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Evolution of 17-β-estradiol, estrone and estrone-sulfate concentrations in late pregnancy of different breeds of mares using Liquid Chromatography and Mass Spectrometry

Joy Ledeck [a], Patrice Dufour [b], Élise Evrard [a], Caroline Le Goff [b], Stéphanie Peeters [b], Flore Brutinel [a], Sophie Egyptien [a], Stéfan Deleuze [a], Étienne Cavalier [b], Jérôme Ponthier [a, *]

[a] Equine Theriogenology, Equine Clinical Sciences Department, FARAH Comparative Veterinary Medicine, Liège University, Belgium
[b] Clinical Chemistry, University Hospital (CHU), Liège University, Belgium

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ABSTRACT

This study describes 17-β-estradiol (E2), estrone (E1) and estrone-sulfate (E1S) concentrations between 4 and 11 months in healthy equine pregnancies of two different breeds using Liquid Chromatography coupled to Mass-Spectrometry (LC-MS).

In 2 stud-farms including 15 Spanish PureBred (SPB) and 11 Showjumping (SJ) types mares, combined thickness of the uterus and the placenta (CTUP) was measured and blood was sampled monthly between 4 and 11 months of gestation. Concentrations of E2, E1 and E1S were assayed with LC-MS in mares with normal CTUP. Effects of breed, day of pregnancy and mare's parity and age on estrogens concentrations were investigated.

Peak of E2 was observed at 5 months (median: 46.4 pg/mL; maximum: 201.5 pg/mL). A strong correlation was observed between E1 and E1S ($p < 0.0001$, $r = 0.85$). Peak of E1 (median: 571.0 pg/mL; maximum: 1641.9 pg/mL) and E1S (median: 573.6 ng/mL; maximum: 997.6 ng/mL) concentrations was observed at the 5th month and then E1S decreased quicker than E1 until the end of pregnancy. Higher E2 and E1 concentrations were observed in SJ than in SPB mares between the 6th and the 8th months. No difference between breeds was observed for E1S monthly evolution.

Estrogen peak values were all observed at 5 months. Unlike recent LC-MS studies, E1S values observed here were in the same range than those previously established using immuno-assays. After the 6th month, E1S decreased quicker than E1. Effect of breed only observed on non-sulfonated estrogens should be further confirmed. These findings confirm that sulfonation activity of the allantochorion may be limited after the 6th month.

1. Introduction

Estrogens are a complex class of steroid hormones, characterized by an aromatic A ring and an alcohol group on the 3-carbon [1]. Mammals use aromatase to transform androgens in estrogens: e.g. estrone (E1) is directly derived from androstenedione [1]. Placental production of sulfonated or non-sulfonated estrogens has been described in many ungulates, including cattle [2], reindeer [3], buffalo [4] and American bison [5,6].

Estrogen production in pregnant mares has been described in the early 1930's (For review: Conley and Ball, 2019 [7]). The onset of this production has been described in 25-days embryos [8]. Later, the equine allantochorion uses fetal androgens to produce sulfonated or non-sulfonated estrogens, including some unusual derivates with an unsatured B-ring: equilin and equilinin [8–15]. Estrogen concentrations increase in mares' serum from about 80 days of pregnancy, peak at 5–6 and 7–8 months for, respectively, estrone and equilin or equilenin [13–15], and then slowly decrease until the foaling [16,17]. The physiological role of estrogens remains unclear during the second part of the equine gestation, but experimental inhibition of their production reduced foal weight at birth [18]. Thus, estrogens have been of interest for late pregnancy

* Corresponding author.
E-mail address: Jerome.Ponthier@uliege.be (J. Ponthier).

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diagnosis [17], fetal well-being assessment [19] and placental pathologies diagnosis [20,21].

For many years, estrogen concentrations were determined using Radioimmunoassays (RIA), Enzyme-Linked Immunoassays (ELISA) or other immuno-assays, but results differed considerably depending on the method used [7,22]. As an example, estrone-sulfate (E1S) concentration assayed by RIA was around 40 ng/mL in the serum of 9-months pregnant mares [23], whereas an ELISA used by another team gave values between 600 and 800 ng/mL for the same period of gestation [18,20]. Recent reviews about pregnant mare endocrinology [7,17,24] asked for standardization of the references' values and suggested to use of Liquid Chromatography coupled to Mass Spectrometry (LC-MS).

Recent studies using LC-MS in pregnant mares described an E1S concentration peak at 26 weeks of gestation with values of ±60 μg/mL [25]. These concentrations are 100 to 1000 times higher than those previously reported with immuno-assays [18,20,23], but this preliminary study was performed on a small number of mares of the same breed [25]. During the validation of our LC-MS method [22], observed values were in the range currently described by immuno-assays: median E1S concentration in late pregnant mares was 108.9 ng/mL with a maximal value of 683.7 ng/mL. Concentrations of E1 and 17-β-Estradiol (E2) were also lower, with respective medians of 154.4 pg/mL and 11.8 pg/mL [22]. However, the 31 mares included in this study [22] were sampled only once after 7 months of pregnancy, thus requiring a dedicated study to describe the reference values throughout the gestation.

Aim of this study was to describe E2, E1 and E1S concentrations and their potential relationships from 4 months until the end of pregnancy on healthy mares using LC-MS. For the validation study [22] and the present report, E2 and E1S were selected because they are frequently assayed in current equine practice, respectively for cycle pathologies and for pregnancy diagnosis. Estrone was selected to learn about the sulfonation metabolism and its limits. The effects of the mare's age, parity and breed on estrogens production were also investigated, using 2 different types of horses that are not cross-bred.

2. Materials and methods

2.1. Animals and sampling

Two stud-farms breeding respectively 17 Spanish PureBred (SPB) and 13 showjumping (SJ) type mares were enrolled in this study. The SJ mares were registered in sBs and BWP, the main Belgian studbooks, or in KWPN, the Dutch studbook, and their mean part of thoroughbred genetics was 36.2 ± 4.11%. Both stud-farms were localized in the same region, 7 km away (in municipality of Manhay, Wallonia, Belgium). The mares were not used for sport and kept outside on ryegrass from April to October. During the winter, they were housed in a barn and fed with wrapped hay and commercial food dedicated to breeding mares. The mares' median body score was 7/9 for SPB and 6/9 for SJ. No embryo-recipient mare was included and all mares were inseminated or naturally mated by stallions of their respective breed. Fetal sex, age and parity of each mare were recorded.

Once a month, in each stud-farm, mares with pregnancies exceeding 4 months were blood sampled Blood samples were collected in serum-tubes by venipuncture of the jugular vein. They were centrifuged (1000×g) within the 2 h and the serum was stored at −80 °C until LC-MS assays. At the same time, transrectal ultrasonography (US) was performed and combined thickness of the uterus and the placenta (CTUP) was measured [26,27] using EXAPad Mini® with a 7.5–10 MHz linear probe (IMV Imaging, Angoulême, France). The mean of the 3 CTUP measures performed in the cervical area was recorded and assigned to the respective estrogen assays in the database. The mare was no longer included in this study when the mean CTUP was increased or the cervical area had heterogenous aspect at US [26,27]. Placentas were examined after birth and mares were excluded a posteriori from this study if the cervical part of the allantochorion was thickened and/or congestive. Adverse events like abortion, prematurity, dysmaturity, weak foal or septicemia also led to the exclusion of the mare.

The ovulation day of the mare was recorded and the duration of pregnancy at the day of sampling was then calculated. Then, each sample was attributed to a month of pregnancy by dividing the day of pregnancy by 30. Samples of the same month were pooled to establish monthly estrogen reference values. For each mare, the day of foaling was recorded and the number of days between sampling and foaling was calculated: each sample was also associated to a month before foaling.

2.2. 17-β-estradiol (E2), estrone (E1) and estrone-sulfate (E1S) assays in serum

Steroids were assayed using the validated Liquid Chromatography and Electrospray Tandem Mass Spectrometry (LC-MS) method previously developed by our team [22]. Powder of E1S sodium salt (Sigma-Aldrich, St. Louis, MO, USA) dissolved in methanol, solutions of E2 (LGC Standards, Luckenwalde, Germany) and E1 (Cerilliant, Round Rock, TX, USA) were used as reference standards [22]. Briefly, 100 μL of serum were extracted with 400 μL of acetonitrile. Extract was evaporated to dryness and then derivatized with dansyl chloride. After derivatization, samples were injected for analysis on a Shimadzu Nexera X2 LC-30AD (Shimadzu Co., Kyoto, Japan) equipped with a BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size; Acquity UPLC, Waters). The HPLC system was connected to a linear combination of triple quadrupole and OrbiTrap mass analyzer, QTrap 6500 (ABSciex, Framingham, Massachusetts, USA) operating in triple-quadrupole mode. The Limits of Quantification (LLOQ) were established at 2.0 pg/mL, 2.0 pg/mL and 0.5 ng/mL for E2, E1 and E1S respectively [22].

2.3. Statistics

Graphpad Prism® was used (version 9.0 for Mac OSX, Graphpad Inc., San Diego, USA) and statistical significance was established at $p < 0.05$ for this double-blind prospective study. Normal distribution of values was tested with Kolmogorov-Smirnov test. Normal distribution was observed for CTUP and age of the mares, thus results are expressed in mean ± standard deviation. An ANOVA1 model with a Tukey post-test was used to determine differences in CTUP according to month of pregnancy. Student *t*-test was used to determine difference in mares' age between breeds and differences in CTUP between breeds for the same month of pregnancy. As parity, E2, E1 and E1S concentrations were not normally distributed, results are expressed in medians, and minimal or maximal value is presented when required for comprehension. Kruskall-Wallis test was used to determine differences in concentration of each hormone between months with a Dunn post-test. This test was also used to determine differences in mares' parity between SPB and SJ, as this parameter was not normally distributed. An ANOVA2 mixed model was used to assess a potential combined effect of age's class and breed or parity's class and breed on estrogen concentrations. For each estrogen (E2, E1 and E1S), a multiple linear regression model was tested (Estrogen concentration ~ Intercept + Fetal sex + Breed + Parity value + Age value + Day of pregnancy). For each hormone concentration,

difference between breeds at the same month of pregnancy was determined using a Mann-Whitney test. Hypothetical difference in fetal sex ratio between breeds was determined using Fisher's exact test.

3. Results

3.1. Clinical and ultrasonographic follow-up of pregnancies

During the protocol, a SPB mare was sold and a SJ mare had to be euthanized for acute laminitis, thus leading to incomplete sets of data. At the 9th month of pregnancy, a SPB mare showed an enlarged CTUP (mean of 3 measures: 8.6 mm) with an heterogenous cervical area of the allantochorion, suggesting placental detachment. She received the current treatments for placentitis (altrenogest and antibiotics) and gave birth to a normal foal, but the placenta was thick and congestive in the cervical area. A SJ mare also had an increased CTUP (mean of 3 measures: 10.6 mm) with an heterogenous aspect at 10 months of pregnancy. She was treated in the same way, but gave birth to a weak foal. Finally, 15 SPB and 11 SJ mares were enrolled in this study: the macroscopic examination of their placentas did not show any abnormalities and their foals were lively at birth.

Mean age of included SPB and SJ mares was respectively 15.4 ± 5.5 years old and 10.3 ± 4.1 years old, whereas median parity was 6 (minimum = 0 and maximum = 14) and 3 (minimum = 0 and maximum = 9), respectively. No significant differences in fetal sex ratio and in mares' age or parity were observed between breeds (mean age: 12.2 ± 5.3 years; median parity: 4 foals before inclusion in this study). Mares were also divided in groups according age and parity: young mares (<12 years old) and old mares (>13 years old); low parity number mares (<4 pregnancies before the ongoing one) and high parity number mares (>5 pregnancies before this one).

The CTUP measures increased gradually during pregnancy and were significantly increased ($p < 0.01$) at 11th month when compared to previous months. No significant difference was observed between the 9th and the 10th month CTUPs, but they tended to be higher ($p < 0.1$) than those observed at 6, 5 and 4 months of pregnancy.

3.2. General description of 17-β-estradiol (E2), estrone (E1) and estrone-sulfate (E1S) during equine pregnancy

Concentrations of E2 and E1 are expressed in pg/mL, whereas E1S concentration is expressed in ng/mL. Concentration of E2 was peaking at the 5th and the 6th months (respective medians 46.4 and 37.4 pg/mL) and those observed during later months were significantly lower. Fig. 1 depicts the E2 evolution from 4 to 11 months of pregnancy and shows a slight non-significant increase in the last month of pregnancy. Concentration of E2 tended ($p = 0.067$) to increase in the 30 last days of pregnancy when data were re-organized to calculate days before foaling.

Peak E1 concentration was also observed in the 5th and the 6th months of pregnancy with respective median concentrations of 571.0 and 504.3 pg/mL, and a maximal single value of 1641.9 pg/mL observed at 5 months. Then, E1 concentration constantly decreased until the 11th month, but was remaining above the LLOQ (11th month median: 80.2 pg/mL). The peak of E1S was observed in the 5th month with a median concentration value of 573.6 ng/mL and a maximal single value of 997.6 ng/mL. Lowest E1S concentrations were observed in the 9th, 10th and 11th months (respectively 105.8, 76.4 and 49.7 ng/mL), but were all above the LLOQ. Fig. 2 shows the evolution of E1 and E1S and statistical differences between monthly concentrations of each estrogen.

A positive significant but weak correlation was observed between E2 and E1 ($p < 0.0001$; $r = 0.63$) and between E2 and E1S ($p < 0.0001$; $r = 0.52$) concentrations. A positive significant strong correlation ($p < 0.0001$; $r = 0.85$) was observed between E1 and E1S concentrations. Assuming that E1 and E1S values were normally distributed, a linear regression ($p < 0.0001$, $R^2 = 0.72$, $Y = 0.7562*X+25.39$) could be drawn (see Fig. 3).

3.3. Age, parity and breed effect for CTUP, 17-β-estradiol (E2), estrone (E1) and estrone-sulfate (E1S)

No difference was observed for CTUP between SPB and SJ mares at the same month of pregnancy. No E1S concentration difference was observed between SPB and SJ mares. ANOVA 2 mixed models showed effect of breed on E2 and E1, whereas no effect of parity or age classes could be observed. For E2 and E1, multiple linear regression models showed an effect of the breed (respectively, $p = 0.0016$ and $p = 0.0003$) and of the day of pregnancy (respectively, $p = 0.0028$ and $p < 0.0001$), whereas no effect of fetal sex, age or parity of the mare was observed. On the contrary, multiple linear regression model showed no effect of the breed on E1S concentration. Median concentration of E2 and E1 was higher (respectively, $p < 0.01$ and $p < 0.05$) in SJ mares between the 6th and the 8th months of pregnancy (see Fig. 4 for E1 evolution).

4. Discussion

To the best of our knowledge, this is the largest set of late pregnant mares' serum samples used to assay estrogens with a LC-MS method.

Concentrations of E2 were above our previously described 2 pg/mL LLOQ [22] and showed a large variation between mares at the same stage of pregnancy that could be partially explained by the potential breed effect observed on E2 concentration. During the entire gestation, concentrations of E2 were low compared to E1 and it can be explained by the important expression of Hydroxysteroid Deshydrogenase 17β2 (HSD17β2) by the endometrium [28]. This enzyme balances E2 production by promoting E1 synthesis, leading to lower E2 concentration than E1 [28], like in the present set of data. The nadir values observed during the 9th and the 10th months were just above those observed with a RIA in estrus mares, whereas peak values of the 5th and 6th months were largely exceeding those values of cycling mares [29,30]. In the last month of gestation, E2 concentration showed a non-significant increase, that can be explained by the E2 peak observed during the last pregnancy days [7,31,32]. Observed E2 peak concentration was below the previously described values obtained by ELISA in pregnant mares [18,31], but in the same range than those previously observed in jennies with a RIA [32]. However, comparison between these different immuno-assays performed by different teams remains hazardous and generalization of LC-MS use could limit these results variations.

Differences in E2 and E1 pregnancy curves were observed, as their correlation was significant but weak. Peak of E1 was observed at the 5th and the 6th months, but constantly decreased thereafter. Concentrations of E1 were also expressed in pg/mL and above the LLOQ whereas non-pregnant mares showed E1 concentration below the LLOQ in our previous report [22]. Between 4 and 11 months, observed concentrations were also dramatically above those observed with RIA in cycling mares [33]. To the best of our knowledge, this is the first report describing E1 references values during equine healthy pregnancies with LC-MS. For many years, E1S concentration has been assayed using different immuno-assays and has been the only late endocrine pregnancy diagnosis in mares [15,18,20,23,34]. However, this study shows that E1 could equally be used for late pregnancy diagnosis in mares, as E1 concentrations in pregnant mares were above those of cycling mares and highly

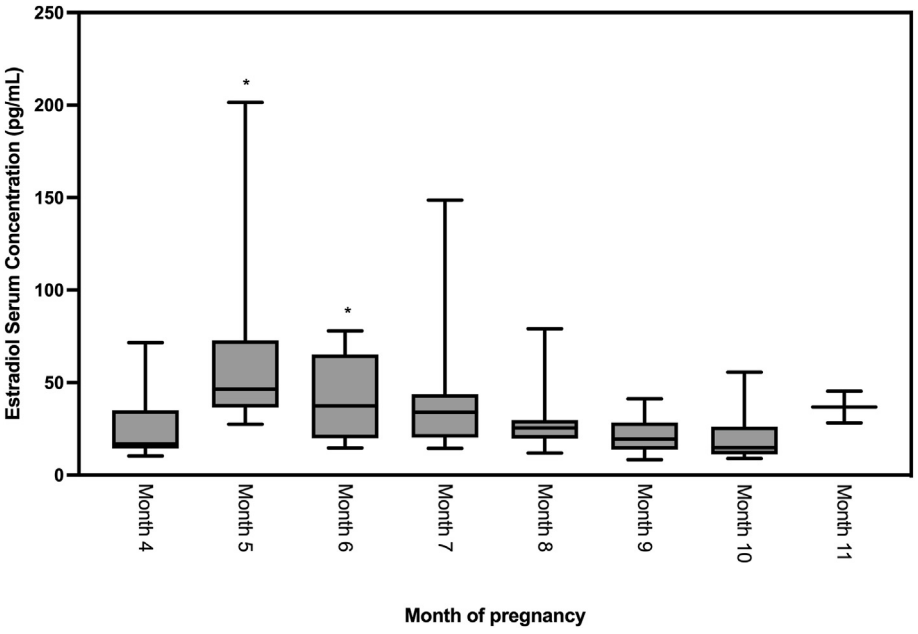

Fig. 1. Evolution of 17-β-estradiol (E2) serum concentration from 4 to 11 months of pregnancy in the general population of mares.
Whiskers show minimal and maximal E2 concentrations (pg/mL), whereas boxes represent 75 and 25 quartiles and the lines inside represent the median values for each month of pregnancy in both Spanish PureBred (SPB) and Showjumping (SJ) type mares. E2 medians with the superscript * are significantly different from others ($p < 0.05$).

positively correlated with E1S, with a nearly linear relation.

In our cohort, E1S concentration peaked at the 5th and the 6th months, and then declined more abruptly than E1, despite their positive and strong correlation. Non-parallel evolution of E1 and E1S production after 7 months could be explained by a quicker decrease of the sulfonation capacity than the androstenedione aromatization activity of the allantochorion. To corroborate this, a recent study [28] reported that expression of sulfotransferases moved from the endometrium to the allantochorion at around 6 months, and that allantochorionic expression of sulfotransferase was lower than in the endometrium. A previous study using RIA [23] described E1S peak at the 6th month of pregnancy, later than our maximal median concentration. This difference could be explained by the different experimental design in terms of sampling timing and assay methods. These authors [23] also observed approximately 4 times lower E1S concentrations (±130 ng/mL) at the peak than in the present set of data. On the other hand, in another study inducing placentitis in 9-month pregnant mares and using immuno-assay [20], E1S concentration in the control group was approximatively 3 times higher than in our cohort. Another recent study using ELISA [18] limited their E1S assays to the 5 lasts months of pregnancy and also observed 2 to 3 times higher values

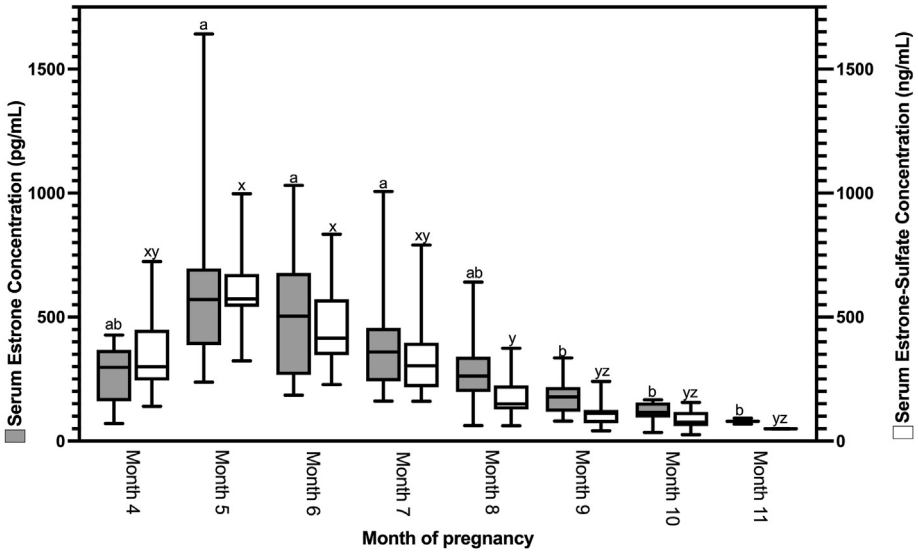

Fig. 2. Evolution of estrone (E1) and estrone-sulfate (E1S) serum concentration from 4 to 11 months of pregnancy in the general population of mares.
Whiskers show minimal and maximal concentration of E1 (■) in pg/mL and E1S (□) in ng/mL, whereas boxes represent 75 and 25 quartiles and the lines inside represent the median values for each month of pregnancy in both Spanish PureBred (SPB) and Showjumping (SJ) type mares. For E1, values with different superscript$^{(a, b)}$ are statistically different ($p < 0.05$). For E1S, values with different superscript$^{(x, y, z)}$ are statistically different ($p < 0.05$).

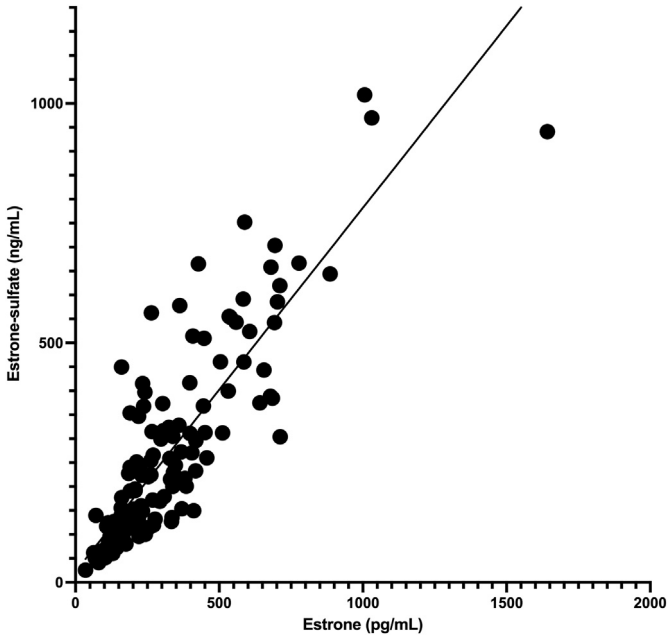

Fig. 3. Linear Regression between estrone (E1) and estrone-sulfate (E1S) concentration in serum of both Spanish PureBred (SPB) and Showjumping (SJ) type pregnant mares from 4 to 11 months.
Data of the Linear Regression: $p < 0.0001$, $R^2 = 0.72$, $Y = 0.7562 * X + 25.39$.

than those reported here for the same month. However, in these reports and in our data, E1S concentrations rarely exceeded 1000 ng/mL, unlike in a recent study using another LC-MS technique that described E1S concentration of ±60 μg/mL at 6.5 months

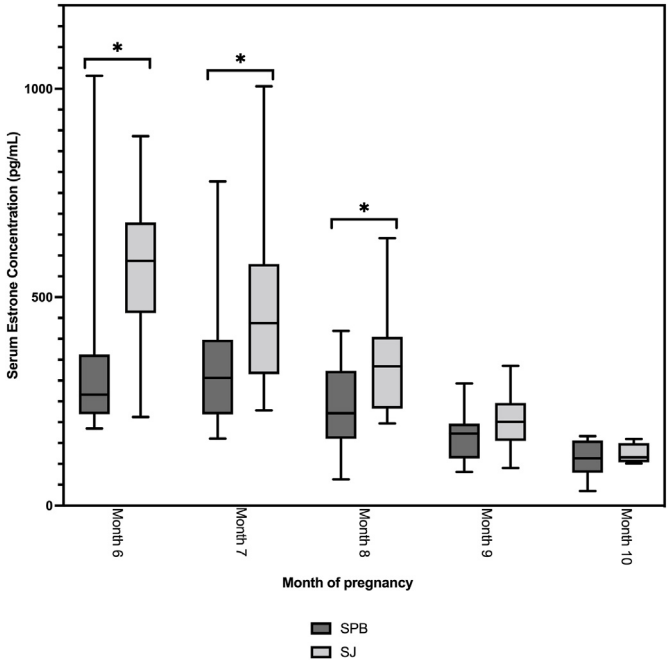

Fig. 4. Comparison of estrone (E1) concentrations between Spanish PureBred (SPB) and Showjumping (SJ) type mares at 6, 7, 8, 9 and 10 months of pregnancy.
Whiskers show minimal and maximal concentration of E1, whereas boxes represent 75 and 25 quartiles and the lines inside represent the median values for each month of pregnancy. The superscript* indicates a statistical ($p < 0.05$) difference in E1 medians between SPB (Spanish PureBred) and SJ (Showjumping) type mares at the same month of pregnancy.

of pregnancy [25]. As previously discussed [22], this important discrepancy could be explained by different sensibilities of the LC-MS devices and settings. However, nutrition, presence of phyto-estrogens [35] or other effects could also have interfered. Exchanging pregnant mare serum samples between teams and comparing estrogen concentrations assayed with different LC-MS methods, devices and settings could help to understand these differences and lead to a standardization of this method.

Among the possible effects that may impact estrogen concentrations, differences in E2 and E1 concentrations were observed between SPB and SJ mares, whereas age and parity effects couldn't be observed on any estrogen concentration during pregnancy. To explain those observed breed differences in non-sulfonated estrogens between the 6th and the 8th months, an eventual breed difference in CTUP was also investigated, but no differences could be observed, despite a recent report [27] suggesting an effect of the breed on feto-maternal unit thickness. To the best of our knowledge, this was the first study designed to compare the CTUPs of different mares' breeds with the same settings. No difference was observed with this protocol between these breeds of similar size.

Present data would lead to hypothesize that sulfonation activity of the allantochorion is limited between 6th and 8th months in both breeds, whereas non-sulfonated estrogen production is higher in SJ than in SPB. Then, their production decreases below the saturation step of the sulfonation activity in both types of mares. As previously reported, the sulfotransferase genes expression is sequential and reduced when the allantochorion replaces the endometrium after the 6th month [28]. This is corroborating our hypothesis of limited sulfonation activity in all breeds after 6 months, whereas SJ produce more E1 and E2 between 6th and 8th month. However, as a limitation, both subpopulations were housed in different stud-farms, with different management, despite being geographically very close. Re-implantation of embryos in recipient mares from a different breed could help to understand these findings. From a clinical point of view, these references values varying between breeds may undermine the feasibility of using E1 and E2 assays between 6 and 8 months for diagnosis of placentitis or of other causes of compromised pregnancy. On the other hand, E1S does not show differences between breeds and is still of interest. However, screening of all estrogens presents in pregnant mares, including sulfonated and specific ones like equilin and equilinin, or maybe other exotics derivates, should be performed to broaden the framework.

5. Conclusion

Using a large number of pregnant mares, peak values of E2, E1 and E1S were all observed at the 5th month. Concentrations of E2 were low and comparable with those observed in estrus, whereas E1 and E1S were dramatically higher. However, in contrast with a recent study, E1S concentration observed with our LC-MS assay was in the ranges previously established with immuno-assays. After the 6th month, E1S was decreasing quicker than E1 and a potential breed effect that should be confirmed was only observed for non-sulfonated estrogens. This strongly suggests a limited sulfonation activity of the allantochorion after the 6th month. As breed effect was not observed for E1S concentration during pregnancy, its assay by standardized LC-MS assays would remain of interest in the diagnosis of placentitis. However, enlarging the array of estrogens assayed would help to understand the endocrinology of the equine pregnancy and the differences between physiologic and pathologic gestations.

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Credit authorship contribution statement

Joy Ledeck: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Methodology, Writing – original draft, Writing – review & editing. **Patrice Dufour:** Conceptualization, Data curation, Formal analysis, Investigation, Validation, Methodology, Writing – original draft, Writing – review & editing. **Élise Evrard:** Investigation, Writing – original draft. **Caroline LE Goff:** Data curation, Investigation, Software, Validation, Writing – original draft, Writing – review & editing. **Stéphanie Peeters:** Data curation, Investigation, Software, Validation, Writing – original draft, Writing – review & editing. **Flore Brutinel:** Data curation, Investigation, Writing – original draft. **Sophie Egyptien:** Data curation, Investigation, Writing – original draft. **Stéfan Deleuze:** Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. **Étienne Cavalier:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft. **Jérôme Ponthier:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

Authors have no conflict of interest to declare.

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