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COMPARISON OF THREE FECAL STEROID METABOLITES FOR PREGNANCY DETECTION USED WITH SINGLE SAMPLING IN BIGHORN SHEEP (*OVIS CANADENSIS*)

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ABSTRACT: We compared three fecal steroid metabolite assays for their usefulness in detecting pregnancy among free-ranging Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) from Bighorn Canyon National Recreation Area, Wyoming and Montana (USA) and captive bighorn ewes at ZooMontana in Billings, Montana. Fecal samples were collected from 11 free-ranging, radio-collared bighorn ewes in late January–May 2001 and from 20 free-ranging, radio-collared ewes in late March to mid-May 2002. Free-ranging ewes were monitored the following spring to determine whether or not they lambed. In addition, two captive ewes were studied at ZooMontana. With three exceptions, free-ranging bighorn ewes that produced lambs had nonspecific progesterone metabolite (iPdG) levels of >1,800 ng/g feces and iPdG levels >7,000 ng/gm feces when samples were collected between early March and mid-May. Samples collected earlier in the year were inconclusive. One false negative was suspected to be the result of sample collection error. Of the captive ewes, nonspecific pregnanediol-3α-glucuronide (PdG) and iPdG followed a predictable curve over the course of the 180-day pregnancies. We conclude that estrone conjugates are not useful in diagnosing pregnancy; however, fecal steroid analysis of PdG and iPdG can be used to accurately determine pregnancy and reproductive function in bighorn sheep. This holds great potential as a noninvasive technique for understanding the role of reproductive disease in wild bighorn sheep.

Key words: Animal reproduction, bighorn sheep, fertility, noninvasive, *Ovis canadensis canadensis,* pregnancy.

INTRODUCTION

Developing noninvasive techniques for gathering biological information from wildlife is a goal of many wildlife managers and biologists. Handling free-ranging wild animals causes stress (DeForge, 1976), may disrupt reproductive events (Ballard and Tobey, 1981; Larsen and Gauthier, 1989), may confound endocrine status (Kirkpatrick et al., 1979), and can be stressful and dangerous to humans (Lasley and Kirkpatrick, 1991). Evaluations of sexual maturity, fertility, and reproductive status are useful and needed by managers to predict potential success of a population and for making general management decisions. Measures of reproductive success are also useful tools to help determine the health of a population (Lasley and Kirkpatrick, 1991). Monitoring reproductive function and reproductive success in free-ranging wildlife by means of fecal and urinary steroids has the potential to provide valuable information on the overall health of the population without the danger and stress of handling animals.

Pregnancy detection by fecal steroid analysis has been successfully applied to a host of ungulate species including moose (*Alces alces;* Monfort et al., 1992; Schwartz et al., 1995), muskoxen (*Ovibos moschatus*; Desaulniers et al., 1989), bison (*Bison bison*; Kirkpatrick et al., 1992, 1993, 1996), *Equus* spp. (Bamberg et al., 1991; Kirkpatrick et al., 1991; Barkuff et al., 1993), caribou (*Rangifer tarandus*; Messier et al., 1990), and black rhinoceros (*Diceros bicornis*; Berkeley et al., 1997). Safar-Hermann et al. (1987) reported using nonspecific radioimmunoassay in four species (red buffalo [*Syncerus caffer nanus*], yak [*Bos mutus*], Grevy's zebra [*Equus grevyi*], and Nubian ibex [*Capra ibex nubiana*]) successfully. Fecal steroid metabolite analysis also has been used successfully to di-

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agnose pregnancy in desert bighorn sheep and Rocky Mountain bighorn sheep (*Ovis canadensis*) using a nonspecific assay for progesterone metabolites (Borjesson et al., 1996) and multiple sampling. The assay used in Borjesson et al. (1996) was for metabolites related to pregnanediol-3α-glucuronide (PdG), coupled with multiple samples (two samples collected 2 wk apart) from 60 days or later in gestation. The Borjesson et al. (1996) method proved to be 100% accurate. But not all wildlife managers and biologists have the time to conduct multiple sampling.

The current bighorn sheep (*O. canadensis canadensis*) population of Bighorn Canyon National Recreation Area (BICA) Wyoming and Montana (USA) is the product of several reintroductions to the surrounding areas (Coates and Schemnitz, 1989). Following a release in 1973 and growth rates near maximum potential of 19.8% per year, the population grew to an estimated peak population of about 211 animals in 1993 and 1994 (Kissell et al., 1996). The population began to decline rapidly in 1995 and 1996. Kissell et al. (1996) noted low ewe:lamb ratios during the decline phase. The population today is estimated at 100±18 (Schoenecker, unpubl. data). Understanding the reproductive dynamics of this herd could help managers and biologists better identify causes for the population decline and monitor current fertility and fecundity of ewes.

Our objectives were to 1) investigate the accuracy of single-sample fecal steroid analysis to predict pregnancy rates in free-ranging Rocky Mountain bighorn ewes, 2) compare the accuracy of three different fecal steroid metabolite assays for detection of pregnancy, and 3) quantify either embryonal loss, late fetal loss, or neonatal loss in free-ranging ewes by comparing pregnancy status with lambing status.

Study area

Free-ranging bighorn ewes were studied in BICA (45°05′00″N, 108°13′00″W). Bighorn Canyon is a National Park Service Unit that encircles a 114-km long reservoir in southeastern Montana and north-central Wyoming (Coates and Schemnitz, 1989). The sheep range also extends into portions of Custer National Forest, Bureau of Land Management lands (Pryor Mountain Wild Horse Range [PMWHR]), and some interspersed private lands in East and West Pryor Mountains. The park is dominated by Bighorn Canyon, a long canyon formed by the Bighorn River. A dam near Fort Smith, Montana, formed what is now Bighorn Lake. A strong precipitation gradient in the park provides 15 cm of rainfall annually at the south upstream end of the park and 45 cm at the north end (Knight et al., 1987). Vertical canyon walls where bighorn ewes lamb are up to 1,700 m high, containing limestone caves and talus slopes. Mountain slopes are forested, but alpine-like meadows, dryland flats, and less vegetated canyons intersperse with forested areas (Gudorf et al., 1996). Elevations range from 900 to 2,500 m (Gudorf et al., 1996). Soils in the precipitous canyon areas originated from sandstone and limestone and dolomite in the nonprecipitous areas (Knight et al., 1987).

Vegetation communities in the park and surrounding lands have been categorized by Knight et al. (1987) and include desert shrubland, sagebrush steppe, basin grassland, juniper woodland, mountain mahogany-juniper woodland, riparian, and coniferous woodland.

Long cold winters and hot dry summers characterize the climate; however, diversity in geography creates locally variable weather conditions (Gudorf et al., 1996). Semiarid conditions along the Dry Head area of the park are contrasted with subalpine zones at higher elevations in the Pryor Mountains.

METHODS

Free-ranging ewes were radio-collared for individual identification and radio-tracking monitoring in February 2000 and January 2001. A single fecal sample was collected from individual free-ranging bighorn ewes in late Janu-

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ary–May 2001 (n=11) and in late March–May 2002 (n=20). Field observers used radio collars to find ewe groups and then waited until collared ewes defecated. Generally observers were able to get close enough to ewe groups to observe fecal deposition with the naked eye. Occasionally observers needed to use binoculars to observe ewes, in which case they would draw a map of the group and landmarks while watching to be able to verify the identity of the fecal sample. Fecal samples for which observers were not 100% sure of the ewe's identity were not used in our analysis. Fecal samples were collected in plastic bags, labeled by individual ewe (radio collar frequency and symbol), and date collected. Samples were frozen and sent to the Science and Conservation Center at ZooMontana (Billings, Montana) for pregnancy determination by fecal steroid analysis. Ewes were then monitored by either boat surveys, ground tracking, and/or fixed-wing aircraft every 2 wk to determine lambing success during the spring lambing season and throughout the summer (May–September).

In order to understand the serial changes in fecal steroid metabolites during the course of gestation, fecal samples from two pregnant captive ewes at ZooMontana were collected monthly from September to parturition in May. Approximate conception dates for the two captive ewes were calculated by counting back 180 days from parturition dates.

Fecal samples were kept frozen at −7.4 C until the time of extraction and assay. Thawed wet feces (0.5 g) were placed in scintillation vials with 5 ml of extraction buffer. The extraction buffer consisted of 450 ml enzyme immunoassay (EIA) buffer (5.42 g NaH_2PO_4·H2O, 8.66 g $NaHPO_4$, 8.7 g NaCl, 1.0 g radioimmunoassay grade bovine serum albumin in 1.0 liter dH_2O), 50 ml EIA wash solution (87.7 g NaCl, 0.5% Tween-20 in 1.0 liter dH_2O), and 500 ml high performance liquid chromatography (HPLC) grade methanol. Samples were vortexed and shaken for 30 min and stored at −15 C overnight, vortexed again in the morning, and shaken for another 30 min. Samples were then centrifuged and the supernatant removed and frozen until assay.

Fecal extracts were assayed by EIA for progesterone metabolites related to PdG and estradiol metabolites, for estrone conjugates (E_1C) as described by Shideler et al. (1991), and for immunoreactive pregnanediol-like progesterone metabolites (iPdG) as described by Kirkpatrick et al. (1991). Quantities of fecal extracts were 20 μl for PdG and iPdG and 40 μl for E_1C. Assays were validated for bighorn sheep by means of testing halving dilutions for parallelism to the standard curve. Reproducibility of assays was calculated by determining inter-assay and intra-assay variation. The antibodies for PdG and E_1C assays were P70 and R583, respectively (courtesy of C. Munro, University of California, Davis, California, USA). The antibody for iPdG was Ab 1284-1, raised against 20δ-hydroxy-4-pregnen-3-one-3 oxime (provided by R. Chatterton, Northwestern University School of Medicine, Evanston, Illinois, USA). Cross-reactivity for the estrone conjugate assay is estrone-3-glucuronide 100%; estrone-3-sulfate 66.6%; estrone 236%; estradiol 17β 7.8%; estradiol-3-glucuronide 3.8%; estradiol-3-sulfate 3.3%; all other steroid metabolites <0.01% (Munro et al., 1991). Cross-reactivity for the PdG assay is PdG 100%; 20α-hydroxyprogesterone 60.7%; pregnanediol 7.3%; all other steroid metabolites <0.01% (Shideler et al., 1991). Cross-reactivity for the iPdG assay is 20α-hydroxy-4-pregnen-3-one 100%; PdG 164%; 20α-hydroxy-4-pregnen-3-one-3-oxime 41%; 20β-hydroxy-5β-pregnan-3-one 10%; 5α-pregnane-3β, 20β-diol 4%; progesterone 2%; androsterone 0.2%; all other steroid metabolites <0.1% (Shideler et al., 1993).

Results are given in ng/g of wet feces. Differences in mean endocrine concentrations were tested for significance with the Mann-Whitney rank sum tests.

RESULTS

Serial halving dilutions of fecal extracts from two captive and two sampled free-ranging ewes revealed parallelism to the standard curve for all three assays. The coefficient of variation for intra-assay variation was 6.0% (n=16), 3.8% (n=19), and 8.1% (n=20) for PdG, E_1C, and iPdG, respectively. The inter-assay coefficient of variation for PdG, E_1C, and iPdG was 6.5% (n=32), 2.8% (n=40), and 11.6% (n=32), respectively. The 50% binding for the PdG, E_1C, and iPdG EIA standard curve averaged 4 ng, 21 pg, and 4 ng, respectively.

Approximate conception dates for the captive ewes at ZooMontana, "Princess" and "Jan" were 11 and 18 November, respectively. Fecal concentrations of iPdG rose steadily from conception until parturition with ranges of 3,000–18,000 ng/g, providing a clear picture of gestational progress in both captive ewes (Figs. 1, 2). Fecal E_1C concentrations did not increase rapidly until after days 67–72 of gestation

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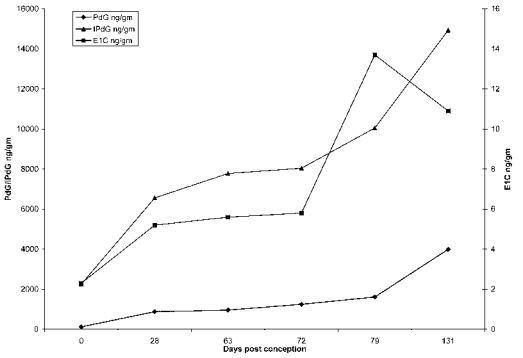


FIGURE 1. Pregnanediol-3-glucuronide (PdG), immunoreactive pregnanediol-3-glucuronide (iPdG), and estrone conjugate (E_1C) levels throughout pregnancy of captive bighorn sheep, "Jan" at ZooMontana in Billings, Montana in 2002. Results are in ng/g wet feces.

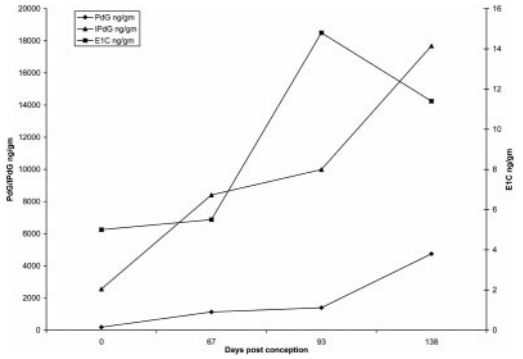


FIGURE 2. Pregnanediol-3-glucuronide (PdG), immunoreactive pregnanediol-3-glucuronide (iPdG), and estrone conjugate (E_1C) levels throughout pregnancy of captive bighorn sheep "Princess" at ZooMontana in Billings, Montana in 2002. Results are in ng/g wet feces.

and then declined from days 73–93 until parturition. However the range was small, from 2 to 15 ng/g feces, providing an accurate picture of pregnancy only for approximately days 70–90 of gestation. Fecal PdG concentrations showed little increase throughout pregnancy, until after days 79–93 of gestation, after which the range of increase was relatively small. Both PdG and iPdG concentrations provided an accurate indicator of pregnancy as early as 28 days postconception, using 1,800 ng/g and 8,000 ng/g, respectively.

In free-ranging ewes, the mean (±SEM) concentration of iPdG in ewes for which lambs were seen was 23,266±2,817 ng/g wet feces, as opposed to 10,592±2,627 in ewes that did not have lambs at their side. The difference in means was significant (P=0.0049). In 2001, there was one false negative, based on an iPdG concentration of 7,485 ng/g wet feces, and one test was inconclusive based on an iPdG concentration of 7,603 ng/g wet feces (Table 1). Pregnancy in the other nine animals was predicted accurately (Table 1). Of three free-ranging ewes sampled in 2002 that were predicted pregnant on the basis of iPdG concentrations (22,468, 18,168, and 32,848 ng/g wet feces), two were seen without lambs but with full mammary glands and extended teats, which suggests that they had both produced lambs that were lost soon after birth (Table 2). The third ewe was never observed leaving her group during the lambing season, as pregnant ewes do, which suggests she was either pregnant and lost the fetus somewhere between the time of fecal collection (4/22/02) and late May, or her pregnancy test was a false positive (Table 2).

Mean fecal concentrations of E_1C were of little value in predicting pregnancy accurately. While the mean "pregnant" concentration of E_1C in ewes with lambs was 11.26±0.56 ng/g wet feces, and while it was 7.79±0.64 ng/g wet feces for ewes with no lambs (P=0.0012), the range between individual ewes was 6.6–15.8 ng/g wet feces for ewes that lambed and 4.4–10.7 ng/g wet feces for ewes without lambs.

Mean fecal concentrations of PdG were 3,143±254.29 ng/g wet feces in animals with lambs at their sides and 1,298±258.15 ng/g wet feces for ewes without lambs (P<0.0001). The range for individual pregnant ewe values was 1,498–4,913 ng/g wet feces and 358–3,432 ng/g wet feces for ewes without lambs. The two values that overlapped were those from the two ewes with full mammary glands and extended teats. The field data and observations from

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TABLE 1. Fecal steroid levels, pregnancy, and lambing results for 11 bighorn sheep sampled in 2001 in Bighorn Canyon National Recreation Area, Montana and Wyoming.

Ewe No.	Date collected	PdG[a] (ng/g)	iPdG[b] (ng/g)	E_1C[c] (ng/g)	Intrepretation	Field result
1	6 March 2001	2,213	10,256	8.4	Pregnant	Lamb
2	2 March 2001	628	5,888	7.1	Not pregnant	No lamb
3	5 March 2001	1,528	8,772	9	Not pregnant	No lamb
4	23 March 2001	3,858	15,534	12.8	Pregnant	Lamb
5	13 March 2001	1,334	7,603	8.9	Questionable	Lamb
6	26 March 2001	1,841	7,485	6.6	Not pregnant	Lamb[d]
7	24 January 2001	1,198	6,452	5.3	Not pregnant	No lamb
8	17 January 2001	1,003	5,261	5.1	Questionable	No lamb[e]
9	2 May 2001	4,332	16,367	11.5	Pregnant	Lamb
10	4 May 2001	1,448	6,252	4.4	Not pregnant	No lamb
11	2 May 2001	475	2,766	6.8	Not pregnant	No lamb

[a] PdG = pregnanediol-3α-glucuronide.
[b] iPdG = immunoreactive pregnanediol-3α-glucuronide.
[c] E_1C = estrone conjugates.
[d] Suspect field sample collection error in this case, since ewe was observed from a distance at the time of fecal collection.
[e] Fecal sample collected too early.

TABLE 2. Fecal steroid levels, pregnancy, and lambing results for 20 bighorn sheep sampled in 2002 in Bighorn Canyon National Recreation Area, Montana and Wyoming.

Ewe No.	Date collected	PdG[a] (ng/g)	iPdG[b] (ng/g)	E_1C[c] (ng/g)	Interpretation	Field result
1	22 April 2002	1,498	15,663	9.6	Pregnant	Lamb
2	22 April 2002	358	4,603	6.5	Not pregnant	No lamb
3	22 April 2002	4,755	47,941	15.8	Pregnant	Lamb
4	14 May 2002	3,717	38,902	13	Pregnant	Lamb
5	22 April 2002	1,860	22,468	10.4	Pregnant	No lamb[d]
6	28 April 2002	721	5,822	9.1	Not pregnant	No lamb
7	22 April 2002	4,913	38,498	11.6	Pregnant	Lamb
8	25 April 2002	3,832	31,568	13.3	Pregnant	Lamb
9	29 April 2002	3,144	25,889	12.1	Pregnant	Lamb
10	22 April 2002	3,432	32,848	10.6	Pregnant	No lamb seen[e]
11	20 April 2002	2,369	24,010	14.2	Pregnant	Lamb
12	22 April 2002	1,667	16,651	9.9	Questionable	Lamb
13	29 April 2002	641	7,805	10.7	Not pregnant	No lamb
14	22 March 2002	3,292	28,546	13.9	Pregnant	Lamb
15	4 April 2002	2,288	18,168	8.5	Pregnant	No lamb seen[e]
16	18 March 2002	2,484	16,654	8	Pregnant	Lamb
17	9 May 2002	3,136	22,367	9.7	Pregnant	Lamb
18	10 May 2002	4,176	30,497	11.1	Pregnant	Lamb
19	29 April 2002	2,492	21,176	10	Pregnant	Lamb
20	22 April 2002	4,178	36,696	13.6	Pregnant	Lamb

[a] PdG = pregnanediol-3α-glucuronide.
[b] iPdG = immunoreactive pregnanediol-3α-glucuronide.
[c] E_1C = estrone conjugates.
[d] Suspect fetal loss or false positive pregnancy test because this ewe was monitored weekly, but not observed lambing or leaving the ewe group to lamb.
[e] Suspect neonatal loss because this ewe was monitored every 2 wk and was observed with full mammary glands, extended teats, and was observed leaving the main ewe group going to a remote location presumably to give birth.

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ground crews indicate that these latter values were in fact from pregnant animals.

Based on visual observations made by field crews that monitored lambs at the sides of ewes for over 2 yr, pregnancy was correctly predicted in 28 of 31 females (93%) using fecal iPdG or PdG concentrations. The remaining three samples (7%) include one false negative, one inconclusive test result, and one false positive, which we believe came from a ewe that terminated the pregnancy before parturition. In this case, the ewe did not leave the larger ewe group, did not go off on her own at any time, and was observed weekly during the time she would have been lambing. We interpret the data as indicating this ewe was pregnant at the time of the pregnancy test but lost the fetus before parturition, although her pregnancy test could have been a false positive. In the case of at least one ewe that was predicted not pregnant but lambed, we suspect field error since defecating ewes were often observed from a distance.

DISCUSSION

The reproductive period (rut for males, estrus for females) is seasonal for most bighorn sheep populations (Turner and Hansen, 1980) and peaks in late November. Gestation is 180 days, after which they give birth to one lamb in late May or early June (Geist, 1971). Thus, fecal sampling, which occurred from 17 January–10 May, would have recovered samples at approximately days 63–176 of gestation, based on a mid-November assumed date of conception. Accuracy of pregnancy diagnosis based on iPdG or PdG was not affected by date of collection. This is consistent with the report by Borjesson et al. (1996) in which samples were collected after 60 days of gestation. This same pattern has been seen in urinary and fecal steroid metabolites in bison (Kirkpatrick et al., 1992), where pregnancy concentrations of PdG are not distinguishable from nonpregnant concentrations until after 90 days gestation, and in cervids, where analysis is most accurate after the second trimester (Stoops et al., 1999). In domestic sheep, the corpus luteum produces relatively small amounts of progesterone during the first 50 days of pregnancy, but after that time the placenta, responding to both luteinizing hormone and prolactin, provides the primary source of progesterone for the remainder of the pregnancy. This is probably why measurable quantities are not seen until after day 60 in sheep.

It is important to note here that neither the iPdG nor the PdG assays are highly specific. The precise progesterone metabolites measured by the two assays is speculative. Cross-reactivities of the iPdG antibody have been reported previously (Kirkpatrick et al., 1991), but the three primary steroids include 20-β-hydroxyprogesterone, or 20-OHP (100%), PdG (164%), and 20-β-hydroxy-5β-pregnan-3-one (41%). Kirkpatrick et al. (1991), using this same assay for pregnancy diagnosis in equids, found that HPLC revealed at least three progesterone metabolites more polar than PdG and that, collectively, the immunoreactive metabolites measured by the iPdG assay positively correlated with blood progesterone.

The relatively low concentrations of metabolites cross-reacting in the PdG assay, compared to values derived from the iPdG assay, suggest both qualitative and quantitative differences in progesterone metabolites measured by the two assays. However, the collective metabolites cross-reacting in the PdG assay have been shown to positively correlate with blood progesterone in ewes (Borjesson et al., 1996).

The failure of E_1C to produce an unambiguous diagnosis of pregnancy suggests that pregnancy results in very little estrone, estrone glucuruonide, and/or estrone sulfate production in wild ewes. In addition, all of these have significant cross-reactivity with the assay's antibody (Shideler et al., 1991). Previous studies with domestic sheep have shown that, although this species produces increasing quantities of estrogens throughout the entire preg-

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nancy, the primary metabolites are probably a nonreactive diconjugate of some sort rather than the collective E_1C conjugates (Lasley, pers. comm.).

It is important to note that in various wildlife species, classic validations of assays for a specific steroid or its metabolite are not always possible or even advantageous. A whole spectrum of reproductive steroid metabolites is produced in mammals, and differences are significant. For example, PdG measurements in the Bovidae are extremely useful but E_1C measurements are not, largely because the primary metabolite of estrogens in these taxa is 17β-estradiol, which does not cross-react with the E_1C antibody. Conversely, PdG measurements in the Equidae are of no value, while E_1C measurements are extremely predictive of blood estrogen concentrations. Because the three assays used in this study cross-react with a significant number of metabolites, it is likely that some important steroids can be quantified. More importantly, for each assay some measurable and correlated reproductive event can be found, e.g., pregnancy, ovulation, blood steroid concentration, or birth of young. It is not vital that the assays be highly quantitative with respect to the specific metabolite because only working estimates are necessary to correlate to the specific reproductive event of interest (Lasley and Kirkpatrick, 1991). In the case of the current study, the confirming reproductive event was parturition and live young, and therefore pregnancy.

In previous studies with bighorn sheep, Borjesson et al. (1996) used multiple sampling and subsequent analysis of immunoreactive PdG metabolites provided to obtain 100% accuracy in diagnosing pregnancy when samples were collected after 60 days gestation. While multiple sampling over time ensures against sampling error or sample anomalies, such sampling presents new logistic challenges to field personnel. Single sampling used in our study yielded an accuracy in excess of 90%. Using the 1,800 ng/g cutoff for the PdG assay results, as reported by Borjesson et al. (1996), accuracy was exactly 90%, and using a cutoff value of 8,000 ng/g for the iPdG assay results, accuracy was also 90%.

The current study suggests that remote pregnancy evaluation of bighorn sheep may be valuable for quantifying fetal or possibly neonatal loss that might normally go undetected. There are diseases that can cause abortion in sheep. *Campylobacter fetus fetus* causes fetal loss in domestic sheep 4–6 wk prior to lambing (Collins and DeLisle, 1985; Salama et al., 1995), which in this study would have resulted in positive pregnancy diagnosis at the time of sampling using the tests described here. Enzootic abortion in ewes (caused by *Chlamydia*) also causes abortion shortly before parturition and could be the cause of fetal loss. Additionally, there is evidence of mountain lion (*Felis concolor*) and coyote (*Canis latrans*) predation on bighorn lambs on this particular range (Schoenecker, unpubl. data).

Our results offer a method for monitoring fertility of bighorn ewes that is noninvasive, safe, effective, and highly accurate. If field error can be kept to a minimum and ewes can be identified individually with preexisting radio collars or other marking devices to facilitate individual identification, much information can be gathered about the fertility and fecundity of bighorn sheep populations using fecal steroid analysis.

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