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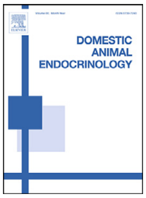

Analysis of progesterone and estrone-sulfate in feces of American Bison using liquid chromatography coupled to mass spectrometry: Technical validation and correlation with blood levels

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ABSTRACT

American Bison's wild nature limits blood sample availability to study its endocrinology. This report describes progesterone (P4) and estrone-sulfate (E1S) assays in American Bison feces using Liquid Chromatography coupled with Mass Spectrometry (LC-MS). In 2 ranches, samples of feces (n = 73) and serum (n = 93) were collected in pregnant and nonpregnant American Bison. Feces samples (250 mg) were extracted with methanol, purified, and concentrated. Then, feces and serum samples were assayed using LC-MS, according to our previously described technique. Fecal matrix homogeneity was determined by measuring steroids in different areas of the sample and concentration evolutions were evaluated after storage at room temperature. During the validation process, lower limits of quantification were 20 pg/g (E1S) and 4 ng/g (P4) by meeting the following criteria: relative standard deviation <15% and relative bias <15%. By measuring hormones in different spots from the same sample, a moderate variability for E1S (coefficient of variation [CV] up to 21.3%) and a high variability for P4 (CV up to 85.5%) were highlighted. Correlation between concentrations in feces and in serum was higher for E1S (r = 0.77) than for P4 (r = 0.65) and P4 could be assayed in pregnant and nonpregnant animals whereas E1S was only present in pregnant. Feces storage at room temperature induced modification of steroid concentrations. The quantification of E1S and, at a lower level, of P4 in feces is an interesting alternative to serum assay to describe the pregnancy-related evolution of these steroids in American Bisons, with feces ideally stored frozen and mixed before the LC-MS procedures.

1. Introduction

Due to the American Bison wild character and its extensive breeding management, blood sample availability is limited [1] and reproductive endocrinology of nonpregnant and pregnant animals is poorly described. Bison's estrus cycle has been estimated to be around 21 d [2,3]. They ex-

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hibit 5 to 6 cycles [3] during the ovulatory season in autumn, whereas an anestrus is observed during the rest of the year (Cervantes et al. [4]). In a natural environment, American Bison calving season is spring (May–July) after around 260 to 285 d of gestation [5–7], although breeding season, grazing areas, and nutrition could modulate this duration [6,7]. The endocrinology of American Bison and bovine gestations seems to share common features, such as the production of pregnancy-associated glycoproteins (PAG) [6,8,9] and estrogens [5,8,10,11].

Recent studies using liquid chromatography and mass spectrometry (LC-MS) in American Bison [8,10] showed that progesterone (P4) was slowly increasing in serum during the first trimester of American Bison pregnancy and seemed to be steady in its 2 last trimesters. However, P4 concentrations were high in some bulls or nonpregnant cows, and a large P4 interindividual variation was observed in pregnant American Bison [8]. Estrone (E1) and estrone-sulfate (E1S) productions were observed in second and third trimesters of pregnancy, but E1 seemed to be a residue of the placental production of E1S [8]. Despite E1S interindividual variability, it was constantly increasing in American Bison sera during pregnancy [8].

However, the collection of blood samples requires gathering and immobilization of American Bison that could induce stress and injuries, as previously suggested [12]. Urinary and fecal sexual steroids assays are potential alternative solutions already tested in early studies on small numbers of American Bison at the third month of pregnancy [5]. In practice, urine is difficult to collect and only total estrogens were assayed in pregnant Bison cows' feces, without information about the type of estrogen produced [5], whereas P4 has been quantified in feces of superovulated American Bison cows [13]. Immuno-assays were used [5,13], and, they are not always properly validated to determine steroids concentration in the species' biological matrix [10,14], which may cause variations in the observed levels. As steroids blood levels in American Bisons were unknown, correlations between fecal and blood concentrations were not assessed [5].

This study aimed to develop a LC-MS method to determine P4 and E1S concentrations in American Bison feces. Correlations between fecal and serum steroids concentrations were determined to evaluate the physiological relevance of the feces assay. The homogeneity of E1S and P4 concentrations in feces was tested and the stability of those concentrations during storage at room temperature was assessed.

2. Material and methods

2.1. Animals and sampling

In the Belgian Ardennes (±50°N), 2 ranches are extensively breeding wild American Bison (*Bison bison*) imported from Montana (USA) since 1998 and 2004, respectively. Animals are freely grazing in meadows previously dedicated to beef cattle. The first breeding herd is divided into 2 separate meadows of 19 and 21 ha, each welcoming 2 sexually mature American Bison bulls and 25 and 26 sexually mature cows. In the second ranch, 10 sexually mature American Bison cows are roaming freely with 1 sexually mature bull in a 15-ha meadow. In both ranches, American Bison are not used to human presence and to manipulations. Once a year, animals are gathered and immobilized for mandatory disease screening in a specific handling/chute system. Between January and February 2019, and between February and March 2020, this opportunity was used to collect blood in 2 dry tubes by venipuncture under the tail of all matured American Bison. Only mature animals which reached sufficient size to be pubescent were sampled. Blood samples were centrifuged (1,000 × g for 10 min) and stored frozen (-80°C) until assays. Feces were also collected by rectal palpation (±50 mL) and were stored frozen (-20°C) within 6 h after collection until assays. Among the 5 American Bison bulls, 2 were sampled in 2019 and 2020. Among the 93 serum samples of American Bison cows, 52 were collected twice on the same animal in 2019 and 2020. Of the 73 feces samples of American Bison cows, 10 were collected twice on the same animal in 2019 and 2020.

Calving days of the American Bison cows were recorded for the year following the sampling procedures. Fertilization day of cows that calved was retrospectively obtained by subtracting 276 d from the calving day. Gestation day on the sampling day was then determined by subtracting the fertilization day of the sampling day. Other events, such as abortion, disease, culling, or prolonged gestation (more than 276 d after sampling) were reported by the breeders during the following year.

2.2. Chemicals and reagents

Reference standard and internal standard solutions were prepared from powder of E1S sodium salt and d_4-estrone-3-sulfate sodium salt (Sigma-Aldrich, St. Louis, MO, USA) dissolved in methanol and solutions of P4 and d_9-progesterone (Cerilliant, Round Rock, TX, USA). Water, acetonitrile, hexane, and methanol (LC-MS grade) were purchased from Biosolve (Biosolve, Dieuze, France). Ammonia solution (25% in water) (Merck KGaA, Darmstadt, Germany) was Suprapur grade and was purchased from Sigma-Aldrich. Homemade quality controls and calibrator samples were made by spiking standards in 500 mg of feces collected from cattle (*Bos taurus*) before extraction.

2.3. Progesterone (P4), Estrone-sulfate (E1S) assays in serum

Sexual steroids (P4, E1S) were assayed with our previously validated and described American Bison LC-MS technique [10]. For P4 and E1S, Lower Limit of Quantification (LLOQ), defined as the lowest concentration in the validation standards that reported relative standard deviation (RSD) and relative bias (RB) lower than 15%, were established at respectively 0.1 ng/mL and 0.5 ng/mL [8,10].

2.4. Feces sample preparation and LC-MS

About 500 mg (± 50 mg, weighed precisely) of calibrator, quality control, or American Bison's feces sample were spiked with 10 µL of internal standard mixture (d_4-E1S at 10 ng/mL and d_9-P4 at 400 ng/mL) before extraction. Two

Table 1
LC-MS conditions.

Parameters	Setting
Apparatus LC	Shimadzu Nexera X2 LC-30 CE (Shimadzu Co., Kyoto, Japan)
Apparatus MS	QTrap 6500 (ABSciex, Framingham, Massachussetts, USA)
Column	BEH C18 column (2.1 mm × 100 mm, 1.7 µm particle size) (Acquity UPLC, Waters)
Column temperature	40°C
Mobile phase (A)	0.02% NH4OH in water
Mobile phase (B)	Acetonitrile
Flow rate	0.4 mL/min
Source	Electrospray (negative and positive mode)
Ion spray voltage	-4,500V & 5,500V
Ion source temperature	650°C

Table 2
Intraday and interday accuracy and precision of estrone 3-sulfate and progesterone. These parameters were measured with validation standards prepared in cattle feces. Validation standards were analyzed in quintuplicate during the first day (intraday variation) and then in triplicate during 3 days (interday variation).

	E1S			P4		
	Target (pg/g)	RSD	RB	Target (ng/g)	RSD	RB
Intraday						
Level 1	20	12.0%	2.9%	4	3.4%	2.1%
Level 2	30	7.8%	-1.9%	6	4.2%	5.6%
Level 3	50	9.0%	2.3%	10	4.5%	6.1%
Level 4	800	5.4%	0.0%	160	3.2%	0.1%
Level 5	1000	7.2%	3.6%	200	4.5%	2.6%
Interday						
Level 1	20	10.9%	8.6%	4	2.2%	-0.4%
Level 2	30	5.5%	6.1%	6	1.8%	1.5%
Level 3	50	11.8%	5.2%	10	3.6%	2.3%
Level 4	800	8.0%	1.6%	160	1.4%	-2.2%
Level 5	1000	5.8%	0.7%	200	3.3%	-0.4%

Precision refers to the relative standard deviation (RSD = Standard deviation × 100/Mean value) and accuracy to relative bias (RB = [Mean of observed concentrations– Spiked concentration] × 100/Spiked concentration)

milliliter of methanol were added to the spiked feces, the mixture was vortexed for 10 S and then sonicitated for 15 min. Mixture was centrifuged (1,000 × g for 5 min) and the supernatant was collected and transferred to a glass tube. The extraction by methanol was repeated once and the methanol fractions were gathered. The methanol extract was washed with 3 mL of hexane and then evaporated to dryness under vacuum at 40°C overnight. The dry extract was then resolubilized in 100 µL of a water/acetonitrile (80/20) mixture and transferred in a 2 mL Eppendorf. The mixture was centrifuged at 16100 × g for 10 min and the supernatant was transferred to LC vials before injection into the LC-MS device.

The LC-MS parameters were exactly the same as those described in [10] except for injection volume which was set at 40 µL in the present study. The LC-MS conditions are gathered in Table 1.

2.5. LC-MS method validation

The whole method process was validated according to the guidelines provided by the USA Food and Drug Administration (FDA). For each validation day, 6-point calibration curves (ranges: 20–1000 pg/g for E1S; 4-200 ng/g for P4) were prepared in duplicate with cattle feces. Integrated peak area ratio between native hormones and marked internal standard was used to calculate the response. Intraday precision and accuracy were evaluated by analyzing 5 levels of validation standards prepared in quintuplicate in cattle feces (standard concentration are gathered in Table 2). Interday precision and accuracy were determined by repeating the analysis 2 more times. Precision corresponds to the relative standard deviation (RSD = standard deviation × 100/Mean value) and accuracy to relative bias (RB = [Mean of observed concentrations – Spiked concentration] × 100/Spiked concentration). Lower and upper limits of quantification were defined as the lowest and the highest concentrations, respectively, in the validation standards that reported RSD and RB lower than 15%. Extraction efficiencies and matrix effects were assessed on 2 levels (low: 50 pg/g for E1S and 10 ng/g for P4 and high: 500 pg/g for E1S and 100 ng/g for P4), each level was assessed in triplicate. Extraction efficiency indicated the percentage of hormone extracted from the matrix and was computed by the ratio of the area measured after extraction and the area determined in post-extraction spiked samples. Matrix effect reflects the effect of the matrix on the compound ionization, this effect could be positive (ionization enhancement) or negative (ionization suppression). Matrix effects were determined by the difference in percent between the ratio of area measured for the compounds and the area determined for the corresponding internal standard in a post-extraction spiked standard and the same ratio for pure standards.

Table 3
Homogeneity of bison fecal samples.

	Bison A		Bison B		Bison C		Bison D	
	E1S (pg/g)	P4 (ng/g)	E1S (pg/g)	P4 (ng/g)	E1S (pg/g)	P4 (ng/g)	E1S (pg/g)	P4 (ng/g)
Top	40.1	6.2	61.8	118.0	117.1	5.9	293.7	58.3
Left	52.5	3.9	50.6	34.7	154.5	4.8	252.1	40.3
Right	44.4	4.1	55.4	32.9	189.3	6.4	287.0	33.6
Bottom	43.2	4.2	51.3	22.1	131.3	4.2	324.3	28.0
CV	11.7%	23.8%	9.4%	85.5%	21.3%	18.9%	10.2%	32.9%

Table 4
Steroid stability in fecal samples.

	Bison A (pregnant)		Bison B (pregnant)		Bison E (not pregnant)		Bison F (male)	
	E1S (pg/g)	P4 (ng/g)	E1S (pg/g)	P4 (ng/g)	E1S (pg/g)	P4 (ng/g)	E1S (pg/g)	P4 (ng/g)
0h	40.1	6.2	61.8	118.0	<20	<4	<20	<4
24 h	132.9	12.9	74.9	>200	<20	20.2	<20	5.9
96h	>1000	< 4	>1000	26.4	<20	<4	<20	<4

2.6. *Homogeneity of fecal matrix and stability during storage at room temperature*

To assess homogeneity of fecal samples, 4 entire and unmixed feces from 4 different pregnant American Bison cows were collected shortly after the emission on the field and steroid hormones were determined in split samples taken from top, bottom, left and right part of each fecal sample. In order to assess stability of steroids in feces, in samples 1 and 2, the left part of the feces was homogenized and an aliquot was left at ambient temperature (15°C–25°C) for 24 h and another for 96 h before being analyzed. Stability at ambient temperature was also assessed in feces from a nonpregnant female and another fecal sample from an American Bison bull.

2.7. *Statistics*

Software R Studio was used (version 3.4.1; R Project for Statistical Computing) and statistical significance was established at $P < 0.05$ for this double-blind prospective study. Normal distribution of values was tested with Shapiro-Wilk test.

The spearman correlation test was used to determine correlations between serum and fecal hormones' concentrations. For E1S, correlation was only tested on the pregnant female cohort since E1S concentrations in serum and feces were below the limit of quantification in all males and nonpregnant females.

3. Results

During the validation process, correlation coefficients (r^2) for the calibration curve were observed to be greater than 0.99. Calibration curves were calculated using 1/x-weighted quadratic regression. The intra- and interday accuracy and precision determined during the validation process are summarized in Table 2. For both steroids and for each concentration tested, RSDs were less than 15% (RSD ranged from 1.4% to 12.0%), while RBs were less than 10% (ranging from -2.2% to 8.6%). Therefore, the limits of quantification (LOQ) of the method were set at 20 pg/g and 4 ng/g for E1S and P4, respectively. Extraction efficiencies were 63% (CV: 22%) and 67% (CV: 11.2%) for E1S in low and high levels samples respectively, while lower extraction efficiencies for P4: 13% (CV: 9.1%) and 9% (CV: 21.8%) were observed in low and high levels samples respectively. Matrix effects were 2.4% (CV: 13.7%) and -11.5% (CV: 5.2%) for E1S in low and high levels samples respectively and 24.5% (CV: 10.4%) and 19.7% (CV: 23.4%) for P4, in low and high levels samples respectively.

Homogeneity of the feces was tested by measuring the steroids in different parts of samples collected from pregnant females. The results are gathered in Table 3. The variation observed for E1S was moderate, (coefficients of variation [CVs] ranging from 9.4%–21.3%), but the variability for P4 was higher since the CVs observed in the 4 samples were above 15% and reached 85.5% in sample B.

Regarding stability, in pregnant females, different kinetics were observed for the 2 molecules: after 24 h at room temperature, both hormone levels increased, but after 96 h, E1S levels continued to increase, while P4 levels decreased. In nonpregnant females and males, E1S levels remained unquantifiable after 24 and 96 h of incubation at room temperature. Conversely, the kinetics of P4 were similar to those observed in pregnant females: an increase of P4 was observed from concentrations below the LOQ at t_0, to measurable levels after 24 h of incubation and then a return to levels below the LOQ after 96h of incubation (Table 4).

Table 5 gathers concentrations of steroids measured in the feces of males, nonpregnant females, and pregnant females. P4 was detected in all samples from pregnant females (median concentration = 21.1 ng/g; range: 5.03–266 ng/g) but also in 2 out of 4 males (median concentration = 5.84 ng/g; range: < 4–5.93 ng/g) and 11 out of 34 nonpregnant females (median concentration = < 4 ng/g; range: < 4–27.8 ng/g). The non-parametric correlation between feces and serum P4 levels was statistically significant ($P < 0.001$) but moderate ($r = 0.65$) with outliers (Fig. 1). E1S is a good biomarker to discriminate pregnant

Table 5
Median and range of concentrations for estrone sulfate (E3S) and progesterone (P4) in feces samples from males, pregnant females, and not pregnant females.

		E1S (pg/g)		P4 (ng/g)	
	n	Median	Range	Median	Range
Males	4	< LOQ	< LOQ–< LOQ	5.84	< LOQ–5.93
Pregnant females	35	250	< LOQ–2698	21.1	5.03–266
Not pregnant females	34	< LOQ	< LOQ–< LOQ	< LOQ	< LOQ–27.8

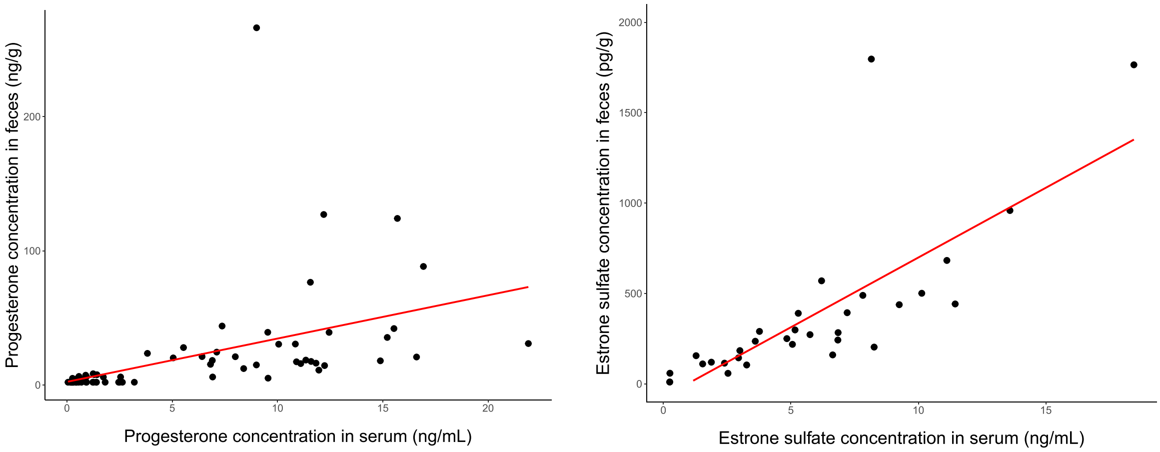

Fig. 1. correlations between progesterone and estrone-sulfate concentration measured in serum and those determined in feces samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

females from nonpregnant females and males, indeed, E1S was not detected in any samples from males or nonpregnant females. In pregnant females, the median concentration was 250 pg/g (range: $<$ 20–2698 pg/g). E1S concentration in feces was below the LOQ in 2 out of the 35 pregnant females showing also E1S and P4 below the LOQ in the serum: one of these American Bison cows was in the first trimester of pregnancy while the other had a spontaneous abortion shortly after the sampling. The correlation between serum and feces E1S concentration in pregnant females is significant ($P < 0.001$) and good ($r = 0.77$) (Fig. 1).

4. Discussion

Our previous study showed that serum E1S is an excellent biomarker to discriminate American Bison cows in their second or third trimester of pregnancy from males and nonpregnant females, whereas data in the first trimester of pregnancy were lacking due to the small number of Bison available in this period [8,10]. However, although serum E1S appears to be a good parameter for diagnosing pregnancy, its use on a large scale for herd monitoring is hampered by the need for immobilization and the associated risks for the animal and the medical team [1]. Using our previous results about steroids concentrations in serum, this study aimed to describe their assay on an easily available matrix: the American Bison feces.

This study demonstrates that quantification of E1S in feces using LC-MS is an efficient method to identify pregnant females. Many studies showed that fecal estrogens (conjugated or not) are a good marker for pregnancy in several species: cow [15], sows [16,17], Red buffalos, yaks, Nubian ibex [18], zebras [18–20], gorillas, sable antelopes, tapirs [19], kiang mares [21], white-tailed deers [22] and caribous [23], while estrone conjugates were not able to discriminate pregnant animals in big horn sheep [24]. However, in all these studies, not-specific immunoassays were used to measure estrogen concentrations, except in [17] where E1S is specifically determined. Therefore, the real identity of the estrogens is difficult to establish and the correlation between serum and feces concentrations was not always assessed.

The E1S median concentration observed in this study for pregnant females (250 pg/mL) was lower than those observed in an earlier study [5], where total sulfonated estrogens were assayed using RIA. All pregnant females presented E1S levels above the LOQ of 20 pg/g in feces, except one that was in the first trimester of pregnancy (the only available animal in this period), and another one that spontaneously aborted shortly after the sampling. None of the male and nonpregnant female feces samples showed measurable levels of E1S. Therefore, if we exclude the case of abortion, the sensitivity of E1S in feces to diagnose second or third-trimester pregnancy was 100% in our population. The high correlation observed between serum and feces E1S concentration was never reported in Bison, nevertheless, [17] reported a high correlation ($r^2 = 0.82$) between the plasma and the fecal concentrations of E1S in sows. Therefore, feces E1S concentration could be a useful tool to estimate the pregnancy period, as it was suggested in serum [8]. Moreover, this diagnosis performance seemed not to be affected by storage at ambient temperature, since E1S levels in feces stored up to 96 h increased in pregnant

American Bison cows and remained below the LOQ in non-pregnant females and males. A long delay between dung emission and sample collection or preanalytical storage in the lab wouldn't interfere with zootechnical results. These promising results should be confirmed and compared to other pregnancy diagnosis methods. Moreover, E1S in feces should also be tested on a larger number of animals in their first trimester of pregnancy.

The association between serum and fecal P4 is moderate, with Figure 1 showing outliers, which is consistent with the previous studies using commercial devices to assay progesterone in different species' feces, with a correlation ranging from 0.57 in hippopotamus to 0.79 in okapis [25]. The poor homogeneity of fecal samples (CV as high as 85% for P4 concentrations was observed in a sample) could partly explain this moderate correlation, in accordance with the heterogeneous distribution of steroids hormones reported in feces, as an example, from brown brocket deer [26]. Therefore, correlation with serum concentration could be improved by a step of homogenization for instance during the collection process before storage at -20°C. Stress-induced P4 production observed in cattle [12] could also explain this lower correlation. Immobilizing American Bisons would lead to variation in serum P4 concentrations depending on the animal's stress, while feces P4 levels wouldn't be immediately modified. Despite the low extraction efficiency for P4, this hormone could be measured in all samples from pregnant females. This low extraction efficiency is due to the wash step during the sample processing, this step was added to enhance the sensitivity for E1S by reducing the background noise signal (the fecal concentrations of E1S are 2 orders of magnitude lower than those of P4) and to increase the lifetime of LC column. Concentrations of P4 observed in the feces of pregnant American Bison cows using LC-MS were in the same range as those observed with ELISA in previously super-ovulated Bisons [13] and below those of early studies using RIA on the small number of pregnant animals [5]. Presence of sulfated progesterone [27] or other P4 metabolites [7] could explain this difference, as our LC-MS method was designed to only assay non-conjugated P4 [10]. Progesterone was chosen because it is present in blood during the bovine pregnancy and the common precursor of the P4 metabolites. However, an increase in progestins (mainly 20-oxo-pregnane metabolites) has been observed early in the pregnancy, making these steroids potential markers of early pregnancy in feces [7]. Unfortunately, the progestins levels were determined by immuno-assays, therefore it is not possible to exactly identify the metabolites measured. A full characterization of progestins in feces using time-of-flight mass spectrometry or nuclear magnetic resonance spectrometry could be a step forward in the identification of such early pregnancy markers.

Kinetics of steroids concentration during feces storage at room temperature doesn't affect the ability of E1S to be used as a pregnancy diagnosis, whereas P4 increases above LOQ in the first 24 h of storage. However, freezing (-20°C) feces as early as possible after collection would be recommended to keep the good correlation with blood levels. With a physiological perspective, observed changes in steroids concentrations during storage at room temperature requires to be investigated. Early studies [28] observed modifications of estrogens concentrations in feces of women receiving antibiotics and already concluded that intestinal flora was involved in estrogen metabolism. More recently, retro-conversion of estrogens in androgens by bacteria [29], re-activation of estrogens in gut [30], de-sulfation of progesterone by gut microbiota have been observed [27] and could explain observed transitory and continuous increase of P4 and E1S, respectively. Initially, the present study using LC-MS methodology was only designed to measured 2 steroids: P4 and E1S. However, LC-MS technology is able to quantify numerous compounds simultaneously, and other steroids could be added to the present methodology to precisely study the steroids kinetic in feces. Describing the sexual steroids metabolism in feces would also help to understand the ethological consequences of E1S increase in feces at ambient temperature. Observed modifications in fecal estrogens could have significance for the American Bison herd behavior [31], but also for their predators tracking them using tramlines' smell.

Determination of steroids concentration in American Bison feces could be a useful tool to study the changes in steroid levels during the pregnancy or the ovulatory cycle. Serum steroids levels are obviously the most representative biomarkers of the hormonal status of the American Bisons, but the practical issues already mentioned [1] limit the weekly sampling of American Bison serum to follow changes in steroid concentrations. Feces collection once a week is much easier and could be considered. The correlation with fecal levels of E1S is good and therefore fecal E1S could be considered a good proxy for pregnancy, while P4 results should be interpreted more carefully. The cost of a LC-MS instrument is an obvious limitation of the present methodology, as not all laboratories can afford such equipment. However, as discussed in our previous publication [10], this limitation is counterbalanced by the greater specificity and sensitivity of the LC-MS compared to immunoassays. Moreover, as a LC-MS equipment is able to quantify a few dozen compounds simultaneously [10,32,33], whereas immuno-assays require a specific kit for each compound assayed. Other steroids such as Estradiol, Estradiol-sulfate, 17α-hydroxyprogesterone (17αOHP), dehydroepiandrosterone (DHEA), and progesterone metabolites, sulfonated or not should be assayed with LC-MS to describe the ovulatory cycle and the complete pregnancy endocrinology.

5. Conclusion

The quantification of E1S and, in a lower level, of P4 in feces is an interesting alternative to serum measurement to describe pregnancy-related changes of these steroids in American Bisons, but also in other wild or domestic ungulates species. This tool could also be used to assess the evolution of other steroids during pregnancy in American Bison and other wild ruminants. Nevertheless, in this last case, particular attention should be paid to the preanalytical process, as an important variability inherent to the feces matrix and to their storage conditions should be considered.

Author's contribution

Patrice Dufour, Vincent Frisée, Goulven Rigaux, Stéfan Deleuze, Jérôme Ponthier and Etienne Cavalier designed the study. Vincent Frisée, Goulven Rigaux, Flore Brutinel, Sophie Egyptien, Philippe Bossaert, Stéfan Deleuze and Jérôme Ponthier sampled the Bison. Patrice Dufour, Caroline Le Goff, Stéphanie Peeters, Jessica Deleersnyder and Etienne Cavalier assayed the samples. All authors analyzed the results. Patrice Dufour wrote the manuscript. Patrice Dufour, Vincent Frisée, Goulven Rigaux, Flore Brutinel, Sophie Egyptien, Philippe Bossaert, Stéfan Deleuze, Etienne Cavalier and Jérôme Ponthier corrected the manuscript.

Declaration of Competing Interest

Authors have no conflict of interest to declare.

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