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ONE-SAMPLE PREGNANCY DIAGNOSIS IN ELK USING FECAL STEROID METABOLITES

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ABSTRACT: Recent research has demonstrated the potential of pregnancy diagnosis in elk (*Cervus elaphus nelsoni*) using immunoassays of fecal steroid concentration. However, multiple samples are required to insure accurate results, limiting its utility for free-ranging animals. We attempted to develop an accurate one-sample pregnancy diagnosis using 153 fecal samples that were collected from free-ranging, radio-collared, adult female elk in Yellowstone National Park (Wyoming, USA) and from captive elk maintained at the Starkey Research Facility (La Grande, Oregon, USA) February through April 1992 and 1997. The pregnancy status of each animal was diagnosed using serum pregnancy-specific protein B (PSPB) assays providing fecal samples from 38 nonpregnant and 115 pregnant animals. Fecal radioimmunoassay (RIA) indicated that mean (±SD) progestagens (P_4) were elevated significantly in pregnant (2.96 ± 1.49 μg/gm) compared to nonpregnant (0.43 ± 0.26 μg/gm) individuals. Confidence intervals (1.96 ± SE) for the two groups were widely separated (nonpregnant 0.34–0.51, pregnant 2.69–3.24) with little overlap in the range of concentrations measured for each group (nonpregnant 0.09–0.98, pregnant 0.90–8.29). These results indicate that fecal progestagens RIA provides a reliable method of noninvasive pregnancy diagnosis using single fecal samples collected from elk during late gestation. However, independent validation of the suggested discrimination criteria should be performed before routine application.

Key words: *Cervus elaphus*, physiology, radioimmunoassay, reproduction, techniques.

INTRODUCTION

The development of noninvasive techniques for assessing pregnancy in uncaptured ungulates is an active area of research that promises to provide biologists with a new and useful tool for the study of reproduction and neonatal mortality (Messier et al., 1990). Methodologies are based on the measurement of reproductive steroids or their metabolic byproducts excreted in feces using immunoassay procedures (Schwarzenberger et al., 1996). Fecal steroids concentrations have been explored in studies on muskoxen (*Ovibos moschatus*) by Desaulniers et al. (1989), caribou (*Rangifer tarandus*) by Messier et al. (1990), feral horses (*Equus caballus*) by Kirkpatrick et al. (1990) and Lucas et al. (1991), moose (*Alces alces*) by Monfort et al. (1993), bison (*Bison bison*) by Kirkpatrick et al. (1993) and bighorn sheep (*Ovis canadensis*) by Borjesson et al. (1996). All of these studies have demonstrated the potential of diagnosing pregnancy using fecal steroid metabolites. However, the ability to discriminate between pregnant and nonpregnant animals from a single fecal sample has varied from 55 to 100% depending on the species, extraction and assay procedures, steroid metabolites measured, and timing of sample collection during gestation.

In an effort to validate this technique for monitoring the long-term reproductive success of cow elk (*Cervus elaphus nelsoni*) in Yellowstone National Park (Wyoming USA) White et al. (1995) used enzyme immunoassay (EIA) to explore the potential of three steroid metabolites for diagnosing pregnancy. They found that free progesterone (P_4) and pregnanediol-3-glucuronide (PdG) concentrations in fecal samples collected during late gestation

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(March and April) were the best indicators of pregnancy status. Discrimination from a single sample was only 74 to 84% correct, leading these investigators to recommend assaying multiple samples in order in ensure accurate results. The collection of numerous fecal samples from a single animal will limit the application of the technique to captive animals and relatively tractable, individually identifiable free-ranging animals. The purpose of this study was to determine if a more reliable single-sample pregnancy diagnosis for elk could be obtained by using alternative procedures.

METHODS

One hundred fifty three fecal samples from adult female elk of known pregnancy status were used in this study. During 12 February to 28 April 1992 fecal samples were collected from 23 individual free-ranging instrumented cow elk occupying the Madison-Firehole winter range in Yellowstone National Park (44°37′N, 110°52′W). An additional 18 animals were sampled 14 March to 8 April 1996 (13 of these animals in common with the 1992 collection). Samples were collected by locating each animal using telemetry and observing the animal until a defecation event was observed (White et al. 1995). One hundred twelve additional fecal samples were collected 8 to 12 April 1996 (n = 57) and 10 April, 1997 (n = 55) from captive adult female elk maintained at the Starkey Research Facility near La Grande (Oregon, USA; 45°30′N, 118°20′W). All samples from each annual Starkey collection originated from different elk, but the same animals were sampled in both years. Descriptions of the Madison-Firehole area and the Starkey Research Facility are provided in Garrott et al. (1996) and Cook et al. (1996), respectively. The pregnancy status of each animal was diagnosed using serum pregnancy-specific protein B (PSPB; Sasser et al., 1986; Hein et al., 1991). Blood samples were collected from free-ranging elk while they were chemically immobilized for the purposes of attaching radio collars (White et al., 1995). Blood samples from captive elk were collected during December with pregnancy status confirmed at parturition in May and June.

Fecal sample processing and extraction procedures were modified from previously described methods (Wasser et al., 1991, 1994; Monfort et al., 1993; Brown et al., 1994; Schwartz et al., 1995). Fecal samples first were dried using a rotary evaporator and 0.1 g feces was boiled (20 min) in 16 × 125-mm glass tubes containing 10 ml of absolute ethanol and approximately 2,000 c.p.m. radio-labeled ^{3}H-progesterone to monitor procedural losses. After boiling, tubes were centrifuged at room temperature (10 min, 1,500 × g) and the ethanol supernate was decanted into clean 16 × 125-mm tubes, evaporated to dryness, and reconstituted in 1 ml methanol. After vortexing, extractants were sonicated (10 min) and 25 µl was withdrawn for radioactive counting to determine extraction efficiency. Fecal P_4 was assayed using an radioimmunoassay (RIA) procedure described previously (Brown et al., 1994; Wasser et al., 1994). Briefly, serial dilutions (range = 1:32 to 1:1024) of a pooled fecal extract from randomly selected female elk of unknown reproductive status yielded a displacement curve parallel to the standard curve. Recovery of known amounts of progesterone (7.5 to 120.0 pg/tube) added to a pool of diluted fecal extract (1:400, 50 µl) was 95% ($y = -1.78 + 1.04x$, $r^2 = 0.99$). Inter-assay coefficients of variation for two separate internal controls were 14% (n = 6, 79 to 95% binding) and 6% (n = 6, 43 to 69% binding). Intra-assay coefficients of variation were <5%, and assay sensitivity was 3.75 pg/100 µl. All hormone concentrations are expressed as mass units of hormone excreted per gm of dry feces. A t-test was used to evaluate differences in mean fecal P_4 concentrations for pregnant versus nonpregnant animals using STATISTICA (StatSoft, Inc., Tulsa, Oklahoma, USA).

RESULTS

PSPB assays indicated that 20 of the 23 elk from Yellowstone sampled in 1992 were pregnant, and 16 of the 18 animals sampled in 1996 were pregnant. Forty-three of the 57 animals from Starkey sampled in 1996 and 36 of the 55 animals sampled in 1997 were pregnant. Thus, of the 153 samples collected, 38 were from nonpregnant animals and 115 were from pregnant animals. Thirty seven of 38 samples from nonpregnant animals had fecal P_4 concentrations <1.0 µg/gm, while 112 of 115 samples from pregnant animals had concentrations ≥1.0 µg/gm. One 1996 sample from a nonpregnant animal from Starkey had a P_4 concentration of 1.26 and a single 1997 sample from a pregnant animal from Starkey had a P_4 concentration of 0.64. These two samples appear to be

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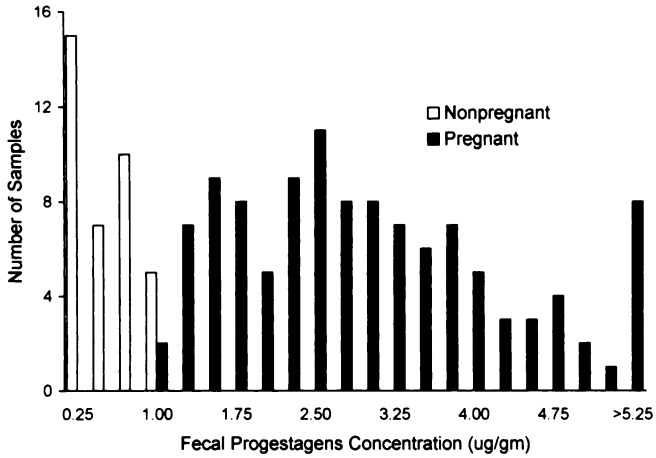


FIGURE 1. Distribution of progestagens (P_4) concentrations (μg/gm dry fecal weight) in 151 fecal samples collected from nonpregnant (n = 37) and pregnant elk (n = 114), collected from free-ranging radio-collared elk in Yellowstone National Park (Wyoming USA) and captive animals maintained at the Starkey Research Facility (La Grande, Oregon USA).

outliers and we suspect errors during sample collection and/or labeling. Mean (±SD) fecal P_4 concentrations, excluding the two apparent outliers, were 0.43 ± 0.26 μg/gm and 2.96 ± 1.49 μg/gm for nonpregnant and pregnant animals, respectively (Fig. 1). Confidence intervals (1.96 ± SE) for the two groups were widely separated (nonpregnant 0.34 to 0.51, pregnant 2.69 to 3.24), indicating higher concentrations for samples from pregnant animals ($P < 0.01$). Overlap in fecal P_4 concentrations for samples from two pregnant animals and two nonpregnant animals was noted in the 0.9 to 0.98 μg/gm range (preg.—0.90, 0.95; nonpreg.—0.96, 0.98).

DISCUSSION

White et al. (1995) demonstrated that fecal concentrations of both PdG and P_4 were significantly higher in samples collected from pregnant elk during late gestation compared to those collected from nonpregnant animals. EIA procedures were employed for measuring fecal steroid concentrations as they felt such assay methodology would be more economical and require less sophisticated laboratory equipment than RIA procedures. However, results of their study indicated that some errors in discrimination between pregnant and nonpregnant animals occurred when pregnancy diagnosis was attempted based on a single fecal sample; false negatives (classifying a nonpregnant animal as pregnant) were the most common error (White et al., 1995). These results led the investigators to conclude that multiple late-gestation fecal samples were needed to accurately assess pregnancy status using the techniques employed in their study. To ascertain whether progestagen metabolites were an inherently poor indicator of pregnancy status, or alternatively if failure to detect pregnancy in a single sample was a consequence of the P_4 EIA employed, we conducted a similar study using an alternative immunoassay. Unlike the previous report (White et al., 1995), the present data indicated that progestagens immunoreactivity provided a reliable single-sample pregnancy test (Fig. 1). However, the fecal P_4 concentrations from two pregnant animals fell within the extreme upper tail of the distribution for nonpregnant animals, indicating a narrow range for potentially ambiguous pregnancy diagnosis.

Selecting a critical P_4 concentration for pregnancy discrimination is a subjective decision and may depend on whether false

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negatives or false positives are more critical to the objectives of a given study. We suggest that when employing the methodology of the present study, only late gestation samples (March–April) should be used, with animals having fecal P_4 concentrations <0.9 μg/gm considered nonpregnant and those with concentrations ≥1.0 μg/gm considered pregnant. Discrimination for samples with concentrations between 0.90–0.99 μg/gm should be regarded inconclusive. Of 105 late-gestation fecal samples collected from radio-collared cow elk in Montana (USA) and Wyoming in separate studies only three fell within the narrow range of P_4 concentrations where diagnosis may be considered uncertain, suggesting that potential ambiguities in diagnosis will be uncommon in field situations. However, we caution that the results of this study and the discrimination criteria suggested should be independently validated before the procedure is routinely applied for pregnancy diagnosis.

Although detailed descriptions of immunoassay methodologies is beyond the scope of this manuscript, the observation that pregnancy diagnosis efficiency varied using two different immunoassays for the same class of hormone warrants discussion. Progesterone, the most biologically potent progestin, can be routinely quantified in blood using any number of specific antisera which are reported to exhibit little or no crossreactivity with other structurally-similar steroids found in blood. However, when progesterone is metabolized and excreted, it is rarely excreted in its native form. Rather, progesterone is generally metabolized to numerous structurally similar steroid molecules, most of which are rather esoteric in strict physiological terms since their structural forms render them biologically impotent. These byproducts of progesterone are primarily important as indirect measures of progesterone in blood circulation. Steroid metabolites can be excreted as conjugated or unconjugated forms; conjugate side-chain moieties such as glucuronides or sulfates are often added during metabolism to facilitate water solubility in the aqueous environments of bile or urine (Lasley and Kirkpatrick, 1991; Schwarzenberger et al., 1996). The situation is further complicated by the fact that the number and distribution of these metabolites vary between species.

Although fecal hormone monitoring techniques are now widely accepted for wildlife research, there remains a great deal of misunderstanding about these methods. For the wildlife biologist, it is critical to understand that all immunoassays are not created equal. The backbone of any immunoassay is the antiserum employed. Each antiserum has a characteristic specificity, or ability to recognize minute three-dimensional structural differences among similar classes of antigens (i.e., steroid hormones). Because antibody production is based upon polyclonal or monoclonal lymphocyte production, no two clones of lymphocytes are likely to produce antibodies with identical specificities. Whether employed in an EIA or RIA, it is the unique specificity of the antibody, and not whether the ligand is enzyme- or radio-labeled, that fundamentally dictates assay performance. Thus, a P_4 immunoassay used by one laboratory will not necessarily recognize the same 'family' of progestagens as an immunoassay used by another laboratory. One P_4 immunoassay might be highly specific (i.e., recognize progesterone and only progesterone), whereas another P_4 immunoassay might be relatively nonspecific (i.e., recognize progesterone and a host of other structurally-related progestins). Furthermore, some antibodies only recognize unconjugated steroids, whereas others recognize only conjugated forms, or both conjugated and unconjugated forms. Thus, when one laboratory reports that their particular immunoassay does not detect elevated steroid during pregnancy, it does not, therefore, follow that another laboratory's immunoassay will provide a similar answer.

Despite the fact that the antibodies utilized in the present study, and by White et

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al. (1995), both presumably are specific for the same class of progesterone metabolites (i.e., 20-oxo-pregnanes), our antiserum appears to provide improved discrimination for pregnancy detection in elk. Clearly, these differences cannot be attributed to the type of immunoassay employed (EIA versus RIA). However, one explanation for this discrepancy is that our antibody may be more specific for major elk pregnancy progestagens than the antiserum employed by White et al. (1995). Alternatively, the extraction method (boiling in ethanol) utilized in the present study may be superior for efficiently extracting elk progestagens compared to the previously employed method (buffer solubilization). In conclusion, it is critical to carefully scrutinize the endocrine methods employed, even when using extraction procedures and/or immunoassays that have been previously reported to be effective for documenting fecal steroid metabolites.

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