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PREGNANCY AND OVULATION DETECTION IN BISON (*BISON BISON*) ASSESSED BY MEANS OF URINARY AND FECAL STEROIDS

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ABSTRACT: Sexually mature bison (*Bison bison*) cows were tested for both pregnancy and ovulation by means of urinary steroid metabolites and fecal steroids. The accuracy of pregnancy diagnosis was determined among 18 bison cows, in approximately the third month of gestation, by means of urinary pregnanediol-3δ-glucuronide (PdG), urinary estrone conjugates (E_1C), and fecal total estrogens (TE). Urinary PdG was 100% accurate, urinary E_1C was 89% accurate, and fecal TE was 100% accurate in predicting pregnancy. Fecal progesterone (P_4) and TE, as well as urinary E_1C and PdG concentrations all increased from conception (August) through January, but significant differences were not apparent until November. During the rutting season ovulation was detected by increases in either urinary PdG or fecal P_4 concentrations. Both pregnancy and ovulation were detected in uncaptured bison with reasonable accuracy by means of urinary and fecal steroids or their metabolites.

Key words: Bison, *Bison bison*, ovulation, pregnancy, reproduction, steroids, urine, feces.

INTRODUCTION

Until recently the study of reproductive physiology in large free-roaming wildlife has required immobilization or capture of animals to measure endocrine levels in blood. During the past decade, diagnosis of pregnancy and non-invasive study of ovarian function in captive exotic species have been based on measurement of urinary steroid metabolites (Loskutoff et al., 1983; Lasley, 1985). Urine can be collected from certain free-roaming wildlife and used in the study of reproduction. Kirkpatrick et al. (1988, 1990, a, b) diagnosed pregnancy in uncaptured feral horses (*Equus caballus*) by measuring urinary estrone conjugates (E_1C) or non-specific progesterone metabolites (iPdG). Collection of serial urine samples has permitted the characterization of conceptive and non-conceptive cycles in feral horses (Lasley and Kirkpatrick, 1991) and North American bison (*Bison bison*) (Kincy et al., 1990; Kirkpatrick et al., 1991b).

While these techniques are successful, collection of urine, even in snow, can be time-consuming. Use of feces for similar studies has such advantages as easily observable elimination by the animal and relatively easy location of the excrement. This strategy has led to successful pregnancy detection in feral horses by means of fecal total estrogens (Kirkpatrick et al., 1990b) and fecal steroid conjugates (Kirkpatrick et al., 1991a).

Very little is known about the reproductive physiology of free-roaming bison. The objectives of this study were to determine if pregnancy could be detected by means of urinary and fecal steroid metabolites, and if ovulation could be detected by means of urinary PdG and fecal progesterone (P_4). The hypotheses tested were (a) concentrations of fecal estrogens and progesterone and urinary estrogen and progesterone metabolites differed significantly between pregnant and non-pregnant animals, and (b) ovulation could be detected by identification of a luteal phase pattern of excretion of fecal progesterone and urinary progesterone metabolites. Our study was based on the desire to understand the mechanisms of reproductive self-regulation and the changes in reproductive success that accompany environmental pressures.

MATERIALS AND METHODS

Animals in this study were part of a herd of approximately 80 bison inhabiting a 200 ha range in northern Wyoming (44°44'N, 109°2'W). The vegetation included predominantly mixed-grass prairie and has been described by Knight et al.

(1987). Cows ranged in age from 3 to 17 yr. 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. Individuals were identified by numbered ear tags.

Experiment 1: During November 1988, bison were observed from a distance of 50 to 100 m, urine samples were collected from 18 cows. Urine was aspirated from pools on the ground or extracted from freshly soaked earth as described by Kirkpatrick et al. (1988). Samples were kept on ice during the day of collection and stored at −7 C without preservatives until tested 2 to 3 mo later. Fecal samples were stored in plastic bags at −7 C until tested.

Urine samples were diluted 1:2 with distilled water (dH_2O); 20 µl of the diluted sample were analyzed for pregnanediol-3δ-glucuronide (PdG) and E_1C. The enzyme immunoassays for PdG and E_1C have been described previously (Kirkpatrick et al., 1990a; Munro et al., 1991) and the PdG assay has been validated in bison (Kincy et al., 1990; Kirkpatrick et al., 1991b). To account for differences in urine concentrations, urine samples were diluted from 1:50 to 1:100 with dH_2O and analyzed for creatinine (Cr) concentrations by the microcolorimetric method of Taussky (1954). Pregnanediol-3-δ-glucuronide and E_1C values were calibrated to Cr values and reported as ng/mg Cr.

Wet fecal samples were weighed and 0.5 g samples were extracted in ethyl acetate/hexane (3:2, V/V) and analyzed for total estrogens by radioimmunoassay (Kirkpatrick et al., 1990b). The cross-reactivity of the anti-estrogen antibody with 17δ-estradiol was 11% (Kirkpatrick et al., 1990b). Approximately 5,000 counts per minute (cpm) of ^{3}H-17β estradiol was added to each fecal sample before extraction in order to estimate recovery of estrogens. Total estrogens were reported as ng/g wet feces. In January 1989, the bison were confined in chutes and tested for pregnancy by rectal palpation; calves were counted during April, May and June. Based on the dates of parturition, 12 of the conceptions had occurred between 15 July and 10 August 1988.

Experiment 2: Fecal samples were collected from six bison cows in September, November 1989 and January, 1990. Weighed samples were extracted with dH_2O, the residue dried and weighed, 0.25 g was rehydrated and extracted with diethyl ether, and redissolved in 95% ethanol by the methods of Desaulniers et al. (1989). Approximately 10,000 cpm of ^{125}I-progesterone was added to each fecal sample before extraction in order to estimate recovery of P_4. Twenty µl of the dHp_2O extract were analyzed for PdG and E_1C. The ethanol extracts were diluted 1:100 to 1:1,000 in dH_2O and 20 µl were assayed for total estrogens by radioimmunoassay (Kirkpatrick et al., 1990b), and for P_4 by enzyme immunoassay (Munro and Stabenfeldt, 1984). All values are given as ng/g dry feces. Mean concentrations for September 1989, November 1989, and January 1990 were tested for significant differences with Student's *t*-test (Freedman et al., 1978). Pregnancy was confirmed by rectal palpation in January 1990, and calf counts were made in April, May and June, 1990. Dates of parturition indicated that five of the six conceptions occurred between 25 July and 15 August.

Experiment 3: Urine and fecal samples were collected approximately every 2 to 4 days during the rutting season from five sexually mature cow bison between 31 July and 31 August 1989. Urine samples were collected, stored, and analyzed for PdG as described in experiment 1. On some days both urine and fecal samples were collected from the same cow but on other days only one type of sample was collected. Fecal samples were weighed and extracted with diethyl ether and the residue redissolved in ethanol as described by Desaulnier et al. (1989). The ethanol extracts were diluted 1:100 in dH_2O and 20 µl were analyzed for P_4 (Munro and Stabenfeldt, 1984). The patterns of urinary PdG and fecal P_4 excretion were recorded for each bison cow and compared by regression analysis; the correlation coefficient for the two hormones was determined (Freedman et al., 1978).

RESULTS

Experiment 1: Mean (±SE) recovery of ^{3}H-17β estradiol from feces was 62.18 (±6.45)%. Fourteen of 18 cows were pregnant during January 1989 as determined by rectal palpation, and all 14 delivered calves during 1989. The total fecal estrogen analysis was the most accurate test for pregnancy, with 1.09 ng/g feces being the lowest value in pregnant cows and 0.20 ng/g being the highest value in a nonpregnant cow (Table 1). Using 1.0 ng/g feces as the minimum value for pregnancy, total fecal estrogen analysis was 100% accurate as a diagnostic test for pregnancy. Urinary PdG analysis was equally accurate. The lowest urinary PdG value for pregnant cows was 54.7 ng/mg Cr while 9.9 ng/mg Cr was the highest value for a

TABLE 1. Urinary pregnanediol-3α-glucuronide (PdG), urinary estrone conjugates (E_1C), and total estrogen concentrations for pregnant bison at three months gestation, and nonpregnant bison.

Bison reproductive group	PdG (ng/mg Cr)	E_1C (ng/mg Cr)	Total fecal estrogens (ng/g feces)
Pregnant ($n = 14$)			
Range	54.7 to 302.9	9.4 to 85.5	1.08 to 1.94
Mean	113.30	26.74	1.54
SE	18.57	5.65	0.08
Nonpregnant ($n = 4$)			
Range	3.6 to 9.9	2.7 to 11.5	0.12 to 0.2
Mean	6.82	5.62	0.16
SE	1.55	2.03	0.02

non-pregnant cow. The correlation coefficient between total fecal estrogens and urinary PdG was $r = 0.70$ ($P < 0.001$). Using a value of >10.0 ng/mg Cr as the minimum PdG value for pregnancy, urinary PdG analysis was 100% accurate in predicting pregnancy. The urinary E_1C analysis was less accurate in predicting pregnancy. The lowest urinary E_1C value for pregnant cows was 9.4 ng/mg Cr and the highest E_1C concentration for a non-pregnant cow was 11.5 ng/mg Cr. The correlation coefficient between total fecal estrogens and urinary E_1C was $r = 0.472$ ($P = 0.049$). Using 10 ng/mg Cr as the minimum E_1C value for pregnancy, urinary E_1C analysis was 89% accurate.

Experiment 2: Urinary E_1C and PdG and fecal P_4 and total estrogen concentrations all rose between the September collection, approximately 1 mo postconception, and January, approximately 4.5 mo postconception (Fig. 1). Urinary E_1C and PdG did not increase significantly ($P < 0.05$) between September and November, but did ($P < 0.001$) between November and January. Both fecal P_4 and total estrogens rose significantly ($P < 0.001$) between September, November, and January.

Experiment 3: Urinary PdG and fecal P_4 concentrations during the 32-day collection period had a pattern consistent with the estrous cycle of the cow (*Bos taurus*) Asdell, 1964) an the European bison (*Bison bonasus*) (Krasinski and Raczynski, 1967). Mean (±SE) recovery of ^{125}I-P_4 from feces was 61.8 (±5.3)%. Basal Fecal P_4 concentrations preceding ovulation ranged between approximately 2,000 to 3,000 ng/g feces and reached a peak of 5,000 to 10,000 ng/g midway through the luteal phase (Fig. 2). These values were similar to those in domestic cows (Desaulniers et al. 1989). Basal urinary PdG concentrations preceding ovulation ranged between 5 to 100 ng/mg Cr and reached a peak of 350 to 1,000 ng/mg Cr at mid-luteal phase (Fig. 2). The correlation coefficient between urinary PdG and fecal P_4 for the five bison cows monitored during the August rut ranged from a low of $r = 0.310$ to a high of $r = 0.922$; however, in all five cases the luteal pattern of P_4 secretion was evident for both hormones. Four of the five bison demonstrated classic estrous behavior which was coincidental with both PdG and P_4 nadirs immediately preceding luteal phase elevation. Four of the five bison had a second estrous cycle during September 1989, and dates of parturition in 1990 corroborate those second estrous cycles as the time of conception.

DISCUSSION

Successful use of urinary steroid metabolites and fecal free steroids to determine pregnancy in the North American bison is based on previous work with related members of the order Artiodactyla. The presence of immunoreactive PdG has been demonstrated in the urine of okapi (*Oka-*

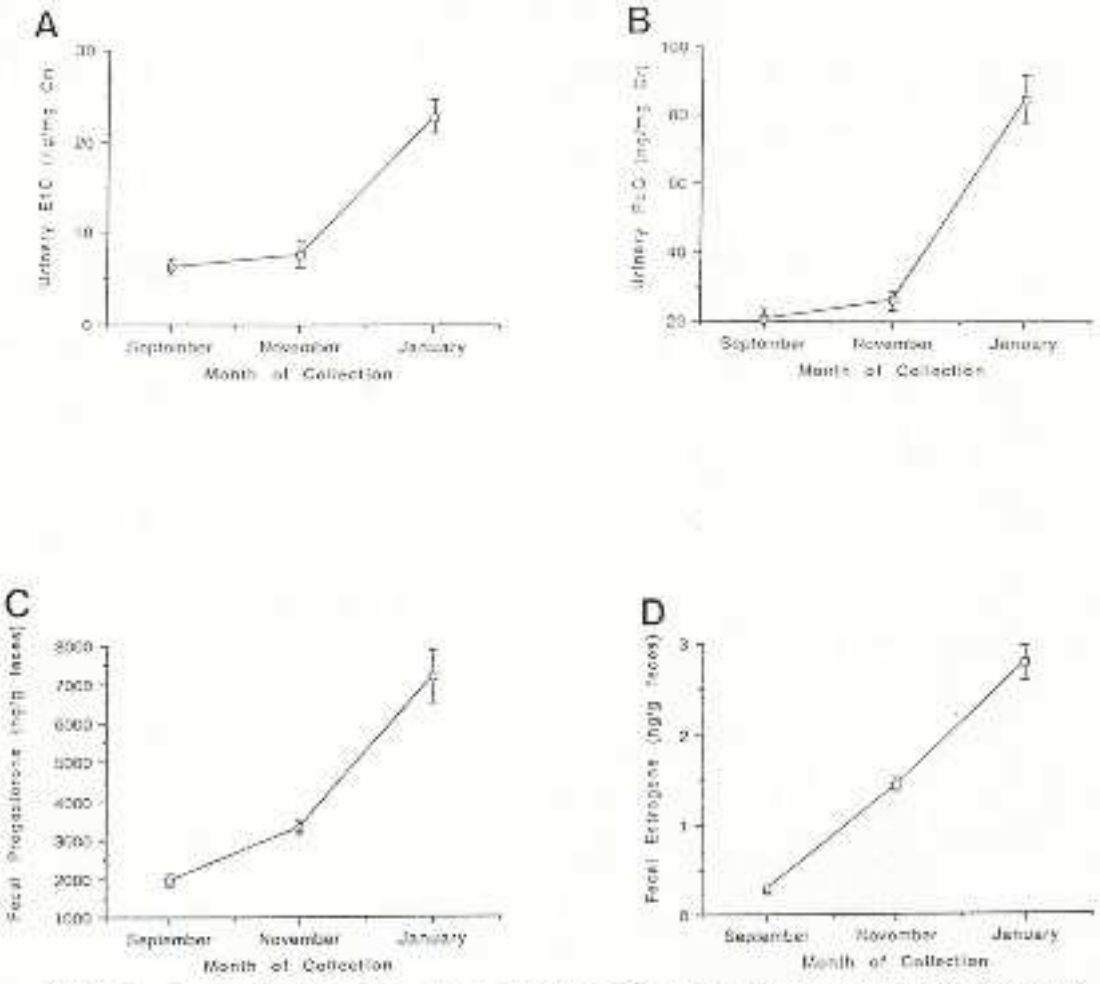

FIGURE 1. Increases in (A) urinary estrone conjugates (E_1C) (B) urinary pregnanediol-3β-glucuronide (PdG), (C) fecal progesterone, and (D) fecal total estrogens through approximately 180 days of pregnancy.

pia johnstoni) (Loskutoff et al., 1982), oryx (*Oryx beisa beisa*), bongo (*Tragelaphus eurycerus*) and domestic cow (Loskutoff et al., 1983), giraffe (*Giraffa camelopardalis*) (Loskutoff et al., 1986), Eld's Deer (*Cervus eldi thamin*) (Monfort et al., 1990) and the North American bison (Kincy et al., 1990; Kirkpatrick et al., 1991b). Additionally, PdG is the major product of P_4 metabolism in domestic cow urine (Feher, 1975), and urinary PdG accurately reflects plasma P_4 in Eld's deer (Monfort et al., 1990) and bison (Kirkpatrick et al., 1991b).

Urinary estrone conjugates are very accurate in the early (>35 days postconception) detection of pregnancy in the Perissodactyla and particularly the Equidae (Kassam and Lasley, 1981; Kasman et al., 1985, 1986, 1988; Czekala et al., 1990), but these steroid metabolites are not useful for early detection of pregnancy among the Bovidae. This is probably because bovids metabolize plasma estrogens to other forms of conjugated compounds. However, by 3 to 4 mo of pregnancy, estrogen concentrations have increased in bovids to the point where estrone conjugates are useful predictors of pregnancy, despite representing only a minor urinary metabolite. This is corroborated by the dramatic increase in fecal estrogens demonstrated by Safer-Hermann et al. (1987) in cows, red

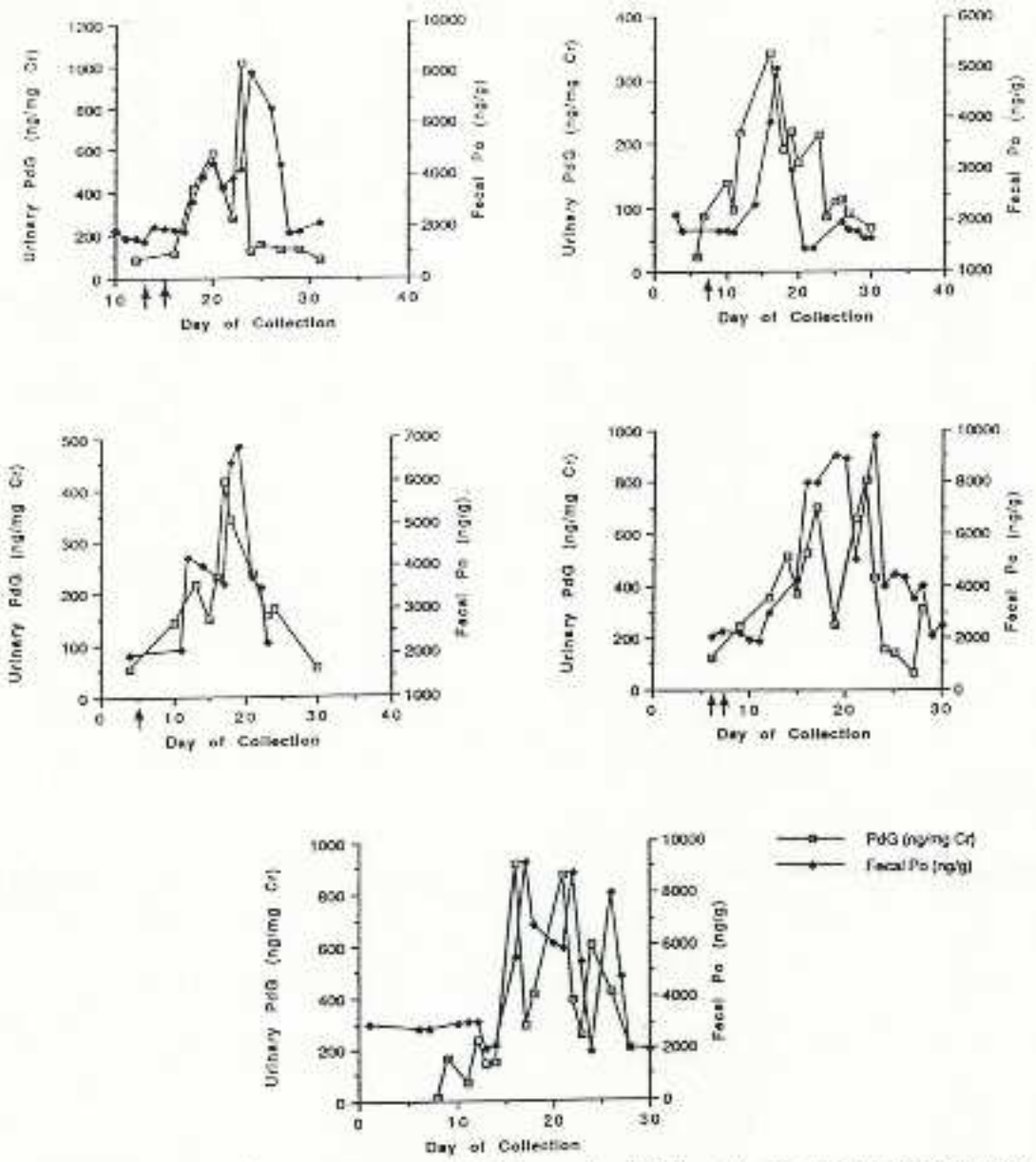

FIGURE 2. Patterns of urinary pregnanediol-3δ-glucuronide (PdG) and fecal progesterone (Fecal Po) excretion during the luteal phase for five bison cows. Arrows indicate the days of observed behavioral estrus.

buffalo (*Syncerus caffer nanus*) and yak (*Bos mutus*). The primary fecal estrogen during pregnancy in cows, after 90 days postconception, is 17δ-estradiol (Mostl et al., 1984), and the elevation of estrogens is great enough that an estrogen radioimmunoassay with only an 11% cross-reactivity with this particular metabolite is sensitive enough for pregnancy detection. The assay in this study, however, was used to measure total estrogens, and the presence of 17δ-estradiol in pregnant bison is only assumed.

Use of urinary PdG, E_2C, or fecal total estrogen concentrations for accurate pregnancy detection in bison was most reliable

after 90 days postconception. The pattern of increase in fecal total estrogens and urinary E_1C in the bison is similar to changes in plasma total estrogens and fecal estrogens seen in the pregnant domestic cow (Desaulniers et al., 1989).

The small number of non-pregnant animals in this study prevented analysis of the full range of values for the steroids measured. Perhaps with a larger number of non-pregnant animals, the range of hormone values would be greater and the accuracy of pregnancy diagnosis might decrease.

Ovulation and formation of the corpus luteum could not be confirmed by rectal palpation or ultrasound in bison of this study. While presence of a fetus could be detected by rectal palpation, the intractable nature of the animals did not permit the sensitive palpation techniques required for detection of corpora lutea. Ultrasound was not available. Despite the inability to confirm ovulation by either of these direct methods, the pattern of P_4 secretion was indicative of ovulation. There can be at least three causes for increases in P_4, including ovulation, formation of a persistent corpus luteum, or extra-ovarian (adrenal) P_4. However, only ovulation and formation of the corpus luteum result in the P_4 pattern seen in these bison. There was an evident 2 to 3 day lag in excretion of fecal P_4 compared to the excretion of urinary PdG. Despite the low correlation coefficient between urinary PdG and fecal P_4 in some of the animals, the luteal phase pattern was clearly evident and a reliable indicator of ovulation.

Several different extraction methods were used for the recovery of steroids or their conjugated metabolites for the three experiments. The ethyl acetate/hexane extraction used in experiment 1 for the recovery of free estrogens was based on the previous successful extraction of fecal estrogens and diagnosis of pregnancy in feral horses (Kirkpatrick et al., 1990b). Extraction of E_1C and PdG required water extraction rather than organic solvent extraction. Conjugated steroid metabolites are excreted primarily by way of the urine, after being resorbed from the gut into the vascular bed; however, a small proportion of the conjugates remain trapped in the gut and are excreted in the feces (Lasley and Kirkpatrick, 1991). These fecal steroid conjugates remain water soluble. Finally, the ether extraction used for progesterone was based on the recent work of Desaulniers et al. (1989), who successfully applied this method to the fecal analysis of P_4 in cows and muskoxen (*Ovibus moschatus*). Tritiated P_4 could not be used for determination of P_4 recovery because large amounts of plant pigments also are removed with the ether extraction, and the interference of the spectrophotometry is too great for liquid scintillation counting.

Validation of urinary and fecal steroid analysis in large intractable animals is difficult and cannot always follow the same procedures used with captive animals (Lasley and Kirkpatrick, 1991). Validation of these methods rests on several assumptions. First, it has previously been demonstrated that there is a strong correlation between plasma P_4 and urinary PdG in bison (Kirkpatrick et al., 1991b); therefore, the correlations between urinary PdG and fecal P_4 demonstrated here (Fig. 2) imply a correlation between plasma and fecal P_4. Second, high performance liquid chromatography previously has been used to validate the presence of PdG as the primary P_4 metabolite in bison urine (Kirkpatrick et al., 1990c). This is supported by the work of Feher (1975), who demonstrated that the primary P_4 metabolite in the domestic cow also was PdG. Third, parallelism between serially diluted samples and the PdG standard curve previously have been demonstrated in bison (Kirkpatrick et al., 1991b). Finally, the ultimate validation of these techniques is a measure of the accuracy in predicting the physiological events in question, namely ovulation and pregnancy, rather than the precise nature of the metabolites being measured.

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