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PREGNANCY DETECTION IN BIGHORN SHEEP (*OVIS CANADENSIS*) USING A FECAL-BASED ENZYME IMMUNOASSAY

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ABSTRACT: We developed and validated an enzyme immunoassay for immunoreactive pregnanediol-3-glucuronide (iPdG) in feces to monitor reproductive status in desert and Rocky Mountain bighorn sheep (*Ovis canadensis*). Fecal iPdG concentrations were strongly correlated (r = 0.71) with serum progesterone concentrations in paired fecal and blood samples collected from 34 free-ranging desert bighorn sheep. In bimonthly fecal samples collected from 12 captive ewes, fecal iPdG profiles were similar between desert and Rocky Mountain bighorn sheep and we selected a pregnancy detection cutoff value of iPdG ≥1.8 ng/mg feces. Fecal iPdG concentrations always exceeded this cutoff value when samples were collected from about day 60 of pregnancy to a few days before parturition, but values <1.8 ng/mg (false negatives) were common for samples collected during the first 60 days of gestation. Although we tested a small number of known pregnant and non-pregnant ewes, the accuracy of the assay was 100% when two samples, collected 2 wk apart, were evaluated for any given ewe. Based on these data, this direct enzyme immunoassay for fecal progesterone metabolites has promise as a diagnostic tool to monitor hormone excretion and pregnancy in a free-ranging ungulate species.

Key words: Bighorn sheep, reproduction, immunoassay, fecal steroids, pregnancy, progesterone.

INTRODUCTION

The ability to accurately assess reproductive success is essential for understanding and managing the dynamics of captive and free-ranging wildlife populations. For example, a decrease in pregnancy, fecundity, and other measures of reproductive success may provide the first detectable sign of a chronic stressor such as enzootic disease (Lasley and Kirkpatrick, 1991). The pregnancy status of wild animals typically has been inferred from direct observations of natality or recruitment, or it has been monitored via palpation, ultrasound, vaginal histology, or determination of serum hormone levels (Loskutoff et al., 1983). However, all of these approaches have limitations. Postnatal estimates of pregnancy rates may be difficult or impossible to obtain for secretive or gregarious species, and differences between conception rates and birth rates will not be detected by this approach. On the other hand, prenatal techniques have traditionally required expensive and stressful restraint operations.

The measurement of fecal steroid metabolites associated with pregnancy theoretically provides a useful alternative for evaluating reproductive status. Radioimmunoassay (RIA) has been successfully used to measure fecal steroids in domestic cattle and horses (Bamberg et al., 1984; Mostl et al., 1984), feral horses (Kirkpatrick et al., 1990), non-human primates (Shideler et al., 1994), caribou (*Rangifer tarandus*) (Messier et al., 1990), muskoxen (*Ovibos moschatus*) (Desaulniers et al., 1989), and many carnivores (Gross et al., 1990). However, RIA procedures have had limited use in field studies because they are labor intensive, require special equipment, and rely heavily on the use of organic solvents and radioisotopes. These problems have recently been overcome by the development of enzyme immunoassays (EIA) that accurately measure progesterone and estrogen metabolites in feces or

urine (Shideler et al., 1990, 1994). Our objective was to develop and evaluate a fecal-based EIA for pregnancy diagnosis in bighorn sheep (*Ovis canadensis*).

MATERIALS AND METHODS

Fecal samples were collected every 2 wk for 9 to 12 mo from five pregnant desert bighorn sheep (DBS) from a captive herd at the Bighorn Institute in Palm Desert, California (USA) (1992 and 1993), and from five pregnant and two non-pregnant Rocky Mountain bighorn sheep (RMBS) from a captive herd at the Deaconess Research Institute in Billings, Montana (USA) (1991 and 1992). Both serum and feces were collected from free-ranging DBS ewes that were captured, radiocollared, and released in October (n = 19) and December (n = 15) 1992 in Anza Borrego Desert State Park (ABDSP), (32°40′ to 33°30′N, 116°20′ to 116°30′W) California. Forty-four additional fecal samples were collected from these 34 radiocollared ewes and 16 other individually identified ewes on an opportunistic basis in 1992 and 1993 by field biologists in ABDSP. Since multiple samples were collected from some individuals, a single sample was selected at random from each of these 50 free-ranging bighorn sheep to use for comparisons with the two captive herds. All fecal samples were collected within 45 min of defecation and frozen within 8 hr. Frequent, direct observation of both captive and free-ranging sheep allowed us to group fecal samples into the following time periods or categories: samples collected anytime after lambing up to about 60 days before breeding (non-pregnant), samples collected from about 60 days pre-breeding through the first 60 days of pregnancy (rut to 60 days), and samples collected from about day 60 of pregnancy through lambing (61 to 180 days).

Serum progesterone (Po) levels were determined for samples collected in ABDSP by the methods of Munro and Stabenfeldt (1984). Metabolites of progesterone, immunoreactive pregnanediol-3-glucuronide (iPdG), were measured in fecal samples using a modification of a competitive EIA described previously by Shideler et al. (1994). Briefly, 1 g of thawed feces was added to 6 ml of phosphate buffer with 40% methanol. Samples were shaken for 24 hr and then diluted 1:20 with phosphate buffer. Samples and an iPdG competitor molecule labeled with horseradish peroxidase were then incubated for 24 hr in 96-well microtiter plates previously coated with a polyclonal antibody directed against iPdG conjugated to bovine serum albumin (Munro et al., 1991). Plates were incubated for 1 hr following substrate addition and then read using an automated plate-reader. Samples were run in triplicate with coefficients of variation (CV) <15% to determine mean iPdG concentrations. Standards and internal controls were included on each plate to ensure quality control and to construct a standard curve (Shideler et al., 1994). Serial dilutions of fecal samples from two pregnant and two non-pregnant ewes were then assayed and examined for parallelism to the working standard curve. Overall assay reproducibility was assessed by calculating both intra-assay and inter-assay CV's. Mann-Whitney and Kruskal-Wallis non-parametric tests (Daniel, 1991) were used to assess differences between mean iPdG concentrations excreted by RMBS, captive DBS, and free-ranging DBS for each of the three time periods. The Spearman rank correlation coefficient was used to assess the association between serum Po and fecal iPdG concentrations for the 34 free-ranging ewes sampled in October and December 1992 in ABDSP (Daniel, 1991).

RESULTS

The inter-assay CVs for the fecal-based EIA were 13% at 47% bound, and 16% at 25% bound (n = 19), while the intra-assay CV = 6.9% when samples were run in triplicate on two plates (n = 24). The 50% bound value for iPdG calculated from the standard curve averaged 15.98 ng/well (n = 19). With the assay, we were capable of accurately detecting fecal iPdG concentrations >0.5 ng/mg feces, and iPdG concentrations <0.5 ng/mg feces were estimated from the standard curve. Parallel dose-response relationships were seen with fecal samples at dilutions of 1:8 to 1:64 when compared to the standard curve. The iPdG concentration could not be determined for test dilutions >1:64, and dilutions <1:8 did not parallel the standard curve due to a matrix effect of fecal contaminants.

All five captive DBS conceived in the fall (October or November) and lambed in late March to mid-April. These ewes' fecal iPdG levels increased during the rut and throughout pregnancy, finally decreasing to low levels within a few days of lambing (Table 1, Fig. 1). Two of seven captive RMBS (BH2, BH5) did not conceive dur-

TABLE 1. Immunoreactive pregnanediol-3-glucuronide (iPdG) concentrations (ng/mg feces) of Rocky Mountain (RMBS) and desert (DBS) bighorn sheep ewes as determined by fecal-based enzyme immunoassay.[a]

	Non-pregnant			Rut to 60 days			60–180 days		
Individual	Mean	SD	Number of samples	Mean	SD	Number of samples	Mean	SD	Number of samples
Captive Rocky Mountain bighorn sheep									
BH1	0.32	0.03	3	0.54	0.24	5	4.30	3.02	7
BH2	0.74	0.30	5	0.61	0.37	5	0.47	0.32	5
BH3	0.57	0.21	3	0.96	0.46	6	4.20	2.84	7
BH4	1.00	0.65	3	1.64	1.48	7	4.75	1.23	6
BH5	0.98	0.41	5	0.94	0.37	4	1.30	1.00	5
BH6	1.43	1.28	4	1.83	1.08	7	7.41	4.88	4
BH7	0.43	0.14	2	1.52	0.89	5	5.36	3.25	8
Total	0.78	0.38	7[b]	1.30	0.47	5[b]	5.20	1.18	5[b]
Captive desert bighorn sheep									
84-01	0.58	0.10	12	0.95	0.56	3	7.43	4.38	6
87-02	0.60	0.43	11	1.97	1.19	5	8.28	3.28	7
86-03	0.64	0.23	12	1.43	0.76	5	5.27	1.38	3
85-04	0.71	0.52	12	1.93	0.86	4	7.46	4.72	7
87-05	0.61	0.20	12	1.62	1.14	4	4.80	1.30	5
Total	0.63	0.05	5[b]	1.58	0.37	5[b]	6.65	1.36	5[b]
Free-ranging DBS Total	0.58	0.33	9[b]	1.55	0.99	24[b]	4.22	2.08	17[b]

[a] Fecal samples were collected in 1991 and 1992 from captive RMBS at the Deaconess Research Institute, Billings, Montana, and in 1992 and 1993 from captive DBS at the Bighorn Institute, Palm Desert, California, and from free-ranging DBS in Anza Borrego Desert State Park, California. Samples were collected at approximately 2 wk intervals for 9 to 12 mo and assigned to the following categories: samples collected anytime after lambing up to about 60 days before breeding (non-pregnant), samples collected from about 60 days pre-breeding through the first 60 days of pregnancy (rut to 60 days), and samples collected from about day 60 of pregnancy through lambing (61 to 180 days). All captive sheep became pregnant except for BH2 and BH5.

[b] The number of samples corresponds to the number of individual ewes sampled for total captive RMBS, total captive DBS, and total free-ranging DBS.

ing their rut (December to January), and their fecal iPdG levels remained relatively low throughout the year. The other five RMBS ewes conceived and four ewes (BH1, BH3, BH4, BH7) lambed in June and July, while the one ewe (BH6) aborted in early June about 6 wk before she was due to lamb. Fecal iPdG levels increased throughout pregnancy for each of these animals, and then returned to baseline levels at the time of lambing ($n = 4$) or abortion ($n = 1$) (Table 1, Figs. 2 and 3). Mean fecal iPdG concentrations were similar ($P > 0.10$) for captive DBS and RMBS during both the non-pregnant and rut-60 days time periods. However, mean fecal iPdG concentrations were significantly higher ($P = 0.04$) in captive DBS than RMBS from day 60 to 180 of pregnancy.

Twenty-nine of 34 free-ranging ewes captured in 1992 in ABDSP were observed with lambs in 1993 and these ewes were used as known-positive controls for evaluation of assay performance. The other five ewes were never observed with lambs and were not considered to be known non-pregnant animals since we did not know whether they became pregnant and aborted or if they had lambs that died soon after birth. These five ewes were not considered further in the evaluation of test performance. Serum Po and fecal iPdG concentrations for the 29 ewes that lambed were significantly higher ($P < 0.01$) for ewes sampled in December ($n = 15$, mean Po = 5.2, SD = 2.5; mean iPdG = 3.4, SD = 1.8) than October ($n = 14$, mean Po = 2.8, SD = 1.1; mean iPdG = 1.8, SD = 0.7). Fecal iPdG concentrations were ≥1.8 ng/mg feces for the 15 pregnant

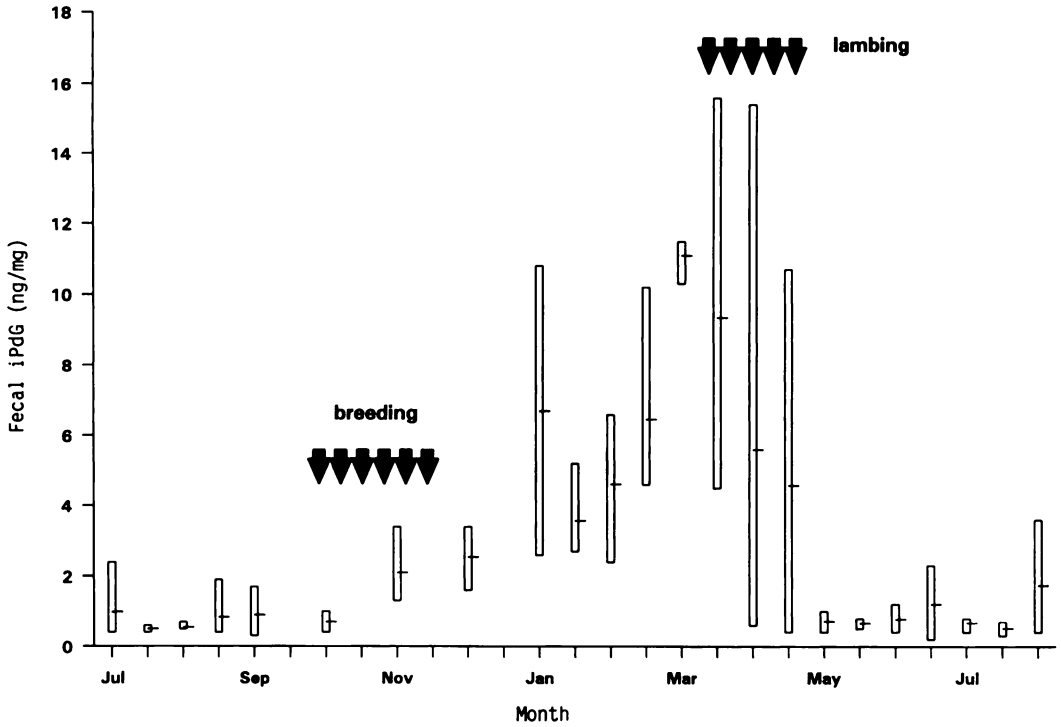

FIGURE 1. Fecal immunoreactive pregnanediol-3-glucuronide (iPdG) levels (range and mean) for five desert bighorn ewes at the Bighorn Institute in Palm Desert, California. Fecal samples were collected at approximately 2-wk intervals and the ewes were bred in the fall of 1992 and lambed in 1993.

ewes sampled in December, while six of 14 pregnant ewes sampled in October had iPdG concentrations <1.8 ng/mg feces. Fecal iPdG concentrations were strongly correlated ($r = 0.71$, $P < 0.01$) with serum Po concentrations, and iPdG concentrations ≥1.8 were always associated with serum Po concentrations >1 ng/ml for the 34 ewes from ABDSP.

Based on these comparisons and results of the fecal iPdG analysis of captive bighorn sheep, we selected a pregnancy detection cutoff value for iPdG of ≥1.8 ng/mg feces. A small number of false positive test results were obtained for non-pregnant ewes both during the breeding and non-breeding seasons, and false negative results were commonly obtained for pregnant ewes sampled in the first 60 days of gestation (Figs. 1 to 3). However, false positive results could always be identified by examining an additional fecal sample collected ≥2 wk after the initial positive sample. False negative results were not obtained for samples collected from about day 61 through 180 days of pregnancy for both captive ($n = 12$) and free-ranging ($n = 29$) bighorn sheep. Fecal iPdG concentrations were similar between free-ranging DBS and both captive DBS and RMBS for samples collected within each of the three time periods ($P > 0.10$ for all comparisons).

DISCUSSION

In this study we demonstrated that a fecal-based competitive EIA can be used to accurately and non-invasively monitor the pregnancy status of captive and free-ranging bighorn sheep. The assay was based on the use of a polyclonal antibody known to bind to iPdG in other species (Munro et al., 1991). Based on our results, this antibody also binds to progesterone metabolites (probably PdG and free pregnanediol) excreted in the feces of bighorn sheep.

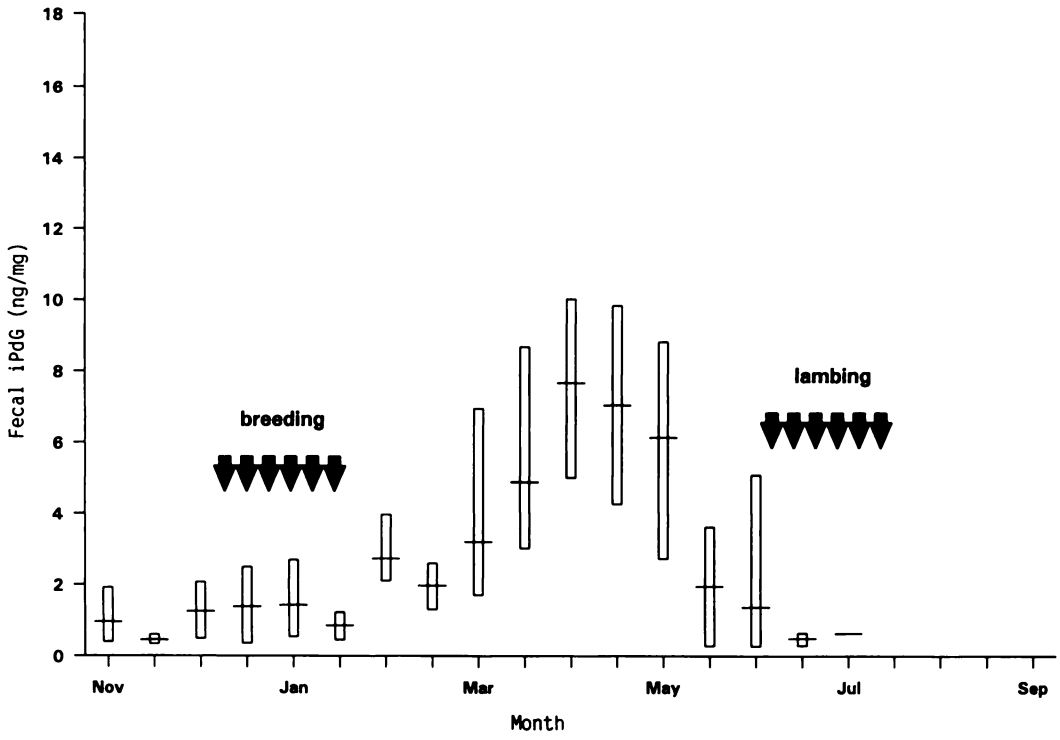

FIGURE 2. Fecal immunoreactive pregnanediol-3-glucuronide (iPdG) levels (mean and range) from four Rocky Mountain bighorn ewes (BH1, BH3, BH4, BH7) that were sampled bimonthly at the Deaconess Institute in Billings, Montana. Fecal samples were collected at approximately 2-wk intervals and the ewes were bred in the winter of 1991 and lambed in 1992.

The EIA was quantitative and reproducible when sample dilutions ranged from 1:8 to 1:64, and the assay was simple, inexpensive, non-radiometric, and appropriate for use in field studies. Shideler et al. (1994) showed this same methodology to be useful for measuring both estrogen and progesterones metabolites in primate species. However, in our study only the iPdG assay could be validated since we obtained non-parallel dose-responses for the estrone metabolites of circulating estradiol and estrone (data not shown). These findings underscore the importance of rigorous assay validation for each hormone metabolite for each new species under investigation.

Using the analysis of fecal iPdG levels in bimonthly samples from captive RMBS and DBS, we delineated the iPdG profiles of pregnant and non-pregnant animals (Table 1, Figs. 1 to 3), and we clearly identified the quantitative relationship between fecal iPdG concentrations and known physiological events such as pregnancy and lambing. Fecal iPdG levels in samples collected during the rut through the first 60 days of pregnancy were not consistently useful for determining the pregnancy status of DBS or RMBS. The increased fecal iPdG concentrations seen occasionally just prior to rut may have been due to a progesterone priming phenomenon similar to that seen in domestic sheep. Since fecal iPdG levels increased steadily during pregnancy, well-timed sample collection (preferably two or more samples after about day 61 of pregnancy), should result in maximum sensitivity and specificity, thus increasing the probability of correct classification of pregnancy status. Using a cutoff of $\geq$1.8 ng/mg feces and two fecal samples collected $\geq$2 wk apart, the assay was 100% accurate except during the first 60

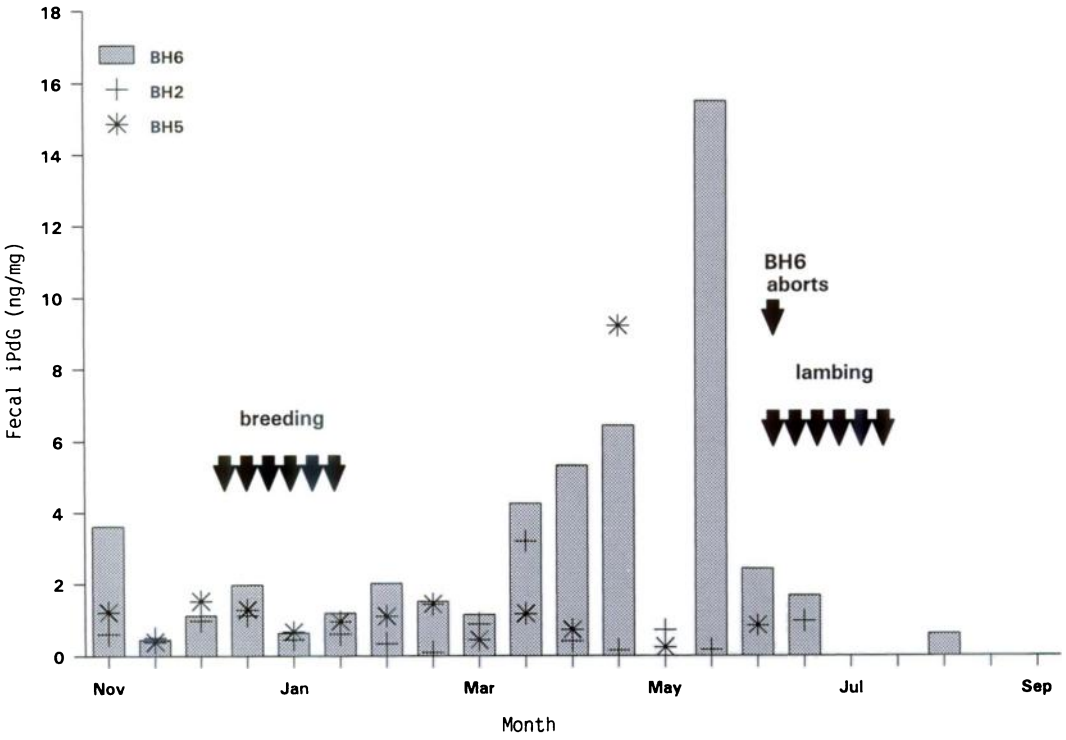

FIGURE 3. Fecal immunoreactive pregnanediol-3-glucuronide (iPdG) levels from three Rocky Mountain bighorn ewes that were sampled bimonthly at the Deaconess Institute in Billings, Montana in 1991 and 1992. Fecal iPdG concentrations are shown for two non-pregnant ewes (BH2, BH5) and one ewe (BH6) that became pregnant and aborted about 6 wk prior to her expected lambing date.

days of gestation. However, this estimate was based on samples collected from only two non-pregnant ewes during the breeding season. We clearly need to test more samples from non-pregnant ewes during the breeding season to investigate the possibility that iPdG values rise above 1.8 ng/mg feces and remain elevated. The timing for appropriate sample collection (≥day 61 of pregnancy) will vary with geographic location since the breeding period differs among bighorn sheep populations. However, it is important to remember that although most ewes within a given herd probably will give birth within a relatively short lambing period, ewes can lamb in any month of the year.

Although captive DBS excreted a significantly higher concentration of iPdG from day 61 to 180 than captive RMBS, this result was probably not biologically significant. Sample size and sampling frequency were low for each group and it is likely that some iPdG peaks were missed; thus our iPdG values probably did not reflect the highest concentration achieved. Also, no differences were detected in the iPdG concentrations of pregnant captive RMBS and free-ranging DBS during this time.

For many free-ranging species, the influence of changes in fecundity and fertility on population dynamics can only be inferred. With small or threatened populations, serial fecal iPdG analyses for individual animals may help identify problems with conception, embryonic loss, or abortion. For example, fecal iPdG levels increased during pregnancy and then returned to baseline levels in a RMBS that aborted during late gestation (Fig. 3). Demographic studies of larger populations also can be enhanced since analysis of fecal iPdG levels provide a non-invasive,

cost-effective method for assessing pregnancy. For example, bighorn sheep numbers in ABDSP have sharply declined presumably due to ongoing poor recruitment and infectious diseases (U.S. Fish and Wildlife Service, 1992; Elliott et al. 1994). Prior to the development of the fecal iPdG assay it was necessary to track ewes on a monthly basis and look for lambs to determine whether the ewes had become pregnant. However, pregnancy status now can be accurately determined based solely on fecal analysis using samples collected >60 days post-rut. Indeed, pregnancy rates were very high for animals sampled in October and December 1992 (≥29 of 34 animals), thus conception rates apparently were not limiting population growth that year. Although our sample sizes were limited, fecal iPdG analysis clearly is a useful tool for assessing pregnancy in bighorn sheep. However, it is important to note that pregnancy rates will not typically equal lamb production and recruitment since abortions and neonatal losses can be expected even within healthy populations.

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