171 for E1S, 23/171 for E1G, 5/171 for E2S, 38/171 for EQS, 17/171 for EQG, and 21/171 for DHEAS. Consistent with the Test group, E2G concentrations were all below the LOQ and were therefore not analysed.

Multivariate models predictions yielded good agreement with observed fetal sex in blocks 7–9 and block 11, with Cohen's kappa coefficients ranging from 0.5 to 0.6. Diagnostic Se (probability that the test correctly identifies a M fetus when it is truly M) increased across blocks 7–11, except for block 10, ranging from 66.7 % to 83.3 %, Sp (probability that the test predicts F when the fetus is F) decreased over the same blocks, from 85.7 % to 75.0 %. Positive predictive values (PPV, probability that a fetus is M given a M test prediction) increased across blocks 7–11, except for block 10, ranging from 75.0 % to 80.0 %, and negative predictive values (NPV, probability that the fetus is F when the test predicts F) from 83.3 % to 87.5 %. Predictive performance was highest at block 11 (accuracy = 80.0 %), with results in block 9 (76.9 %) also indicating strong accuracy. For blocks 7 and 8, Cohen's kappa coefficients were 0.5, indicating consistent reliability in sex prediction, with an accuracy of 75.0 %. Performance was decreased in blocks 5 (accuracy = 47.1 %), 6 (accuracy = 47.1 %), 10 (accuracy = 36.9 %), and 12 (accuracy = 61.1 %); but provided useful insights into optimal gestational timing for fetal-sex determination. Evolution of Se, Sp, PPV, NPV, and accuracies are presented in Table 5.

Based on the comparison between predicted and observed fetal sex, no differences in predictive accuracy were observed between mares previously included in the Test group and mares never seen before, both overall across the pregnancy (regardless of fetal sex, seen vs. never seen, $p = 0.50$) and when stratifying by fetal sex (F, seen vs. never seen, $p = 0.62$; M, seen vs. never seen, $p = 0.67$). Within mares previously included in the Test group, predictive accuracy seems to not differ between correctly predicted F and M fetuses across the pregnancy ($p = 0.80$). This was similar for mares never included in the Test group ($p = 0.59$). Differences in proportions of correct predictions for either sex were not observed with Fisher's tests, as well as between previous inclusion of the mare or not (≥ 0.67). Logistic regression confirmed that neither fetal sex ($p = 0.99$) nor mare previously inclusion of the mare or not ($p = 0.43$) significantly influenced the probability of correct fetal-sex determination.

4. Discussion

The primary objective of this study was to investigate sex-related differences in maternal conjugated steroid profiles throughout equine gestation and to assess their potential for non-invasive fetal-sex prediction. Over five-year period, 141 pregnancies were longitudinally monitored, constituting one of the largest equine cohorts reported to date, with 52.5 % M and 47.5 % F fetuses. These proportions did not significantly differ from the expected 1:1 ratio and were consistent with previously reported equine sex ratios (Aurich and Schneider, 2014). Mare-specific data, including age, parity, and breed, had no significant effect on fetal sex ratio, contrasting with earlier studies suggesting an influence of maternal age (Kuhl et al., 2015).

Table 4
Comparative performance of univariate and multivariate hormonal models for prediction of fetal sex in mares in the test group.

Model Type (AUC)	Hormone	Block 5	Block 6	Block 7	Block 8	Block 9	Block 10	Block 11
Univariate	*E2S*	0.821	0.759	0.813	0.804	0.721	0.710	0.683
	EQG						0.597	0.629
	E1G	0.778	0.726	0.764	0.741	0.723	0.660	
	DHEAS	0.862						
	EQS	0.826	0.768	0.668				
	E1S	0.845	0.753	0.865	0.862	0.756	0.710	
Multivariate	*All steroids*	0.858	0.912	0.865	0.907	0.846	0.824	0.747

This table compares the predictive performance of univariate versus multivariate hormonal models for fetal-sex determination in mares across gestational blocks in the test group. Gestational weeks were assigned sequentially (week 1 = days 0–7, week 2 = days 8–14, etc.), and samples were grouped into 4-weeks (28-days) blocks for analysis. The test group included 119 mare pregnancies from 2020 to 2023. For univariate models, each row corresponds to a single hormone (estrone 3-sulfate (E1S), estrone β-D-glucuronide (E1G), 17β-estradiol 3-O-sulfate (E2S), 17β-dihydro-equilin 3-sulfate (EQS), equilin 3-O-β-D-glucuronide (EQG), and dehydroepiandrosterone 3-sulfate (DHEAS)) and reports the area under the curve (AUC) for each gestational block, reflecting the ability of that hormone to predict the fetal sex alone (0 = no discrimination, 1 = perfect discrimination). The multivariate model integrates all measured steroids simultaneously, with AUC values indicating the combined discriminative power of the panel. Underlined AUC values indicate performances that did not differ significantly from the corresponding multivariate model.

Table 5
Diagnosis performance of multivariate fetal sex-prediction models across gestational blocks in the equine validation group.

	Number of pregnancies (n)	Number of males (M)	Number of females (F)	McNemar p-value	Kappa Cohen (95 % CI)	*Se* (95 % CI)	*Sp* (95 % CI)	*VPP* (95 % CI)	*VPN* (95 % CI)	*Accuracy*
Block 5	17	10 M	7 F	0.74	-0.12 (-0.58–0.34)	60 (26.2–87.8)	28.6 (3.70–71.0)	47.1 (23.0–72.2)	54.6 (23.4–83.3)	47,1 %
Block 6	17	9 M	8 F	0.74	-0.06 (-0.53–0.42)	44.4 (13.7–78.8)	50.0 (15.7–84.3)	47.1 (23.0–72.2)	50.0 (15.7–84.3)	47,1 %
Block 7	16	9 M	7 F	0.32	0.51 (0.10–0.91)	66.7 (29.9–92.5)	85.7 (42.1–99.6)	75.0 (47.6–92.7)	85.7 (42.1–99.6)	75.0 %
Block 8	16	10 M	6 F	0.32	0.50 (0.09–0.91)	70 (34.8–93.3)	83.3 (35.9–99.6)	75.0 (47.6–92.7)	87.5 (47.4–99.7)	75.0 %
Block 9	13	8 M	5 F	0.56	0.53 (0.07–0.99)	75 (34.9–96.8)	80 (28.4–99.5)	76.9 (46.2–95.0)	85.7 (42.1–99.6)	76,9 %
Block 10	19	12 M	7 F	0.0005	0	0	100 (59−100)	36.8 (16.3–61.6)	0	36,8 %
Block 11	20	12 M	8 F	1	0.58 (0.22–0.95)	83.3 (51.6–97.9)	75.0 (34.9–96.8)	80.0 (56.3–94.3)	83.3 (51.6–97.9)	80.0 %

This table summarizes the diagnostic performance of multivariate fetal-sex prediction models across gestational blocks in the equine validation group. Gestational weeks were assigned sequentially (week 1 = days 0–7, week 2 = days 8–14, etc.), and samples were grouped into 4-weeks (28-days) blocks for analysis. The validation group included 22 pregnancies from 2024. For each block, n indicates the number of pregnancies, and the fetal-sex distribution is reported as the number of male (M) and female (F) foals. Discordance between predicted and observed fetal sex was assessed using McNemar's test (significant if $p < 0.05$). Agreement beyond chance was quantified with Cohen's Kappa (0 = no agreement, 1 = perfect), which ranged from 0 (block 10, no agreement) to 0.58 (block 11, moderate agreement). Sensitivity (Se, %) represents the proportion of correctly predicted M fetuses (0–83.3 %), specificity (Sp, %) the proportion of correctly predicted F fetuses (28.6–100 %), positive predictive value (VPP, %) the probability that a predicted M is truly M (0–80 %), negative predictive value (VPN, %) the probability that a predicted F is truly F (36.8–87.5 %), and overall accuracy (%) the proportion of correct predictions (36.8–80 %).

Most previous investigations into equine steroid endocrinology have relied on immunoassays, which are limited by cross-reactivity and analytical Se and Sp (Senger and Phillip, 1997; Conley and Ball, 2019; Raeside, 1976). The GC-MS/MS method has also been used for steroid analysis in equine studies, particularly in doping control (Wong et al., 2017). However, the complex sample preparation and prolonged analysis restrict its practical use in large-scale equine studies. In contrast, the present study used a rigorously validated in-house LC-MS/MS method, following CLSI guidelines, enabling accurate and simultaneous quantification of DHEAS and multiple conjugated estrogens. The method demonstrated excellent linearity, precision, and accuracy, confirming that the observed hormonal variations reflect true biological differences rather than analytical bias. Moreover, pre-centrifugation stability experiments indicated that whole blood samples could be stored at + 4 °C for up to eight hours prior to centrifugation without loss of analyte integrity, offering practical flexibility for sample handling in field conditions.

A methodological limitation lies in the current LOQ for E2G (2.0 ng/mL), which may not capture physiologically relevant concentrations. To date, circulating E2G concentrations have not been reported in pregnant mares. In non-pregnant mares, E2G accounts for only a small proportion of total conjugated estrogen urinary excretion as measured by immunoassay (Monfort et al., 1991), suggesting that its production represents a minor metabolic pathway. This likely explains its low circulating levels and precluded further analysis of potential associations with other conjugated estrogens or DHEAS.

In contrast, significant correlations were observed between E1S and E1G from gestational blocks 5–12, except for block 6. These correlations likely reflect their common precursor (E1) and conjugation enzymatic pathways via sulfotransferases and UDP-glucuronosyltransferases (Raeside, 2017; Loux et al., 2020). Given that E2S and E2G also derive from a common precursor (E2) and are conjugated through the same enzymatic systems, similar correlation patterns would be expected. Strong correlations were observed between E1S, E2S, and DHEAS, particularly at mid-gestation. The E1S-E2S association persisted from blocks 5–11, except during block 6, aligning with previous reports (Legacki et al., 2019). This highlights the metabolic coupling of these estrogen sulfates, explained by their shared precursor, dehydroepiandrosterone (DHEA) (Raeside, 2017; Rance and Park, 1978; Legacki et al., 2019; Bhavnani et al., 1969). The transient absence of correlation at block 6 may correspond to the peak activity of placental cytochrome P450 aromatase around six months of gestation (Loux et al., 2020), which could temporarily disrupt estrogen production and conjugation, thereby decoupling circulating concentrations. In contrast, neither EQS nor EQG correlated with other conjugated steroids, suggesting regulation through distinct pathways. Equiline derivatives originate from 7-dehydro-DHEA, rather than DHEA (Pashen et al., 1982; Numazawa and Osawa, 1987; Bhavnani, 1988), which may explain their independence from classical estrogens. Moreover, despite their common precursor, EQS and EQG themselves were uncorrelated, likely reflecting divergent conjugation mechanisms, with EQS formed through sulfation of dihydroequilin and EQG via direct glucuronidation (Bhavnani, 1988). These observations highlight differences in the regulation of glucuronidation and sulfation pathways for some atypical steroids, warranting further investigation.

Temporal dynamics of conjugated steroids followed non-linear gestational trajectories characterized by progressive rises, defined peaks at specific gestational windows, and subsequent declines, consistent with previous observations (Legacki et al., 2019; Ledeck et al., 2022). The timing of peak concentrations for E1S, E1G, E2S, and EQS differed between fetal sexes, indicating that both gestational stage and fetal sex jointly shape maternal conjugated steroid profiles. In the present study, maternal E1S, E1G, and E2S concentrations peaked earlier in F-bearing pregnancies (block 5) than in M-bearing pregnancies (E1S and E1G at block 7, and E2S at block 6). An earlier peak in EQS concentration (at block 8) was also observed in F-bearing pregnancies when compared to M-bearing pregnancies (at block 9). These findings align with previous reports not accounting for potential effects of fetal sex (Legacki et al., 2019), which reported E1S and E2S peaks at weeks 26 and 24, respectively. For both sexes, EQG and DHEAS reached maximal concentrations at blocks 8 and 7, respectively. The LC-MS/MS study by Legacki et al. (2019) reported that maternal DHEAS increases only between 7 and 9 weeks of gestation and remains consistently low thereafter, a pattern that contrasts with the clear peak observed at block 7 (25–28 weeks) in the present study. This discrepancy may be explained by the small sample size (n = 4) and the high individual variability in DHEAS concentrations observed among their mares. A temporal shift was already reported between E1 and equilin, peaking respectively at 5–6 months and 7–8 months of gestation (Cox, 1975; Savard, Burdulis,1961). The observed synchrony between conjugated steroids and their unconjugated counterparts (Rance and Park, 1978; Cox, 1975; Ledeck et al., 2022) supports that placental conjugation is sufficiently active throughout gestation to metabolize available precursors or temporally coordinated with native steroid production.

Using LC-MS/MS, this study provides, for the first time, evidence of fetal sex-related differences in conjugated steroid production. After a similar gonadal development in both sexes early in gestation (Barreto et al., 2018), interstitial cell hyperplasia occurs in the fetal ovarian cortex between days 90 and 140 and has been suggested to be influenced by equine chorionic gonadotropin concentrations (eCG) (Barreto et al., 2018). Reported differences in eCG production may be influenced by fetal sex (Mönke and Franz, 1985; Wilsher and Allen, 2011), potentially affecting asynchronous gonadal growth and the production of androgen precursors for estrogen synthesis (Raeside, 2017). Consequently, peaks in E1S, E1G, and E2S occurred earlier in F-bearing pregnancies (block 5), whereas they appear later in M-bearing pregnancies. From block 6 onward, E1S, E1G, and E2S were higher in M-bearing pregnancies, whereas DHEAS no longer showed sex-related differences. Although fetal testes contain fewer interstitial cells, they produce more C21-steroid precursors per gram of tissue than fetal ovaries (Tait et al., 1983), suggesting greater steroidogenic efficiency and explaining the higher maternal conjugated estrogen concentrations later in gestation. Conversely, EQS and EQG were consistently lower in M-bearing pregnancies, highlighting differences in the regulation of atypical estrogen biosynthesis that remain to be elucidated.

This study indicate that E1S concentrations were significantly different between M- and F-bearing mares from mid- to late gestation (113–280 days), with AUC ranging from 0.710 to 0.865, according to blocks. By comparison, de Lara et al. (2025) reported an AUC of 0.87 for P4 at 8 months (block 9), and Busato et al. (2021) reported AUCs of 0.77 and 0.79 for testosterone at months 5 and 8 (blocks 6 and 9), respectively. With comparable AUCs, our approach allows fetal-sex prediction over a longer gestational period, indicating that

E1S may be a reliable biomarker. Its reduced accuracy in early and late gestation likely reflects the smaller sample size of mares during these periods. Including additional early-gestation pregnancies could improve prediction accuracy and provide further insight into the effects of fetal sex on conjugated steroid kinetics. Multivariate models generally improved discrimination in late gestation, whereas univariate models based on E1S, E2S, EQS, or DHEAS provided comparable accuracy during mid-gestation.

Currently, US remains the reference method for fetal-sex determination in equids, using either TR or TA approaches depending on gestational age (Holder, 2011). However, accuracy depends on the operator experience, fetal position, and the timing of the examination (Curran and Ginther, 1989, 1993; Curran,1992). Three periods have been defined for fetal-sex determination in mares: early (55–90 days), mid (90–150 days), and late (> 150 days), using TR US for the first two and TA US for the last (Curran and Ginther, 1989; Renaudin et al., 1997; Holder, 2014; Van de Velde et al., 2018). Transabdominal examinations are not recommended beyond 210 days of gestation due to limited fetal visualization (Curran and Ginther, 1989; Renaudin et al., 1997, 1999; Holder, 2014; Mari et al., 2002; Van de Velde et al., 2018). Our LC-MS/MS-based approach, using multivariate models, complements these imaging modalities, enabling reliable fetal-sex determination (75–80 % accuracy between 169 and 308 days of gestation) beyond the gestational limits of TA US. Reported accuracies for US vary widely, from 65 % to > 90 % by experienced veterinarians (Mari et al., 2002; Resende et al., 2014; Bucca,2005), possibly reflecting that multiple examinations are often performed throughout gestation (Livini, 2010; Renaudin et al., 1999). Transrectal US accuracy is highest in early gestation, reaching 81 % within the optimal window of 59–68 days, but declines in mid-gestation, with correct diagnoses reported in 49 % of cases around 90 days and 26 % after 160 days (Livini, 2010; Renaudin et al., 1999). A large-scale study using TA US for late fetal sexing (Tönissen et al., 2016) reported performances ranging from 71 % at 4 months, to 58 % at 5 months, 49 % at 6 months, 49 % at 7 months, and 54 % at 8 months. Moreover, these studies often exclude inconclusive TA US examinations, with up to 16 % of cases resulting in an indeterminate diagnosis (Tönissen et al., 2016), whereas our hormonal method is applicable to nearly all mares.

To the best of our knowledge, this is the first study to include an independent validation cohort. Our Validation group (n = 22) demonstrated that serum hormonal analysis reliably predicts fetal sex from mid- to late pregnancy (169–308 days), with accuracies ranging from 75 % to 80 %. Lower accuracies observed during early pregnancy (blocks 5–6 = 47.1 %), are consistent with TA US, achieving slightly higher values over the same period (49.0–58.0 %). Accuracies then increased to 75.0 % in later blocks, generally matching or exceeding those reported for TA US during the corresponding mid- to late-gestation periods (49.0–54.0 %) (Tönissen et al., 2016).

Furthermore, accuracy seems to be fetal-sex dependent, with F fetuses identified more accurately in late pregnancy using US (Van de Velde et al., 2018). Interestingly, our study showed that accuracy for F fetuses exceeded that for M in late gestation (blocks 10 and 11). In contrast, our LC-MS/MS-based model showed higher accuracy for predicting M fetuses throughout most of gestation.

Isolation of cffDNA from maternal serum offers another non-invasive alternative, but it is feasible only during the last trimester, later than the time frame accessible with our LC-MS/MS method. Reported accuracy for cffDNA-based fetal sexing averages 85 % with a Se of 72 % (de Leon et al., 2012), depending on PCR methodology (de Leon et al., 2012; Kadivar et al., 2021). Its performance is equivalent to, or slightly lower than, what is observed with our multivariate hormonal models described here for the same gestational period, with accuracies (76.9–80.0 %) and Se (75.0–83.3 %) in our independent validation cohort. Improved understanding of cffDNA kinetics in maternal circulation (Allen and Stewart, 2002) could contribute to improve its accuracy for fetal-gender determination.

As a limitation, our Validation group included only 22 pregnancies, as priority was given to maximize the number of mares in the Test group in order to build the most robust statistical model and to explore potential threshold ranges for fetal-sex prediction. This design may have slightly reduced predictive accuracy and warrants replication in a larger, independent population. Early gestational data were also limited, restricting assessment before four months of pregnancy. Neither prior inclusion of mares in the Test group nor fetal-sex distribution seems to influence predictive accuracy in the Validation group. Given its small sample size, differences may have gone undetected and statistical power was limited. Future work should investigate whether prior inclusion or sex-dependent effects become apparent with larger cohorts and explore additional steroid markers to strengthen predictive models.

The analytical LC-MS/MS method for conjugated steroid profiling offers dual applications in equine practice. It enables early, non-invasive fetal-sex determination through simple blood collection and sample shipment to a laboratory, allowing use by any veterinarian in the field. Knowledge of fetal sex during gestation can assist breeders in optimizing their herd management, mating strategies, and sales planning, particularly for high-value genetic crosses. The method is rapid, and minimally invasive compared to the US, requiring no specialized equipment or extensive training, though its predictive value remains preliminary. Moreover, longitudinal steroids monitoring could serve as a biomarker of fetal well-being or placental pathologies, offering new opportunities for early diagnosis and intervention in gestational pathologies such as placentitis, ultimately improving reproductive outcomes.

5. Conclusion

To the best of our knowledge, this study represents the first longitudinal investigation in a large cohort of mares integrating validated and precise quantification of maternal serum conjugated steroids by LC-MS/MS with an assessment of fetal sex-specific dynamics throughout equine gestation. Our results demonstrate that these steroid profiles provide reliable, non-invasive biomarkers for fetal-sex determination from mid- to late gestation. Mare-specific data, including age, parity, and breed, did not significantly influence fetal sex or conjugated steroids patterns. On the other hand, sex-specific hormonal trajectories were observed during equine gestation. In M-bearing pregnancies, concentrations of classical conjugated estrogens were lower during early mid-gestation (block 5) and higher thereafter, while atypical estrogens (EQS and EQG) remained consistently lower in M fetuses throughout gestation. The univariate E1S model exhibits strong predictive performance across most of gestation, but multiplexed conjugated steroid profiling are achieving higher overall accuracy (75–80 %) in our independent validation cohort across blocks 7–11

(169–308 days of gestation except block 10 = 253–280). This conjugated steroid LC-MS/MS assays offers a minimally invasive, field-applicable alternative to conventional fetal-sexing methods, which are often more invasive, require specialized veterinary expertise, or have limited accuracy.

Future studies with larger cohorts and earlier gestational sampling are warranted to evaluate the feasibility of fetal-sex determination during the first four months of pregnancy. Combining hormonal profiling with complementary approaches, such as US or cffDNA analysis, may further improve predictive accuracy throughout gestation. Additionally, investigation of other steroid markers and their enzymatic pathways will improve understanding of feto-placental steroid metabolism under physiological conditions and refine predictive models for early and reliable fetal sexing in equine practice.

CRediT authorship contribution statement

Thomas Dubrowski: Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Ledeck Joy Christel:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Stéphanie Peeters:** Writing – review & editing, Methodology. **Matthieu Schoumacher:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Sophie Egyptien:** Writing – review & editing. **Caroline Le Goff:** Writing – review & editing, Methodology. **Etienne Cavalier:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Stéfan Deleuze:** Writing – review & editing. **Jérôme Ponthier:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

Ethical statement

This study was conducted in accordance with the Ethics Committee for Animal Experimentation of the University of Liege (protocol no. 21–2392), and samples were collected following informed consent from the animal owners.

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Declaration of Competing Interest

All authors of the paper entitled: "Equine fetal sex determination from mid-gestation to term using serum conjugated steroid profiling by liquid chromatography-tandem mass spectrometry" have no conflict of interest to declare.

This study was performed in accordance with the Ethics Committee for Animal Experimentation of the University of Liège (protocol no. 21–2392), and samples were collected with the informed consent of the animal owners.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2026.108098.

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