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American Bison (*Bison bison*) reproductive endocrinology: serum Pregnancy Associated Glycoproteins (PAG), Progesterone, Estrone and Estrone-Sulfate in non pregnant animals and during gestation

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ABSTRACT

This study describes concentrations of Pregnancy Associated Glycoproteins (PAG), progesterone (P4), estrone (E1) and estrone-sulfate (E1S) in American Bison sera. In 2 ranches, mature American Bison were sampled once a year for 2 yr. Subsequent American Bison cows calving days were reported. PAG concentration was determined by Radio-Immuno Assay, whereas P4, E1 and E1S were assayed using Liquid Chromatography and Mass Spectrometry. Concentrations were compared between American Bison bulls (B, n = 7), Nonpregnant cows (NP, n = 32), first (1TP, n = 3), second (2TP, n = 26) and third (3TP, n = 15) trimester of pregnancy. Seven American Bison bulls and 92 cows were sampled, 51 calved during these 2 yr. Calving occurred mostly in spring (74.5%), but also in summer (13.7%) and fall (11.8%). PAG and P4 were higher in 2TP and 3TP than B and NP ($P< 0.0001$). P4 was non-basal in B and NP. E1 and E1S were correlated ($P< 0.0001$; r = 0.76) and increased in 2TP and 3TP when compared with B and NP ($P< 0.01$). Moreover, E1S was higher in 3TP than in 2TP ($P< 0.0001$) and correlated to pregnancy day ($P< 0.0001$; r = 0.60). Breeding American Bison in Belgium induces a calving seasonality loss. P4 slowly increases in 1TP and remains steady and high in 2 and 3TP. P4 non-basal and variable concentrations in B or NP disable its use as gestation marker. American Bison produce PAG in the 2 and 3TP, but Estrone-sulfate assay seems to be the best pregnancy marker during the 2 last trimesters as it could help to estimate the gestation period.

1. Introduction

Although they were recently nearly extinct, American Bison (*Bison bison bison* or *Bison bison athabascae*) are reinvading their natural environment and bred in natural parks or for meat production. Due to American Bison wild character [1] and its extensive breeding management, reproductive endocrinology of nonpregnant and pregnant

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animals is poorly described. Bison estrus cycle has been estimated to be around 21 d [2,3]. They exhibit 5–6 cycles [2] during the ovulatory season in autumn [4], whereas an anestrus is observed during the other periods of the year [4]. In a natural environment, American Bison calving season is spring (May to July) after around 275 d of gestation [5,6], although weather or grazing areas and nutrition could modulate this duration [6]. The endocrinology of American Bison and bovine gestations seems to share common features, such as Pregnancy Associated Glycoproteins (PAG) [6,7] and estrogens [5,8] production.

Radio Immuno Assay (RIA) or Enzyme-Linked ImmunoSorbent Assay (ELISA) for PAG have been validated in American Bison placental extracts [7,9–11]. These assays are suitable in serum, but their evolution of concentrations during pregnancy is still unknown. Semiquantitative BIOPRYN ELISA for Pregnancy-Specific Protein-B (PSPB) [12], one of the glycoproteins of the PAG family [13], has recently been described for pregnancy diagnosis in American Bison, but the evolution of PSPB concentration was not precisely reported during pregnancy [6].

Urinary and fecal sexual steroids have been assayed in early studies on small numbers of 3-mo pregnant American Bison cows [3,5], but the type of estrogens and their blood concentrations were not reported [5]. Moreover, most of these studies used steroids dedicated immunoassays [5]: they were not necessarily validated for this species' biological matrix, and this can lead to variations in the observed steroids concentrations [14,15]. The use of Liquid Chromatography and Electrospray Tandem Mass Spectrometry (LC-MS), has recently been described by our team for specific steroids assays in different species [15] and could decrease intrinsic results' variations.

As reproductive endocrinology of American Bison still requires investigations, this report aims to describe PAG, progesterone (P4), estrone (E1) and estrone-sulfate (E1S) in American Bison bulls, nonpregnant cows and during their pregnancies using a large number of animals sampled. The zootechnical results of ranches extensively breeding American Bison (*Bison bison*) in Belgium are also presented.

2. Materials 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, that are mainly covered by ray grass, clover and fescue. During the winter, wrapped hay collected on similar pastures during the spring is given twice a day, ad libitum. The first ranch herd is divided in 2 separate meadows of 19 and 21ha, each welcoming 2 sexually mature American Bison bulls and respectively 25 or 26 sexually mature cows. In the second ranch, 10 sexually mature American Bison cows are roaming freely with 1 sexually mature bull in a 15ha 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 diseases 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 matures American Bison. Blood samples were centrifuged (1000 x g) and stored frozen (-80°C) until assays.

Among the 5 American Bison bulls, 2 were sampled in 2019 and 2020. Of the 92 samples collected on American Bison cows, 52 were obtained from the same animal sampled in 2019 and 2020. Calving days of the American Bison cows were recorded for the year following the sampling procedures. Fertilization day of American Bison cows that calved was retrospectively obtained by subtracting 276 d of the calving day. Gestation day on blood sampling day was then determined by subtracting the fertilization day of the sampling day. Other events, such as abortion, disease, culling or late birth (more than 276 d after sampling) were reported by the breeders during the following year.

2.2. PAG assays in serum

Assays for PAG were performed on pregnant, nonpregnant cows and males: the last 2 groups were used as negative control. The caprine RIA-706 assay developed with the previously described method [16] was used to measure PAG concentrations, as a preliminary study showed it was allowing the best detection of PAG epitopes sampled in closely related species, as bovine or American Bison. Pure boPAG 67kDa preparation was used as standard and tracer. Iodination (Na-I125, Amersham Pharmacia Biotech, Uppsala, Sweden) was carried out according to the Chloramine T method previously described [17]. Shortly, the samples were first assayed in a preincubated system with standard curve ranging from 0.2 to 25ng/mL. Samples with PAG concentrations above the estimated standard dose of 25ng/mL at which the percentage B/B0 was 20% were reassayed in non-preincubated systems, with a standard curve ranging from 0.8 to 100ng/mL after an overnight incubation. The minimum detection limit (MDL), calculated as the mean concentration plus twice the standard deviation of 20 duplicates of the zero (B0) standard [18], was 0.1ng/mL. The intra- and inter-assay coefficients were 2.9% and 7.3%, respectively.

2.3. Progesterone (P4), Estrone (E1) and Estrone-Sulfate (E1S) assays in serum

Steroids were assayed using the Liquid Chromatography and Electrospray Tandem Mass Spectrometry (LC-MS) technique previously validated [15] using 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. Analyst 1.6.2 was used for data acquisition and processing. For P4, E1 and E1S, Lower Limit of Quantification (LLOQ), defined as the lowest concentration in the validation standards that reported Relative Standard Deviation and Rela-

Table 1
Monthly distribution (%) of calving observed in 2019 and 2020.

Winter			Spring			Summer			Fall		
January	February	March	April	May	June	July	August	September	October	November	December
0	0	0	37.3[a]	25.5[a]	11.8	7.8	2.0	3.9	3.9	7.8	0

Births are expressed in percent (%).
[a] Expresses a significantively increased number of calving when compared to a monthly equivalent repartition of calving.

tive Bias lower than 15% [15], were established at respectively 0.1ng/mL, 2.0pg/mL and 0.5ng/mL.

2.4. 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*. Results are expressed as mean ± standard deviation.

Fertility was calculated as the number of mature American Bison cows giving birth to a live calf within the year divided by the total number of mature American Bison cows, excluding the culled females. As their gestation day at the time of sampling could not be retrospectively determined by calculations, aborted and culled animals were excluded for the calving seasonality assessment and the hormonal curves establishments. *Khi-2 test* was used to determine if the distribution of calving differed between months. American Bison samples were divided into 5 classes: Bulls, used as negative controls (B); Nonpregnant, when no calving was observed within the next 276 d after sampling (NP); first trimester of pregnancy (1TP), from day 1 to day 92; second trimester of pregnancy (2TP), from day 93 to day 183; third trimester of pregnancy (3TP), from day 184 to the end. As only 1 sample was available per year and per animal, an *ANOVA* for non-repeated measures was performed to determine differences in concentration between groups for each hormone assayed, with a *Tukey post-test*. *Pearson test* was used to determine correlations between hormones concentrations or between hormones concentrations and pregnancy day.

3. Results

3.1. Breeding results and seasonality

During the 2-yr study, 99 matures American Bison serum samples were collected and 7 of them were originating from American Bison bulls. Among the 92 females' samples, 32 were originating from nonpregnant animals. Four females were culled within the following year and 5 American Bison cows (5.7%) aborted some weeks after sampling procedures, without causal relationship. Sampled American Bison cows gave births to 51 calves: 59.0% of Bison cows produced a calf per year in the first ranch and 50.0% in the second. No twin pregnancy was observed during our study. Table 1 shows the monthly pattern of calving during the 2-yr study: in studied Belgian ranches, American Bison births were mostly observed in spring (74.5%), but also in summer (13.7%) and early fall (11.8%). As sampling occurred in January and February 2019 and in February and March 2020, pregnant animals' samples were mostly collected in 2 and 3TP (respectively: n = 26 and n = 15), whereas only 3 samples were collected in 1TP. Body score evaluation using bovine standards [19], was evaluated at 5 for the first ranch herd and 3 for the second.

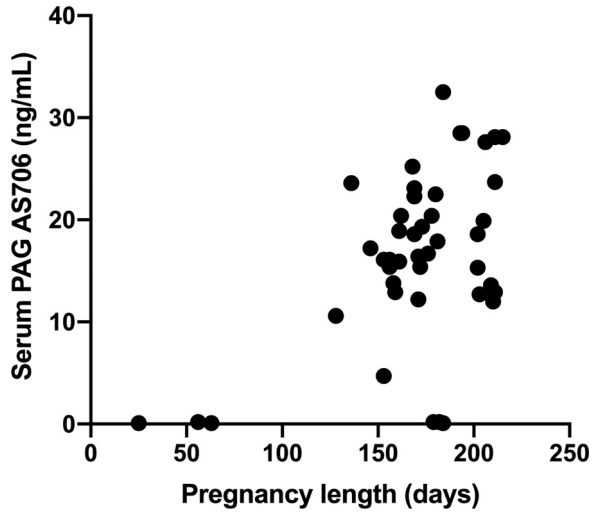

Fig. 1. Pregnancy Associated Glycoproteins (PAG) concentration assayed with AS706 antibodies in ng/mL evolution during American Bison pregnancy (in days). Each dot represents the PAG concentration (ng/mL) observed in a different pregnant American Bison cow for its day of pregnancy at sampling.

3.2. Pregnancy Associated Glycoproteins (PAG) evolution during Bison pregnancy

Nearly basal PAG concentrations did not differ ($P > 0.05$) between B (0.21 ± 0.12ng/mL) and NP (0.25 ± 0.62ng/mL), that were used as negative controls. PAG concentrations in the 3 samples of 1TP American Bison cows were also basal (day 25 = 0.1ng/mL; day 56 = 0.2ng/mL; day 63 = 0.1ng/mL) and similar to those observed in B and NP. PAG significantly increased ($P< 0.0001$) in 2 and 3TP, when compared to B and NP groups. However, PAG concentrations did not differ ($P > 0.05$) between 2 and 3TP and standard variation was very large in those 2 periods (respectively 16.00 ± 6.42ng/mL and 20.14 ± 8.99ng/mL). The evolution of PAG during pregnancy is summarized in Figure 1.

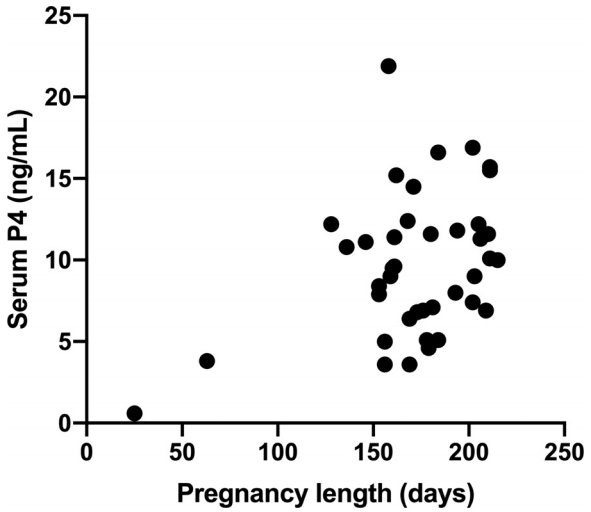

Fig. 2. Progesterone (P4) concentration assayed by Liquid Chromatography coupled to Mass Spectrometry in ng/mL evolution during American Bison pregnancy in days. Each dot represents the progesterone concentration (ng/mL) observed in a different pregnant American Bison cow for its day of pregnancy at sampling.

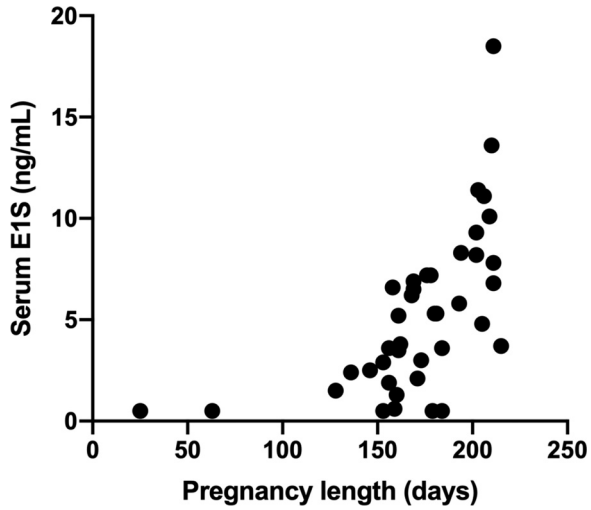

Fig. 3. Estrone-Sulfate (E1S) concentration assayed by Liquid Chromatography coupled to Mass Spectrometry in ng/mL evolution during American Bison pregnancy in days. Each dot represents the estrone-sulfate concentration (ng/mL) observed in a different pregnant American Bison cow for its day of pregnancy at sampling.

3.3. Progesterone (P4) evolution during bison pregnancy

Progesterone concentrations did not differ ($P > 0.05$) between B and NP and showed a large variability (respectively, mean ± SD: 1.22 ± 0.87ng/mL and 1.56 ± 2.26ng/mL; median 1.30 and 0.90ng/mL; 25–75 quartile 0.28–1.90 and 0.40–1.70ng/mL). In those groups, P4 concentrations were low, but non-basal. In the NP group, 20 samples were above the lowest P4 concentration observed in the pregnant Bison cows, whereas 12 were below. As previously mentioned, only a few values were available in 1TP, but they are suggesting a slow P4 increase (Fig. 2). Progesterone concentrations were increased in 2 and 3TP when compared to B and NP ($P < 0.0001$), but did not differ between 2 and 3TP, and also showed large variability (respectively 9.33 ± 4.29ng/mL and 11.21 ± 3.68ng/mL; $P > 0.05$). A significant positive, but weak correlation was observed between PAG and P4 ($P < 0.0001$; $r = 0.52$) in all the pregnant American Bison cows.

3.4. Estrone (E1) and Estrone Sulfate (E1S) evolution during bison pregnancy

Estrone was basal in B and NP and did not differ between these 2 groups ($P > 0.05$; respectively 2.0 ± 0.0pg/mL and 2.47±2.89pg/mL with a 2.0ng/mL median). In 1TP, E1 concentrations of the few samples were under the LLOQ values (2.0pg/mL). In 2 and 3TP, E1 concentrations differed (respectively 28.96 ± 21.29pg/mL and 49.40 ± 31.97pg/mL; $P < 0.05$) and were both significantly higher than in B and NP ($P < 0.01$). A strong correlation ($P < 0.0001$; $r = 0.76$) was observed between E1 and E1S in pregnant American Bison cows.

In B and NP groups, E1S concentrations were basal and did not differ ($P > 0.05$) between the 2 groups (respectively 0.50 ± 0.00ng/mL and 0.52 ± 0.12ng/mL). The 3 values observed in 1TP were similar to those observed in B and NP. Concentrations in E1S were increased in 2 and 3TP when compared with B and NP (for the different group comparisons, $P < 0.01$). In 2TP, E1S concentration was lower ($P < 0.0001$) than in 3TP (respectively 3.76 ± 2.29ng/mL and 8.23 ± 4.46ng/mL) and there was a significant moderate positive correlation between the day of pregnancy at sampling and E1S concentration ($P < 0.0001$; $r = 0.60$). Figure 3 depicts the evolution of E1S during pregnancy.

4. Discussion

To the best of our knowledge, this is the largest number of American Bison blood samples used to compare hormonal levels between males, nonpregnant and pregnant females at various gestation days.

American Bison bulls blood samples, used as negative controls, provide new information. Values of E1 and E1S matched the previously described LLOQ [15], demonstrating that male American Bison do not produce high estrogens levels, as observed in cattle [20], but unlike stallion or boar [21,22]. Concentrations of P4 observed in American Bison bulls were above the described LLOQ [15] and similar to reported bovine bulls concentrations assayed with immuno-assays [23,24]. Stress-related progesterone production is observed 15 min after electro-ejaculation in Bovine bulls [24]. Thus, the gathering and/or the immobilization in the specific handling/chute system could lead to a stress related P4 increase in the more anxious American Bison bulls and explain the observed high variability.

A large proportion of American Bison cows were nonpregnant, and some issues, as the transient escape of the second ranch' American Bison bull, could explain the low fertility observed in this herd. However, the overall observed fertility is below the beef cow breeding standards [25], but abortion and calving rates per year and per fe-

male should only be compared between North-American and Belgian ranches breeding American Bison. Western-Europe rainy temperate climate and over-grazing due to smaller meadows' surfaces could interfere with the American Bison fertility. In the NP group, PAG, E1 and E1S concentrations were similar to their respective MDL or LLOQ [15,18], making these hormones potential pregnancy markers. In NP animals, P4 concentrations were above the LLOQ [15] and showed high variability, despite our LC-MS assay good reproducibility [15], thus discarding the use of P4 for pregnancy diagnosis, like in other species [8,26,27]. Sampling occurred in January, February or early March, when most of the American Bison NP cows should be in anestrus [4]. This variation in P4 levels could also be related to stress induced adrenal progesterone production [28], as discussed for bulls. However, 20 of 32 of these cows had P4 levels above the lowest concentration observed during pregnancy, suggesting they were in diestrus. The breeding season seemed to be extended beyond fall in Belgium, as births occurred mainly in spring (April and May), but were observed until November, as a consequence of a February mating. This difference with wild American Bison, which are mainly calving from May to July [5,6], is difficult to explain, because light and melatonin related seasonality [26] has not clearly been established yet in American Bison. The Belgian oceanic climate with temperate and rainy winters could explain nonseasonal breeding and calving. Moreover, lower energy intakes due to over-grazing of meadows in fall could also decrease the hypothlamo-hypophysis axis activity [26] and delay the breeding season. As a consequence, this loss of births' seasonality leads to rise some young calves in mud and cold weather. Moreover, culling decision could be inappropriate, as American Bison cows in early gestation could be considered as nonpregnant by palpation, ultrasonography or available hormonal assays when the herd is gathered in late winter or early spring for mandatory disease screening.

Thanks to this loss of breeding seasonality, samples were collected from American Bison cows at different periods of pregnancy. However, as a limitation of the once-a-year sampling, only tendencies could be described with the few samples collected in 1TP resulting from unlikely mating in winter. Concentration in P4 slowly increased at the beginning of pregnancy, as observed in other ungulates like Reindeers [29], but it could also be related to individual variations. Levels of E1 and E1S were also nearly basal, a priori disabling their use as early gestation marker, but requiring more samples in 1TP. The PAG level was low and basal in 1TP samples, whereas PAG increase with a concentration above 1ng/mL around day 20 in Reindeer [29] and at day 23 in Water Buffaloes [30]. In dairy cows, the first short rise of PAG occurs at day 24 of pregnancy [31] and is followed by a second increase around day 70 [13]. In the present report, a transient peak could have been missed, due to the limited data available in 1TP. An assay for a specific glycoprotein of the PAG family, the PSPB [13], has been validated in American Bison [6] and showed higher concentration (1.42 $\pm$ 1.29ng/mL) in early pregnancy (0–45 d) than our total PAG assay. Despite our caprine PAG RIA kit showed the best detection of the American Bison PAG epitopes, the different sensitivities of the assays could explain those diverging results. Moreover, high PSPB concentration variability was observed during this period [6] and information about PSPB evolution in time and levels in non-pregnant animals were not reported [6]. More data about the PAG evolution in early American Bison pregnancy are required to use our PAG RIA and to explain the differences with the semiquantitative BIO-PRYN ELISA for PSPB [6].

In 2 and 3TP, PAG concentrations were dramatically below those observed at peak in dairy cows [32], but were similar to those observed in Water Buffaloes [30]. Total PAG concentrations observed in our study during the 2 last trimesters of American Bison pregnancies were above those observed with the specific PSPB assay in mid and late gestation [6], conversely to the observations in 1TP. In the present cohort of American Bison, like in Water Buffaloes [30], no PAG peak was observed during the 2 last trimesters of pregnancy. This could also results from the large variability in PAG concentrations observed in American Bison pregnant cows, which could be explained by the mating season, and, as reported in dairy cows or goats, the parity [13,33] or the fetal sex [32]. Moreover, as a limitation, no samples were available in the end of 3TP, and it could hide a late PAG or P4 peak in American Bison pregnant cows, as P4 interindividual variability was large and didn't show either a peak in 2 or 3TP. The good reproducibility of our LC-MS cannot explain this variability [15], but factors modulating the molecular regulation of the placental P4 production could be involved, like nutritional stress that has been already described in bovine [34–36] and stress-related adrenal production [28]. Like in other species [8,26,27], P4 production in nonpregnant cows disables its use as an specific pregnancy marker in American Bison. Constant and high P4 levels observed in 2 and 3TP and correlation between PAG and P4 at the end of pregnancy suggest that placenta could contribute to P4 production in the 2 last trimesters of American Bison pregnancy.

The high E1 and E1S concentrations in 2 and 3TP, as well as their basal value in NP animals, make these hormones potential pregnancy markers after day 93 in American Bison. The strong correlation between E1 and E1S in pregnant American Bison and the low E1 concentrations (in pg/mL) when compared with E1S (in ng/mL) suggest that E1 should be considered as a trace of estrogens metabolism. E1S production has been described in late pregnancy of mares [37], reindeer [29], ewes [38], goats [33], buffalos [39], cows [40], and many other ungulates [41]. Estrogen production in pregnant American Bison was reported in early studies [5], but without identifying the involved estrogens, and only in feces of a small number of animals with approximative assessment of the pregnancy day. As observed in Buffaloes [39] and in various breeds of cattle [40], present data show that blood estrogens level are rising in the 2TP and continue to increase in the 3TP. In American Bison, E1S production seems to be correlated with the pregnancy day. In 2TP, E1S concentration was higher in American Bison than in beef cows [40]. At 220 d, E1S levels in American Bison were similar those of beef cows [40], but below those observed in Buffaloes [39]. However, Immuno-Assays used in cows [40] and buffaloes [39] precludes any definitive comparisons with our LC-MS results. The LC-MS method developed by our team

[15] doesn't explain the interindividual variability observed for the same pregnancy period in American Bison samples. Calf weight at birth or other factors, as described for beef cattle [40], are more likely to be involved in this variability. Origin of E1S has been located in the placenta of many ungulates, like horses [27] and more precisely in the cotyledonary portion of the placentome in ruminants [42]. The evolutionary proximity of the studied species and the similar evolution of E1S during pregnancy suggest that estrogens are also mainly originating from the placenta during the American Bison pregnancy.

5. Conclusion

As a conclusion, breeding extensively American Bison in Belgium resulted in less than 60% of the females giving birth per year. Lengthening of the breeding season leading to a loss of births' seasonality is also observed and is jeopardizing the life of calves born in autumn. In American Bison like in many other species, progesterone assay is not useful for pregnancy diagnosis. Estrone-sulfate seems to be a pregnancy marker of interest during the 2 last trimesters as its assay could give an estimation of the gestation period, in opposition to PAG that does not increase significantly during the last 6 mo of American Bison pregnancy.

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Authors' contributions

Vincent Frisée, Goulven Rigaux, Stéfan Deleuze, Etienne Cavalier and Jérôme Ponthier designed the study. Vincent Frisée, Goulven Rigaux, Flore Brutinel, Sophie Egyptien, Philippe Bossaert, Stéfan Deleuze and Jérôme Ponthier sampled the Bison. Patrice Dufour, Olimpia Barbato and Etienne Cavalier assayed the samples. All authors analyzed the results. Jérôme Ponthier and Olimpia Barbato wrote the manuscript. Vincent Frisée, Goulven Rigaux, Patrice Dufour, Olimpia Barbato, Flore Brutinel, Sophie Egyptien, Philippe Bossaert, Stéfan Deleuze, Etienne Cavalier and Jérôme Ponthier corrected the manuscript.

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