A SERUM PREGNANCY TEST WITH A SPECIFIC RADIOIMMUNOASSAY FOR MOOSE AND ELK PREGNANCY-SPECIFIC PROTEIN B

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Abstract: A double-antibody radioimmunoassay (RIA) specific for elk (*Cervus elaphus*) and moose (*Alces alces*) pregnancy-specific protein B (PSPB) was established. Sheep anti-moose PSPB was used for the first antibody and purified placental moose PSPB (mPSPB) was used as a standard. This assay was shown to quantify moose and elk PSPB in serum. When used to detect pregnancy in elk near 40 days after artificial insemination, there was agreement with a bovine RIA at 96%. Accuracy of both RIA's was 93% compared to calving observation. The PSPB concentration in serum of moose increased steadily from 40 to 100–150 days during gestation, but remained steady or decreased slightly between 150 and 190 days. The concentration of PSPB in serum of moose bearing twin fetuses was significantly different and higher than it was in moose bearing a single fetus. A cut-off point of 365 ng/mL PSPB in serum was chosen to separate moose bearing single or twin fetuses at approximately 10 weeks before parturition. The accuracy of detection of singles and twins was 90.5%. Based on this RIA, pregnancy can be detected in elk and moose and prediction of single or twin pregnancies in moose is possible.

JOURNAL OF WILDLIFE MANAGEMENT 64(2):492–499

Key words: *Alces alces*, *Cervus elaphus*, elk, moose, pregnancy-specific protein B, pregnancy test, radioimmunoassay.

Pregnancy can be detected in cows by inserting the hand into the rectum and palpating through the rectal and uterine walls for the amniotic vesicle or cotyledons within the uterus (Wisnicky et al. 1948). Hulet (1972) described a rectal-abdominal palpation method for pregnancy testing in sheep. Ultrasonic detection of pregnancy has been developed recently and can obtain nearly 100% accuracy after 40 days of gestation.

Proteins produced by the placenta have been used to detect pregnancy for a long time. Human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG) are reliable pregnancy markers for women (Marshall et al. 1968) and mares (Cole and Hart 1942), respectively. Progesterone assay can be used to test pregnancy in ruminants, but the assay is not pregnancy-specific and the accuracy of detection of pregnant animals is not reliable.

A double-antibody RIA to detect PSPB in sera of pregnant cows has been developed and validated (Sasser et al. 1986). This highly sensitive assay was mostly used to detect PSPB in cows from day 28 to the end of gestation. It serves as an accurate means of early pregnancy detection (greater than 95% accuracy after 28 days of pregnancy; Sasser et al. 1986, Humblot et al. 1988*b*), as an indicator of embryonic mortality (Humblot et al. 1988*a*), and as a possible indicator of fetal twins (Willard et al. 1995, Dobson et al. 1993, Vasques et al. 1995, Patel et al. 1995).

Since PSPB has been found in all species of ruminants tested (Sasser and Ruder 1987), the RIA for bovine PSPB (bPSPB) has also been developed successfully to detect pregnancy in mountain goat (*Oreamnos americanus*; Houston et al. 1986), mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus* Wood et al. 1986), *sheep (Ovis aries*; Ruder et al. 1988), red deer (*Cervus elaphus*; Haigh et al. 1988), muskoxen (*Ovibos moschatus*; Rowell et al. 1989), goat (*Capra hircus*; Humblot et al. 1990), wood bison (*Bison bison athabascae*; Haigh et al. 1991), moose (Haigh et al. 1993, Stephenson et al. 1995), fallow deer (*Dama dama*; Wilker et al. 1993, Willard et al. 1994*c*), elk (Willard et al. 1994*b*, Noyes et al. 1997), sika deer (*Cervus nippon*; Willard et al. 1994*a*,

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1996), and caribou (*Rangifer tarandus*; Russell et al. 1998). Even though the accuracy of these assays is similar to the bovine test, it has been shown that the PSPB of other ruminants cross react incompletely with the antibodies which were developed against bPSPB. Therefore, it is not possible to quantify PSPB in serum of other ruminants using the bovine assay.

Ovine PSPB (oPSPB) was isolated from sheep placenta and an antiserum that was specific to oPSPB was developed. The antiserum was used instead of the anti-bPSPB and this heterologous bovine assay could quantify PSPB in sheep and domestic goat serum (Willard et al. 1995). We have isolated, purified, and partially characterized PSPB from elk and moose placenta (Huang et al. 1999). If a specific RIA can be established to quantify PSPB in serum of elk and moose, prediction of fetal numbers in moose and determining the age of fetuses in moose and elk might be possible. Estimates of in-utero twinning reflect the nutritional condition of individuals within a population. Franzmann and Schwartz (1985) suggested that twinning is related to habitat quality and it is manifested through effects of body condition on reproductive performance. Therefore, the objective of this study was to develop a specific double-antibody RIA for elk and moose PSPB that would quantify PSPB in sera of these animals and, perhaps, other cervids.

STUDY AREA AND METHODS

Antisera Production

Elk PSPB (ePSPB) mPSPB were isolated and purified as described previously (Huang et al. 1999), and was used as the antigen for developing antisera. Two mature sheep were each immunized by intradermal injection in the 2 axillary and 2 inguinal regions of the body with 1 mg mPSPB or 0.5 mg ePSPB protein, which was emulsified with Freund's complete adjuvant. A booster immunization of the same amount of PSPB was given 4 weeks later with Freund's incomplete adjuvant. Blood was drawn 2 weeks after the booster injection. Antisera were obtained after centrifugation and stored frozen until use. Sheep anti-mPSPB and ePSPB IgG were isolated from these sera by ImmunoPure immobilized protein G (Pierce, Rockford, Illinois, USA). The second antibody was similarly raised in a goat by injection of sheep IgG (Sigma, St. Louis, Missouri, USA).

Radioimmunoassay and Validation

Bovine PSPB (laboratory preparation, R-37) was radioiodinated with ^{125}I as a tracer (Sasser et al. 1986). Moose or elk PSPB (collected after the affinity column purification step; Huang et al. 1999) was used as standard and was added to nonpregnant moose or elk serum and phosphate buffered saline (200 and 300 μL, respectively). The protein concentration of the PSPB standard was determined by Micro BCA Protein Assay (Pierce, Rockford, Illinois, USA). The RIA procedures are exactly as described for a bPSPB RIA (Sasser et al. 1986).

Accuracy of measurement of PSPB in serum by RIA was determined by assaying known amounts of mPSPB (0.7, 1.4, 2.8, 5.6, 11.2 ng/mL diluted in nonpregnant moose serum) and ePSPB (0.5, 1, 2, 4, 8 ng/mL diluted in nonpregnant elk serum). Interassay precision was calculated by determining the coefficient of variation (CV) for the repeated measurement of the same 2 sera (elk serum near 52% binding and moose serum near 100% binding) in 6 different assays. Intraassay precision was calculated by determining CV for repeated measurements (n = 10) of a pregnant moose serum pool (near 77% binding) and a pregnant elk serum pool (near 27% binding) within an assay. Sensitivity of the assay was determined by measurement of serial dilution of serum pools from pregnant moose and elk to determine the smallest amount of serum antigen that was distinguishable from the buffer control. Specificity of the assay was evaluated by measuring the cross-reactivity of anti-mPSPB serum with various proteins, hormones, and pools of sera from pregnant and nonpregnant elk, moose, and other ruminant animals. The following 12 proteins or hormones used were: α-fetoprotein, hCG, PMSG, fetuin, ovine follicle stimulating hormone (oFSH), ovine thyrotropin (oTSH), ovine luteinizing hormone (oLH), ovine growth hormone (oGH), ovine prolactin (oPRL), bovine prolactin (bPRL), bovine growth hormone (bGH), and bovine thyrotropin (bTSH). Besides elk and moose, the sera pools of red deer, reindeer (*Rangifer tarandus*), caribou, mule deer, and bison (*Bison bison*) were also tested for cross-reactivity with anti-mPSPB.

Pregnancy Detection in Elk

Sixty-seven samples of domestic elk sera collected at approximately 40 days after breeding

(compliments of BioTracking, Moscow, Idaho, USA) were tested for PSPB by mPSPB RIA and results were compared to the detection results with the bPSPB RIA. The results were verified for 28 animals by observation of calving.

Gestational Serum Profile of Moose PSPB

The mPSPB concentration in serum was measured between 28 and 192 days of gestation from captive adult moose bearing single or twin fetuses. Animals were reared at the Kenai Moose Research Center on the Kenai Peninsula, Alaska (60°N, 150°W), and were fed formulated pelleted rations ad libitum during winter (Oct to May) and natural browse from June to September. Moose were immobilized during September, November, January, March, and April, 1997–98, and January and March 1994 with carfentanil citrate-xylazine hydrochloride and reversed with naltrexone (Schmitt and Dalton 1987) and tolazoline hydrochloride (Schwartz et al. 1997). Serum was obtained from blood collected by jugular venipuncture and stored frozen at −20°C.

Transrectal ultrasonography was used to detect the presence and number of fetuses during immobilization in early November (Stephenson et al. 1995). Scanning was conducted with an Aloka 210 or 500 (Aloka, Wallingford, Connecticut, USA) portable ultrasound unit with a 5 MHz linear-array transducer. Exact or approximate breeding dates were determined by daily monitoring. Multiple cow moose were confined with a single bull in a 4-ha pen during the rut (late Sep to early Oct). Exact calving dates and number of fetuses born were determined by daily monitoring of known pregnant cows during the calving period (late May).

Enumerating Moose Fetuses

During March, 1998, free ranging moose in Denali National Park and Yukon Flats National Wildlife Refuge, Alaska, were immobilized from a helicopter (Bell 206B, Bell Helicopter Textron, Fort Worth, Texas, USA) by administering carfentanil citrate-xylazine hydrochloride with Palmer Cap-Chur (Leichhardt, New South Wales, Australia) equipment using 3-cc darts. Anesthesia was reversed with naltrexone. Captured moose were radiocollared and serum was collected by jugular venipuncture and frozen at −20°C. During the calving season (late May), radiocollared adult females were monitored daily using fixed-wing aircraft to determine parturition date and number of births. Sixty-three sera samples were collected and PSPB content was determined by RIA.

Statistical Analysis

An analysis of parallelism between the slope of each curve from dilutions of serum and the standard was done as follows. We (1) fit a linear regression to the log transformed data for each curve, (2) assessed the fit of the lines to data points, and (3) compared the slopes of each serum curve with the standard curve using dummy variable regression techniques (Neter et al. 1983). A Student's *t*-test was used (1) to compare differences between serum concentration of PSPB in moose bearing single or twin fetuses and (2) to compare buffer control containing no PSPB with points on the standard curve or various concentrations of potential cross reacting hormones and proteins. All computations were carried out using SAS (1985). An alpha value equal to 0.05 was used to determine the significant difference.

RESULTS

Assay Development

Sheep anti-mPSPB and anti-ePSPB, used at a dilution of 1:20,000 and 1:5,000, respectively in the RIA, bound 25% of radiolabeled bPSPB (40,000 dpm) in the absence of unlabeled PSPB. Specific activity of ^{125}I-bPSPB was 90–110 μCi/μg. The inhibition curve of mPSPB standard ranged from 0.3125 to 40 ng per tube and bound 97 to 7% of the buffer control (Fig. 1). The inhibition curve for ePSPB was not examined.

The regression equation for the accuracy of measurement of mPSPB was $Y = 0.96X + 0.06$ with a correlation coefficient of 0.99. For e-PSPB, the regression equation was $Y = 0.97X + 0.19$ with a correlation coefficient of 0.99. The interassay CV for 6 assays was 2.6% (at 52% binding level) and 1.3% (at 100% binding level). The intraassay CV's for 10 replicates of serum with a high PSPB content (27% binding) and a low PSPB content (77% binding) were 2.4% and 1.3%, respectively. The sensitivity for testing moose or elk PSPB was 250 pg and 500 pg per assay tube, respectively. For specificity, none of the 12 proteins or hormones tested in quantities up to 1 μg per tube inhibited binding of radiolabeled bPSPB ($P > 0.05$). No inhibition of binding was observed with nonpregnant moose or elk serum (Fig. 1). Dilution of serum

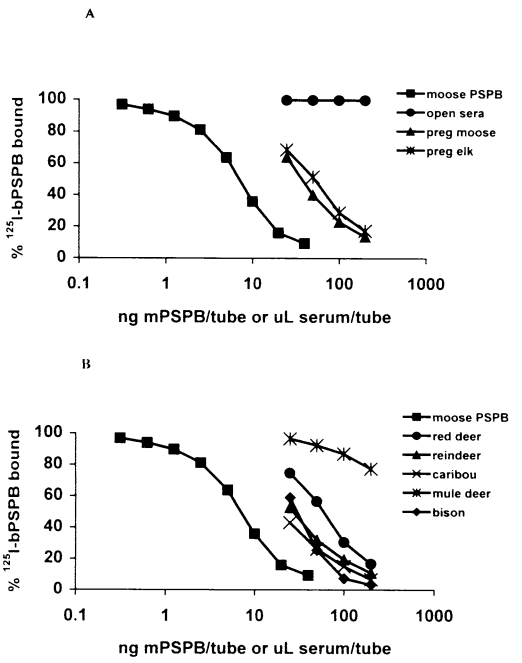

Fig. 1. Evaluation of parallelism between the varying amounts of moose PSPB (standard curve, ng/tube) and (Graph A) varying amounts of sera (μL/tube) from pregnant (preg) or nonpregnant (open) moose and elk and (Graph B) varying amounts of pregnancy sera from 5 species of animals.

from pregnant elk, moose, reindeer, mule deer and caribou resulted in inhibition curves with slopes similar ($P > 0.05$) to the mPSPB standard curve (Fig. 1). Dilution of serum from pregnant bison and red deer did not inhibit binding in a manner parallel to the mPSPB standard curve ($P < 0.05$; Fig. 1).

Pregnancy Detection of Elk

In the bPSPB assay, 48 elk were classified as pregnant and 19 nonpregnant. When tested in the mPSPB assay, 47 of the 48 were classified as pregnant and 17 of the 19 were classified nonpregnant. The agreement of mPSPB RIA with bPSPB RIA for pregnant and nonpregnant elk was 98% and 89%, respectively. Twenty-eight of 67 of the tests were verified by observing calving. For these 28, both the bPSPB and the mPSPB RIA classified 26 as pregnant and 2 as nonpregnant; the accuracy of both assays was 93%.

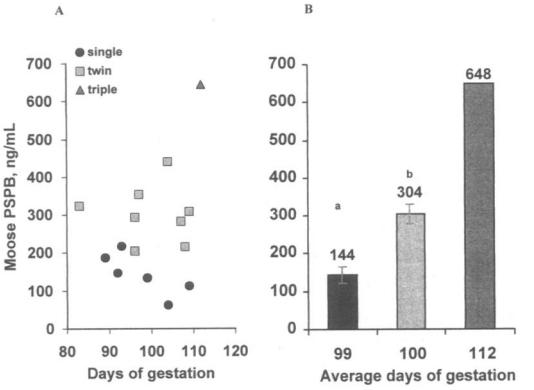

Fig. 3. The comparison of PSPB concentration in serum among moose bearing single ($n = 6$), twin ($n = 8$) or triple ($n = 1$) fetuses. Sera were collected from individual moose at 83 to 112 days of gestation (Jan 1997). Graph A shows individual values. Graph B shows the mean mPSPB concentration (n = numbers above the bars) at average days of gestation in each group for single, twin and triplicate fetuses. The letters a, b designate a significant difference (t-test, $P < 0.001$) in serum concentration.

Gestational Serum Profile of Moose PSPB

The mPSPB concentrations in serum were compared between 2 moose with normal pregnancies bearing single or twin fetuses during the 4 stages of gestation. Mean mPSPB concen-

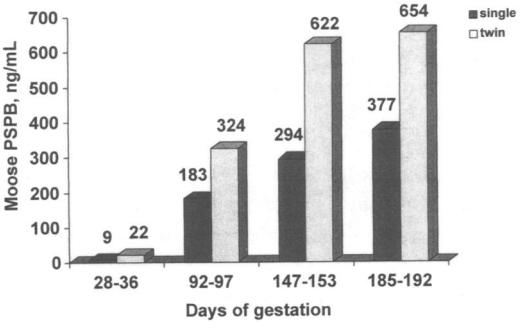

Fig. 2. Comparison of mPSPB concentration between moose bearing single and twin fetuses at 4 different periods of gestation. Means are of 2 moose for each group that had similar dates of sampling and normal pregnancies.

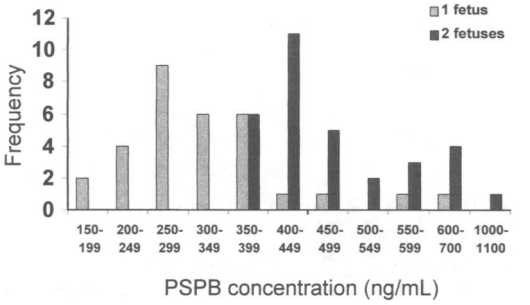

Fig. 4. Distribution of 63 moose bearing single or twin fetuses by serum PSPB concentration tested by mPSPB RIA. The average PSPB amount in moose bearing a single fetus was 321 ± 19 ng/ml and that of moose bearing twin fetuses was 491 ± 25 ng/mL. Sera samples were collected in March 1998.

tration in serum of moose bearing twin fetuses was higher than for those bearing a single fetus (Fig. 2). From approximate days 32 to days 100 to 150 of pregnancy, the concentration of m-PSPB in serum increased in a linear fashion. From 150 to 190 days, this concentration continued to increase in some moose while it remained the same or slightly decreased in others. A third moose bearing twin fetuses had a similar profile but aborted after 190 days of gestation and was not included in the data of Fig. 2.

The serum of a single moose bearing twin fetuses was tested on days 36, 97, 152 and 192 of gestation and at day 14 after parturition. The PSPB concentration in serum was 25, 354, 555, 550, and 16 ng/mL at the respective times.

At approximately 100 days of gestation (range 83 to 112 days), the concentration of mPSPB in serum of moose bearing a single fetus averaged 144 ± 23 ng/mL ($\bar{x}$ ± SE); those bearing twin fetuses averaged 304 ± 26 ng/mL (*t*-test, $P < 0.001$; Fig. 3). A single animal with triplets had a serum PSPB concentration of 648 ng/mL.

Detection of Moose Fetal Numbers

Thirty-one moose were observed with 1 calf and 32 were observed with twins. The mPSPB concentration in sera of moose bearing 1 fetus ranged from 165 ng/mL to 700 ng/mL ($\bar{x}$ = 321 ± 19 ng/mL); that of moose bearing twin fetuses ranged from 353 ng/mL to 1085 ng/mL ($\bar{x}$ = 491 ± 25 ng/mL; Fig. 4). The means were significantly different (*t*-test, $P < 0.001$). A cut-off point was established at 365 ng/mL so moose with <365 ng/mL were categorized as bearing a single fetus; if higher it carried twin fetuses. Using this cut-off point, the categorization of a single fetus was 84% correct and of twin fetuses was 97% correct. The overall accuracy of detection for fetal numbers for all 63 moose samples was 90.5%.

DISCUSSION

We have previously (Huang et al. 1999) isolated elk and moose PSPB from the placenta. Although PSPB after the final step of affinity column purification tested more nearly pure than PSPB after the preceding Sephadex G-75 gel filtration step, we still chose to use the latter PSPB as the antigen. Our rational was that there was a limited amount of PSPB after the affinity column isolation step compared to the Sephadex G-75 isolation step and all variants for PSPB after Sephadex G-75 were readily recognized by anti-bPSPB in Western blotting procedures.

We used both elk and mPSPB as antigen to separately immunize sheep. The immunoresponse to mPSPB was stronger than that to e-PSPB. This likely occurred because more m-PSPB protein was available for injection. Parallelism between the standard curve and dilutions of sera from pregnant elk or moose (data not shown) suggest that either antisera could be used to develop and assay to quantify PSPB of either species. The moose RIA was chosen for further development because (1) antisera titer was higher, (2) it was desirable to use the assay to predict twining in moose and a specific test would be more likely to do so, and (3) the mPSPB RIA likely would quantify ePSPB for possible dating of fetal age.

Both interassay and intraassay CV's were very low and highly acceptable as was the sensitivity for detection of elk and moose PSPB. There was no immunological cross-reactivity with the 12 proteins or hormones which were tested. This was similar to that found for the oPSPB RIA (Willard et al. 1995) and the bovine PAG RIA (Zoli et al. 1991, 1992), but different from the bPSPB RIA (Sasser et al. 1986) in which there was minor cross-reactivity to bovine luteinizing hormone and follicle stimulation hormone. Inhibition of binding in a manner parallel to the mPSPB standard curve by the dilutions of sera of pregnant elk, moose, caribou, mule deer, and reindeer provides a quantitative PSPB RIA for these cervids. Dilution of serum from pregnant bison and red deer did not inhibit binding in a manner parallel to the m-PSPB standard curve. The result of bison is reasonable because bison do not belong to the cervid family. Lack of parallelism between mPSPB standard curve and serum dilution of pregnant red deer was not expected and will be tested in subsequent studies.

The bovine RIA has been used reliably for detection of pregnancy in elk. The fact that the current moose RIA had close agreement with the bovine assay lends credibility to validity of the moose assay. In addition, both assays agreed in prediction of pregnancy at 40 days after artificial insemination in 28 elk in which the pregnancy outcome was known. The same 2 of 28 animals were missed by both assays. One of the 2 had a high concentration of PSPB in serum and likely aborted after the blood sample was collected. The other animal had a negative test

for PSPB in the sample collected 40 days after artificial insemination but calved 30 days after expected time of calving. She likely did not conceive to artificial insemination but conceived to a clean-up bull at the subsequent estrus.

It is possible that the mPSPB RIA can detect pregnancy earlier than 40 days after conception in elk; however, earlier samples are not available. Specific RIA's for ovine (Willard et al. 1995) and bovine (Sasser et al. 1986, Humblot et al. 1988*b*) PSPB detected pregnancy reliably at 21 days and 28 to 30 days after conception, respectively. The bPSPB RIA has not been able to detect pregnancy earlier than 40 days in elk. The current, specific cervid RIA may provide for an earlier test and this hypothesis will be examined in subsequent research. Additionally, the serum profile throughout pregnancy needs to be determined for elk to determine if concentration of PSPB can be used to estimate the age of the fetus.

During pregnancy in moose, mPSPB concentration in serum increased linearly from days 28–36 until near the end of the second trimester, and then increased only slightly. This profile is similar to that of sheep (Willard et al. 1995) in which it increased linearly and then remained the same during the last trimester. In cattle, concentration continues to increase until 2 to 3 weeks before parturition, during which time it increases 5 fold. Further sampling is needed in moose to determine changes that occur near time of parturition.

At 21 days after parturition in cattle, bPSPB concentration was 78 ng/mL of sera and it remained detectable until 87 days in some animals (Kiracofe et al. 1993). In contrast, it was last detectable in sheep at 13 ± 2.3 days postpartum. Similarly in our study, 1 moose bearing twin fetuses had 16 ng/mL of serum at 2 weeks after parturition. A larger sample size is needed to characterize postpartum PSPB in moose, but it may to be cleared sooner in moose than in cattle.

During 30 to 190 days of gestation, the PSPB concentration in moose bearing twin fetuses was higher than moose bearing a single fetus. However, the sample size was small. With a larger sample size, it may be possible to categorize moose as bearing single or twin fetuses at various stages of gestation. The ability to distinguish between moose with twin fetuses or a single fetus is important to wildlife biologists who are studying habitat quality. In this study, sera from 63 moose were collected in March, about 10–12 weeks before parturition. The overall accuracy for detecting twin or single fetal-bearing moose was 90.5% using a cutoff point of 365 ng PSPB/ml of serum. For twin pregnancies, the accuracy was 97%. Thus, the assay is reliable for predicting single or twin pregnancies in March for moose. Fetal numbers were predicted correctly in 82% of sheep by oPSPB RIA (Willard et al. 1995). Although the mPSPB RIA was accurate at 90.5% overall and 97% for twins, prediction of single pregnancies was lower at 84%. The RIA categorized some moose bearing a single fetus as bearing twin fetuses. There are at least 3 possible reasons for this. First, the placenta produces PSPB and a larger placenta could produce more PSPB. So, it is possible that a moose bearing a single large fetus may produce more PSPB than normal. Second, current data suggest that abnormal pregnancies in moose could cause an abnormal PSPB level in serum. Some moose aborted late in gestation or delivered stillbirths and might have had abnormal amounts of PSPB in their sera. Finally, calving number was determined by observation from an airplane at low altitude in the wild. Neonates may be lost to predators or may not be visible due to dense vegetation. However, data are reasonably reliable since calving observations were monitored daily in most cases during the season until parturition was identified.

Osborne et al. (1996) describe an assay for pregnancy associated glycoprotein −1 (PAG-1) in serum of white-tailed deer. They used an RIA for bovine PAG-1. The gene structure for PAG-1 and PSPB are exactly the same. Thus, they have the same bovine assay that we found could not be used for a quantitative assay of this pregnancy protein in deer. We found no parallelism between the bPSPB standard curve and deer serum (Wood et al. 1986). The Osborne manuscript claims that the test was quantitative but proof of validation of the assay in deer serum was not presented. In addition, the change in serum concentration throughout gestation was minimal and ranged from approximately 1 to 8 ng PAG-1/mL of serum. This small change would result from an incomplete cross-reaction of deer PAG-1 with anti-sera. Because of incomplete cross-reaction, detection of twin pregnancies due to more PAG-1 in sera may not be accurate. Certainly, if a protein in sera cross-reacted incompletely with antibodies, a "yes or

no" pregnancy test is valid but a quantitative test is not possible. Until this white-tailed deer PAG-1 assay is validated, interpretation of those data are not possible.

MANAGEMENT IMPLICATIONS

The development of a specific, quantitative RIA for cervidae PSPB will allow one to quantify circulating levels of the protein in serum, during gestation in any cervid. The quantity in serum at specific dates in gestation could provide 2 sets of information. First, it may permit estimation of fetal age or date of conception if concentration varies with age of gestation. Second, it will facilitate classifying moose (or other twin-bearing species) by fetal numbers in the uterus, and may thus allow biologists to evaluate habitat quality.

ACKNOWLEDGMENTS

We thank BioTracking, Moscow, Idaho, USA for providing sera from artificially inseminated elk and for assay of samples with the bPSPB RIA. We thank B. Hawk for typing this manuscript, and K. J. Hundertmark, J. A. Crouse, S. R. Rickabaugh, L. Adams and M. Bertram for assistance in collecting samples from moose. Research support was provided by the Idaho Agricultural Experiment Station, University of Idaho, the Federal Aid in Wildlife Restoration, and the Department of Fish and Wildlife and US Forest Service, LaGrande, Oregon, USA.

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Received 26 January 1999.
Accepted 31 August 1999.
Associate Editor: Rattner.