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Oestrous cycle of the North American bison (*Bison bison*) characterized by urinary pregnanediol-3-glucuronide

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Summary. An enzyme immunoassay for urinary pregnanediol-3α-glucuronide (PdG) was evaluated for the indirect measurement of progesterone metabolites during the oestrous cycle and early pregnancy of uncaptured North American bison. Comparisons between plasma progesterone and urinary PdG, dose–response parallelism between the standard curve and diluted urine samples and high-performance liquid co-chromatography revealed that PdG was a primary immunoreactive urinary metabolite of progesterone in bison. Urine samples were collected directly from the soil from 29 bison cows during the August rutting season and analysed for PdG. Eight bison cows demonstrated complete oestrous cycles ranging from 19 to 26 days (mean cycle length = 23·12 ± 0·76 days) and behavioural oestrus among four of these cows correlated with PdG nadirs. Mean PdG nadirs were 63·62 ± 21·61 ng/mg urinary creatinine (Cr) and mean peak midluteal values were 546·01 ± 130·73 ng/mg Cr. Seven of eight became pregnant, indicating that bison exhibit a second seasonal oestrus. Eighteen other bison cows were pregnant prior to the beginning of the study and demonstrated non-cyclic increased PdG concentrations (> 200 ng/mg Cr) during the 30-day course of collection. Three cows ovulated and became pregnant during the 30-day collection period and then exhibited increasing urinary PdG concentrations. This report demonstrates that ovarian function in uncaptured bison can be monitored by means of urinary PdG and that both ovulatory cycles and early pregnancy can be detected.

Keywords: oestrous cycle; progesterone metabolites; urine; bison

Introduction

The North American bison (*Bison bison*) is found free-roaming on numerous preserves and in commercial herds throughout North America. In its free-roaming state, the reproductive biology of these large ungulates has been the subject of numerous studies (Lott, 1979; Robitaille & Prescott, 1981; Sambraus, 1981; Lott & Galland, 1985; Rutberg, 1986a, b; Maher & Byers, 1987), but all have been confined to behavioural observations or examination of tissues from slaughtered animals. Bison cows first breed at 2 years of age (McHugh, 1958; Fuller, 1961; Halloran, 1968; Haugen, 1974) and fecundity is age-dependent. Based on examination of corpora lutea (148 ovaries) from slaughtered bison, Haugen (1974) claimed that a second oestrus and ovulation per year is rare among bison; and Rutberg (1986a) did not observe cows from the National Bison Range breeding more than once per season. Kransinski & Raczynski (1967) reported that among European bison (*Bison bonasus*) behavioural manifestations of oestrus lasted 1–3 days and that oestrus recurred every 18–22 days.

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Despite the interest in bison by wildlife biologists and the increasing commercial value of the animals, endocrine characterization of their oestrous cycle has not been reported. Because the species is intractable, sequential blood sampling is impractical. This study characterized the oestrous cycle of the bison without capture or handling, by monitoring urinary steroid metabolites. A urinary metabolite of progesterone was measured, which would indicate formation of the corpus luteum and, by implication, the occurrence of a previous ovulation. The rationale for this study is based on the need to determine the occurrence of ovulation among free-roaming bison in order to determine the mechanisms of reproductive self-regulation.

Materials and Methods

Assay validation

Comparison of plasma progesterone and urinary pregnanediol-3α-glucuronide. To validate the indirect measurement of plasma progesterone through the use of urinary pregnanediol-3α-glucuronide (PdG), paired blood and urine samples were collected from five sexually mature bison cows killed during a controlled hunt in Montana, USA, just north of the Yellowstone National Park boundary (45°N latitude), during December 1988 and January and February 1989. Blood samples were collected from pooled blood in the carcasses within minutes of death and the plasma was separated by centrifugation and stored frozen. Urine samples were collected by puncturing the bladder with a 10 ml syringe and an 18 gauge needle and withdrawing urine, which was also stored frozen. Plasma samples were assayed for progesterone using an enzyme immunoassay (EIA) described by Munro & Stabenfeldt (1984). Recovery of [^{3}H]progesterone from plasma was 86 ± 7·4% (n = 5). The intra-assay coefficient of variation was 4·9%. Urinary PdG immunoreactivity was determined by EIA as described by Shideler *et al.* (1990) and Kirkpatrick *et al.* (1990) with the following modifications. The urine samples were diluted 1:1 with distilled H_2O (dH_2O) and 20 µl of the dilution was assayed. The anti-PdG antiserum (P70) was diluted to 1:10 000 and produced a viable standard curve between 0·07 and 10 ng. The inter- (n = 17) and intra-assay (n = 10) coefficients of variation were 14·54 and 7·60%, respectively. The specificity of the PdG antiserum, determined by EIA was pregnanediol-3α-glucuronide, 100%; 20α-dihydro[hydroxy]progesterone, 60·7%; pregnanediol, 7·3%; 20β-dihydro[hydroxy]progesterone, 2·5%. All other steroids or steroid conjugates cross-react at less than 0·1%. The PdG standard was 5β-pregnane-3α,20α-diol glucuronide (Sigma Biochemical, St Louis, MO, USA). Plasma progesterone and urinary PdG concentrations were compared for the five bison cows.

High-performance liquid co-chromatography. To establish the specificity of the PdG EIA for bison urine, urine samples from two pregnant (approximately 4 months pregnant) and one non-pregnant bison were analysed by high-performance liquid co-chromatography (HPLC) as described by Kirkpatrick *et al.* (1990). Samples of 1 ml were extracted twice, each time with 3·5 ml of diethyl ether, to remove free steroids; 1 ml of methanol:ethanol (1:1) was added to the urine, the mixture was vortexed, cooled to −5°C, centrifuged (1500 *g* for 10 min) to remove salts and the supernatant was removed for HPLC. Approximately 1000 c.p.m. of 6,7[^{3}H]pregnanediol-3ß-glucuronide (20–40 Ci/mmol; Courtauld Institute, London, UK) was added to serve as a co-chromatography marker and the supernatant was reduced to ~1·0 ml volume under N_2. Recovery of [^{3}H]PdG was 83·3 ± 4·2% (n = 4). After 25 µl was injected onto a 15-cm HPLC column (ALTEX, Ultrasphere-ODS, dp 5µ), 1·0-ml fractions were collected during a MeOH/H_2O gradient (10–65% MeOH over 40 min). From each fraction, 200 µl was mixed with 5·0 ml of scintillation cocktail (Ready Protein, Beckman, USA) and radioactivity was measured to monitor PdG recovery. The remaining 800 µl was evaporated, redissolved in 100 µl of dH_2O and assayed for PdG as described above. Fractions containing immunoreactive PdG were compared with fractions containing [^{3}H]PdG.

Parallelism to the standard curve. The quantitative nature of the PdG EIA for bison urine was established by developing a dose–response curve to five urine samples. Samples were diluted 1:1, 1:2, 1:4, 1:8, with dH_2O, measured for PdG as described and compared with the assay standards.

Longitudinal urine sampling and processing

The assay was applied to 377 urine samples collected during the August 1989 rutting season, over a 30-day period from 29 sexually mature cow bison inhabiting ~200 ha in northern Wyoming. The cows ranged in age from 3 to 17 years and 27 were of proven fertility and were part of a total herd of 80 bison. Six sexually mature bulls were present among the herd at all times. Water was available from natural sources *ad libitum* and feed was natural grass. The bison were observed from a distance of 50–100 m and urine samples were collected once a day if possible and at a minimum of every 2–5 days between 06:00 and 18:00 h. Urine samples were aspirated from pools on the ground or extracted directly from the soil immediately after a urination as described by Kirkpatrick *et al.* (1988). All cows were identified by numbered colour-coded ear tags, which had been placed on them when calves, and by a variety of identifying physical characteristics. Samples were placed on ice immediately after collection and stored frozen in plastic vials

within 2–3 h of collection. Urine samples were analysed for PdG concentrations as described above; urinary creatinine (Cr) was measured by the nanocolorimetric method of Taussky (1954). Urinary PdG was indexed to creatinine concentrations and reported as ng/mg Cr to account for differences in urine concentrations. The pattern of PdG excretion for each animal was plotted and compared with behavioural observations, which were recorded daily. Particular attention was given to oestrous behaviour as described by McHugh (1958), and calving dates for April, May and June 1990 were recorded for each of the 29 bison cows. Urine samples with Cr values below 0·1 mg/ml (181, or 21·1% of the total collected) were considered to be too low for accurate measurement of PdG and excluded from the results. Behavioural observations were not available for all animals in this study.

Results

Plasma progesterone and urinary PdG concentrations from the five bison killed in the controlled hunt ranged from 0·9 to 10·5 ng/ml and 10·02 to 536·61 ng/mg Cr, respectively (Table 1). The correlation coefficient between plasma progesterone and urinary PdG concentrations was $r = 0·93$, and suggests that in bison cows PdG accurately reflects plasma progesterone secretion.

Table 1. Plasma progesterone and urinary pregnanediol-3-glucuronide (PdG) in five bison cows

Bison no.	Age (years)	Plasma progesterone (ng/ml)	Urine PdG (ng/ml Cr)
81	3	0·9	18·90
40	5	1·8	26·16
95	2	2·8	10·02
79*	8	4·1	325·71
88*	2	10·5	536·61

*Pregnant cow, defined by the presence of a fetus.

Both 6,7[^{3}H]PdG (fractions 32 to 34) and immunoreactive urinary PdG from pregnant and non-pregnant cows eluted in the same fractions in HPLC, indicating that the PdG EIA was predominantly specific for PdG (Fig. 1).

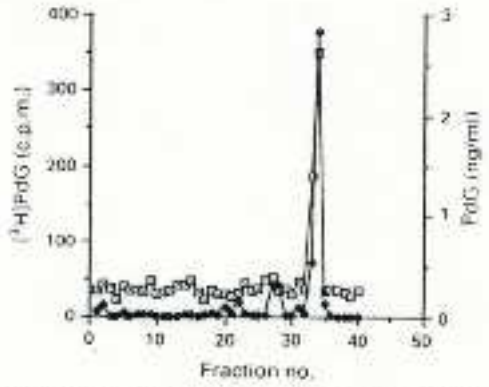

Fig. 1. High-performance liquid co-chromatography profiles of [^{3}H]pregnanediol-3-glucuronide (PdG, ■) and urinary immunoreactive PdG (●) from a pregnant bison cow; [^{3}H]PdG and the immunoreactive PdG eluted in fractions 32–34.

The five urine samples ranged from 6·2–25·5% binding at the 1:1 dilution to 39·6–75·6% at the 1:8 dilution (Fig. 2).

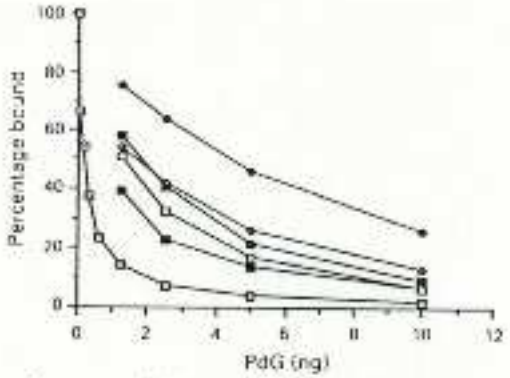

Fig. 2. Standard curve of pregnanediol-3-glucuronide (PdG) assay and serial dilutions (1:1 to 1:8) of five randomly selected cow bison urine samples; each sample shown by a different symbol.

Eight bison demonstrated clear PdG nadirs, which were followed by PdG increases. Oestrous behaviour was coincidental with the PdG nadir for four of the cows and Fig. 3 illustrates the pattern of PdG excretion in two of these cows. The length of the luteal cycles for all eight cows displaying a PdG rise, measured by the number of days between PdG nadirs, ranged from 19 to 26 days with a mean of 23·12 ± 0·76 (s.e.m.). A composite urinary PdG profile for these eight cows is illustrated in Fig. 4. The PdG nadirs for individual cows ranged between 55·7 and 123·3 ng/mg Cr, with a mean of 63·62 ± 21·61 at the beginning of each cycle and 55·7 ± 20·2 ng/mg Cr at the end of each cycle. Peak values for individual cows during midluteal phase ranged from 364·28 to 1080 ng/mg Cr, with a mean of 546·01 ± 130·73 ng/mg Cr. Seven of these cows became pregnant after 1 September 1989.

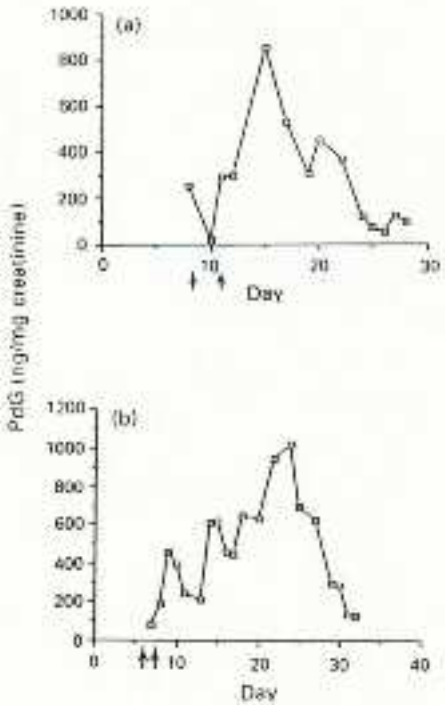

Fig. 3. Luteal-phase urinary patterns of pregnanediol-3-glucuronide (PdG) for two bison cows: (a) No. 401, (b) No. 424; (†) days of observed behavioural oestrus.

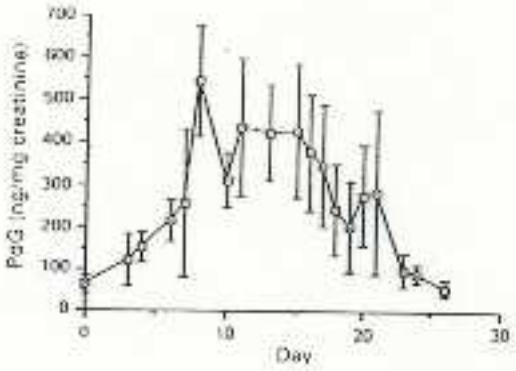

Fig. 4. Mean (±s.e.m.) urinary pregnanediol-3-glucuronide (PdG) for eight bison cows collected at a minimum of once every 3 days between 31 July and 30 August. Day 0 represents alignment of the PdG nadir for each cow.

Eighteen cows did not display a clear oestrous cycle based on urinary PdG, but instead showed non-cyclic, continually increased PdG concentrations (> 200 ng/mg Cr) throughout the collection period, which ended on 31 August. Based on a gestation length of 270 days (Asdell, 1964) and the retrospective assessment of parturition dates, these cows were already pregnant when the study began. The non-cyclic, increased urinary PdG concentrations presumably reflect progesterone production during early pregnancy. Another three cows had clear urinary PdG nadirs and became pregnant during the study period. These cows then demonstrated increasing urinary PdG concentrations until the end of the 30-day collection period.

Discussion

The oestrous cycle of 19–26 days reflected by urinary PdG in this study approximates the 18–22 day cycle reported for European bison (Krasinski & Raczynski, 1967). As daily urine sample collections were not always possible for each animal, several days occurred between some collections. The lengths of the oestrous cycles reported here are, therefore, approximate; but they fall within the values reported on the basis of behavioural observation (Krasinski & Raczynski, 1967) for a subspecies of North American bison and for the oestrous cycle of the closely related and interbreeding domestic cow (*Bos taurus*) (18–24 days; mean = 21·28 ± 0·66) (Asdell, 1964).

Although neither HPLC nor co-chromatography alone can be used as evidence for the molecular structure of an immunoreactive substance, together they provide evidence that chemical identity is likely to exist between standard and unknown. This is particularly true for most of the common metabolites of progesterone, as most have been characterized and shown not to co-chromatograph with PdG using HPLC (Liskowski & Wolfe, 1972; Loskutoff *et al.*, 1983; Shideler *et al.*, 1985).

Although increased concentrations of progestins, as indicated by urinary PdG, cannot be used as proof of ovulation this is the most likely conclusion when the observed patterns fit the predicted profile of the oestrous cycle. There are four major causes for progestin increases: ovulation, luteinized follicles, persistent corpora lutea and extra-ovarian progestins, but only ovulation and the formation of a corpus luteum result in a cyclic rise and fall in plasma progesterone. In this study, increases in PdG for 23 days, separated by periodic nadirs, are consistent with ovulatory cycles of other Artiodactyla, including the domestic cow.

Four of the cows exhibiting oestrous cycles on the basis of urinary PdG manifested behavioural signs of oestrus, including the constant raising and swishing of the tail and sniffing of the vulva by bulls and other cows (McHugh, 1958) on or about the time of preluteal-phase PdG nadirs. Behavioural manifestations of oestrus lasted ~1·5–3 days, which is similar to European bison

(Krasinski & Raczynski, 1967). This study focused on endocrinological patterns, but the correspondence between the time of PdG nadirs and behavioural oestrus suggests that the evaluation of urinary steroid metabolites can be applied to behavioural, as well as to reproductive studies.

Despite studies suggesting that bison are not polyoestrous (Haugen, 1974; Rutberg, 1986b), seven of the eight cows that demonstrated a clear luteal phase and complete oestrous cycle became pregnant during their second cycle and subsequently produced calves. The dates of calving for these cows confirm conception during September 1989 and support the existence of a second seasonal oestrus.

Urinary steroid metabolites to study reproductive function in free-roaming uncaptured ungulates should be used with caution. It is important to be certain that the particular urinary metabolite being evaluated reflects a specific identifiable plasma steroid. Unfortunately, it is not always possible to collect paired blood and urine samples to validate the relationship between plasma and urinary hormones; only five matched samples of blood and urine were available from bison cows. However, the high degree of correlation between plasma progesterone and urinary PdG supports the contention that, in bison cows, PdG is a major urinary metabolite of progesterone and a valid indirect measurement of this ovarian steroid. The HPL co-chromatogram indicates that the EIA used in this study was, in fact, measuring PdG. This is further supported by studies of the metabolic fate of [^{14}C]progesterone in domestic cows (Purdy *et al.*, 1980; Clemens & Estergreen, 1982), in which significant quantities of labelled 5β-pregnane-3α,20β-diol appeared in the liver and the kidney. Feher (1975) demonstrated that PdG was the major urinary progesterone metabolite in cows. In other species, including man (Munro *et al.*, 1991) and horse (Kirkpatrick *et al.*, 1990), there is a strong correlation between urinary PdG or PdG-like metabolites and plasma progesterone. The similarity between the standard curve and the five unextracted serially diluted urine samples indicates that soil minerals were not interfering to any significant degree with the assay and that there were no other major metabolites with significant cross-reactivity with the anti-PdG antibody at dilutions betwen 1:1 and 1:8. Previous studies have demonstrated that recovery of [^{3}H]labelled steroid conjugates from soil is >90% (Kirkpatrick *et al.*, 1988).

In some cases, dramatic decreases of PdG in the middle of the luteal phase curve were produced from samples with creatinine values at the low end of the range. In the diagnosis of pregnancy in horses by means of urinary oestrone conjugates, samples with creatinine values of <0·1 mg/ml are not considered reliable. The bison in this study showed great variation in daily creatinine concentrations, which generally reflected day-to-day activity patterns. On some days, the herd moved constantly, stopping only to graze, and not watering at all; on other days it spent several hours watering. An alternative explanation for the erratic luteal-phase PdG profiles is found in the work of Plotka *et al.* (1967), Dobrowolski *et al.* (1968) and Donaldson *et al.* (1970), all of whom reported a midluteal decline in plasma progesterone in domestic cows. Despite the unevenness of the daily values, oestrous cycles can be clearly tracked using urinary PdG.

This study details an approach to the study of reproduction in uncaptured bison that eliminates the need for capture, immobilization or blood collection. Major advantages of this method include a rapid (<6 h) direct non-radiometric EIA procedure, which uses <50 µl of urine for both the PdG and creatinine evaluations. Female bison can be monitored without the alteration of normal physiological patterns that might result from the stress of capture or handling. It is now possible to monitor the occurrence of ovulation and early pregnancy in bison, events that may be helpful in understanding ovarian dysfunction, persistent corpora lutea, early pregnancy failure and other events which may be associated with the phenomenon of ungulate reproductive self-regulation.

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