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TECHNICAL REPORT

A Versatile Enzyme Immunoassay for the Determination of Progestogens in Feces and Serum

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The ability of zoos to monitor the reproductive status of their animals can vastly improve the effectiveness of husbandry/management practices, and noninvasive methods such as fecal steroid analysis are the easiest to apply in a zoo setting. Furthermore, enzyme immunoassay (EIA) is preferred to radioimmunoassay (RIA) as the method of quantifying hormones because EIAs do not involve the use, storage, and disposal of radioactive materials. However, progesterone is excreted in the feces as predominantly unconjugated metabolites (progestogens) and, until recently, antibodies able to cross-react with a variety of progestogens were used primarily in RIAs. An EIA using a broad-spectrum progestogen antibody is described and applied to serum and/or fecal samples from female African elephants, black rhinoceros, white rhinoceros, okapi, and hippopotami. The clear progestogen profiles generated in these species suggest that the described EIA would be as versatile as the RIA using the same antibody and could be a practical and economical alternative to RIAs for monitoring gonadal function via progestogen analysis in zoo species. Zoo Biol 20:227–236, 2001.

Key words: enzyme immunoassay; progestogens; fecal analysis; zoo species

INTRODUCTION

Practical, reliable methods for monitoring gonadal function are often essential for assessing the reproductive status of individual animals, diagnosing fertility problems, and for developing and using assisted reproductive technology when natural breeding fails and/or genetic management is improved with cryopreservation of germ

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plasm. Circulating free steroid concentrations are considered to be the most accurate reflection of gonadal function; however, the regular collection of blood samples can be difficult in non-tractable animals.

In the past two decades, great strides have been made in developing non-invasive methods for monitoring ovarian function in a variety of species by the immunoassay of urinary and fecal steroid metabolites [see reviews: Lasley and Kirkpatrick, 1991; Schwarzenberger et al., 1996a; Brown et al., 1997]. Although radioimmunoassay (RIA) has been the predominant analytical technique used in the past, there are many drawbacks to its use. RIAs use radioactive materials that require special licenses to use, specialized and expensive equipment to detect, and are a health hazard in use and disposal. As a result, enzyme immunoassays (EIAs) are the analytical endocrine technique of choice in zoo and field situations because they circumvent the need for radioactive materials or bulky, expensive equipment.

Most of the easily available EIAs use antibodies specific for unmetabolized steroid hormones or conjugates of steroid metabolites, which makes them most applicable to the measurement of steroid hormones circulating in the blood or steroid metabolites excreted in the urine, respectively [Czekala et al., 1986; Lasley and Kirkpatrick, 1991; Munro et al., 1991]. However, feces are generally easier to collect than serum or urine; therefore, fecal steroid analysis is often the preferred method of non-invasively monitoring ovarian function in exotic animals in captivity and the wild. In herbivores, steroid hormones are excreted in the feces predominantly as unconjugated metabolites [Schwarzenberger et al., 1996a]; thus, EIAs used for blood or urine hormone analysis are not necessarily appropriate for fecal hormone analysis.

One antibody in particular has shown great promise for monitoring progesterone and progesterone metabolites (progestogens) in the feces of a variety of species. The monoclonal antibody (Quidel clone no. 425) was produced against 4-pregnen-11-ol-3, 20-dione hemisuccinate:bovine serum albumin (BSA) [Grieger et al., 1990]. It has been used in RIAs to monitor luteal function/pregnancy via fecal progestogen analysis in a variety of felid species [Graham and Brown, 1997; Brown et al., 2000], African wild dogs [*Lycaon pictus*; Monfort et al., 1997], maned wolves [*Chrysocyon brachyrus*; Velloso et al., 1998], moose [*Alces alces*; Monfort et al., 1993], scimitar-horned oryx [*Oryx dammah*; Morrow and Monfort, 1998], elk [*Cervus elaphus*; Garrott et al., 1998], Sable antelope [*Hippotragus niger*; Thompson et al., 1998], baboons [*Papio sp*; Wasser et al., 1988], and white rhinoceros [*Ceratotherium simium*; Radcliffe et al., 1997; Patton et al., 1999].

The goal of the present study was to adapt the Quidel clone no. 425 antibody to an EIA system that could be used to monitor progestogens in the serum and/or feces of a variety of species. We present serum and/or fecal progestogen profiles from African elephants (*Loxodonta africana*), black rhinoceros (*Diceros bicornis*), white rhinoceros (*Ceratotherium simium*), okapi (*Okapia johnstoni*) and hippopotamus (*Hippopotamus amphibius*) to demonstrate the versatility of the described EIA in measuring the variety of progestogens found in a range of non-domestic species.

MATERIALS AND METHODS

Blood Sample Collection and Processing

Blood samples were collected two to seven times per week from female African elephants (n = 3) housed at Disney's Animal Kingdom. Samples were collected

in heparinized tubes, centrifuged (2,500 rpm, 15 minutes), and the serum was stored at –20°C until analysis. Serum samples were diluted 1:4 in assay buffer (0.02 mol/L tromethamine [Trizma], 0.300 mol/L NaCl, 0.1% BSA, 0.1% Tween 80; pH 7.5) before EIA.

Fecal Sample Collection and Processing

Fecal samples were collected two to seven times per week as part of ongoing endocrine studies on female African elephants (n = 3), black rhinoceros (n = 2), white rhinoceros (n = 4), okapi (n = 1), and Nile hippopotami (n = 9) housed at Disney's Animal Kingdom. All fecal samples were collected within 14 hours of defecation and stored at –20°C until analysis. The fecal extraction protocol was simplified from previously published methods [Graham et al., 1993;Schwarzenberger et al., 1993]. In brief, 0.5 g of feces was placed into a 7-mL glass vial (Fisher Scientific, Pittsburgh, PA), and 5 mL 80% MeOH was added to extract the steroid metabolites. The vials were capped and placed on a shaker (Eberbach Corp., Ann Arbor, MI) for 12 to 14 hours (overnight). Samples were then centrifuged (2,500 rpm, 15 minutes), and the supernatant containing steroid metabolites was poured off and stored at –20°C until analysis. Fecal extracts were diluted in assay buffer before analysis.

Enzyme Immunoassay

The progestogen EIA protocol was a modification of previously published protocols [Schwarzenberger et al., 1991, 1996b]. The optimum concentrations and dilutions of antibodies and biotin label were determined by checkerboard titration. In brief, microtiter plates (Nunc; Fisher Scientific) were coated with affinity purified goat anti-mouse gamma globulin (50 µg/plate; Sigma Chemicals, St. Louis, MO) dissolved in coating buffer (0.015 mol/L Na_2CO_3, 0.035 mol/L $NaHCO_3$; pH 9.6) and incubated overnight at room temperature. Wells were emptied and refilled with a second coating buffer containing preservative (0.02 mol/L Trizma, 0.300 mol/L NaCl, 1.0% BSA, 0.01% NaN_3; pH 7.5) and stored at room temperature. Plates were used within 2 weeks of coating.

Coated plates were washed (0.04% Tween 20), and 50 µL of diluted sample and standards were dispensed. Biotinylated progesterone (1:750,000; supplied by F. Schwarzenberger, Vienna, Austria) was dispensed followed by 100 µl of primary antibody (Quidel clone no. 425, 1:400,000; final purification by C. Munro, Davis, CA). Plates were incubated overnight at room temperature. After incubation, plates were washed and 200 µL of streptavidin-peroxidase conjugate (1 µL in 30 mL assay buffer; Roche Molecular Biochemicals, Indianapolis, IN) were added to each well. After incubation (45 minutes; room temperature) plates were washed and 200 µL of substrate solution (0.5 mL of 0.016 mol/L tetramethylbenzidine in dimethyl sulfoxide and 100 µL 0.175 mol/L H_2O_2 diluted in 24 mL 0.01 mol/L $C_2H_3O_2Na$; pH 5.0) was added to each well. After incubation (45 minutes, room temperature) the enzyme reaction was stopped with 50 µL of stop solution (3 mol/L H_2SO_4). The optical density was measured at 450 nm.

Progesterone was used as standard, and serial dilutions of serum and serial dilutions of fecal extracts from each species gave displacement curves parallel to that of the standard curve. The cross-reactivity of the CL no. 425 antibody as used in the described EIA is given in Table 1. The sensitivity of the assay was 15 pg/well. Interassay coefficient of variation was 12.6% (40% binding) and 15.8 (70% binding) (n = 76 plates).

TABLE 1. Cross-reactivity of the Quidel clone no. 425 progestogen antibody to various progesterone metabolites (relative to the binding of progesterone)

Progesterone metabolite	Common name	Cross-reactivity (%)
4-Pregnen-3,20-dione	Progesterone	100.0
4-Pregnen-3α-o1-20-one		188.0
4-Pregnen-3β-o1-20-one		172.0
4-Pregnen-11α-o1-3,20-dione		147.0
5α-Pregnan-3β-o1-20-one		94.0
5α-Pregnan-3α-o1-20-one		64.0
5α-Pregnan-3,20-dione		55.0
5β-Pregnan-3β-o1-20-one		12.5
5β-Pregnan-3,20-dione		8.0
4-Pregnen-11β-o1-3,20-dione		2.7
5β-Pregnan-3α-o1-20-one		2.5
5β-Pregnan-3α,20α-diol	Pregnanediol	<0.1
5α-Pregnan-3α,20β-diol		<0.1
5β-Pregnan-3,17-dione	Androstenedione	<0.1
5β-Pregnan-11β,21-diol-3,20-dione	Corticosterone	<0.1

RESULTS

EIA of unextracted serum from African elephants revealed temporal changes in serum progestogens indicative of ovarian cyclicity (Fig. 1a). These temporal changes were mirrored by changes in fecal progestogen concentrations (Fig. 1b).

Fecal analysis in black rhinoceros, white rhinoceros, and okapi revealed temporal changes in fecal progestogens indicative of ovulation and corpora lutea function (Figs. 2a, b and 3a). Elevations in fecal progestogens were observed during presumptive luteal phases and pregnancy in the Nile hippopotamus (Fig. 3b).

DISCUSSION

Data on the ovarian function of wildlife species can inform husbandry/breeding management. This is especially true for those species for which there is still limited management experience. For example, monitoring reproductive hormones can help establish the onset of puberty and estrous cyclicity, determine the optimum time to pair animals for breeding, and diagnose pregnancy and predict parturition.

The most accurate reflection of ovarian function is circulating free-steroid concentrations. Using the described progestogen EIA, serum analysis indicated that the African elephant had a cycle length of approximately 100 days, which is similar to the cycle length reported in the literature [Hodges, 1998]. Characterization of the progestogen antibody used in the EIA indicated it has a high cross-reactivity with 5α-reduced progesterone metabolites, which are the major progestogens found in the serum of elephants [Hodges et al., 1997]. This likely explains the greater than 10-fold higher concentrations of serum progestogens measured in African elephant with the described EIA compared with those quantified with antibodies specific for progesterone [Hodges, 1998]. The concentrations of progestogens measured in the serum of African elephants in the present study were more similar to those measured with antibodies specific for 5α-reduced progesterone [Heistermann et al., 1997].

For many non-domestic species, regular blood collection can be difficult in captive situations and nearly impossible in the wild. An alternative to blood hormone

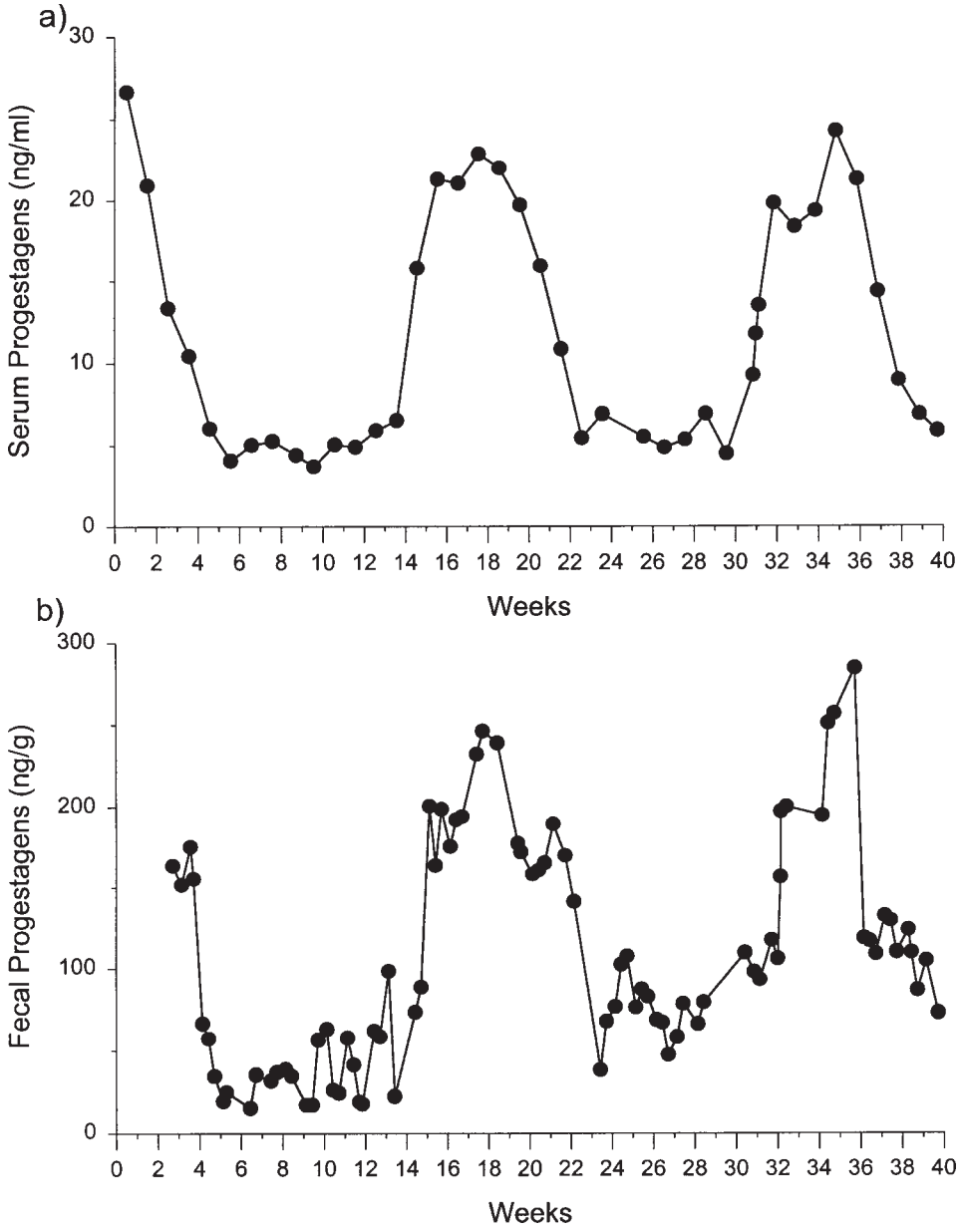

Fig. 1. Serum **(a)** and fecal **(b)** progestogen concentrations in an African elephant.

analysis is fecal hormone analysis, and 5α-reduced progestogens are the predominant progesterone metabolites found in the feces of many mammalian species [Schwarzenberger et al., 1996a]. Fecal progestogen analysis indicated a cycle length of 3 to 4 weeks in the black rhinoceros, which is similar to the cycle lengths previously reported for this species [Hindle et al., 1992], and a cycle length of approximately 2 weeks in the okapi, which also agrees with previous reports [Loskutoff et

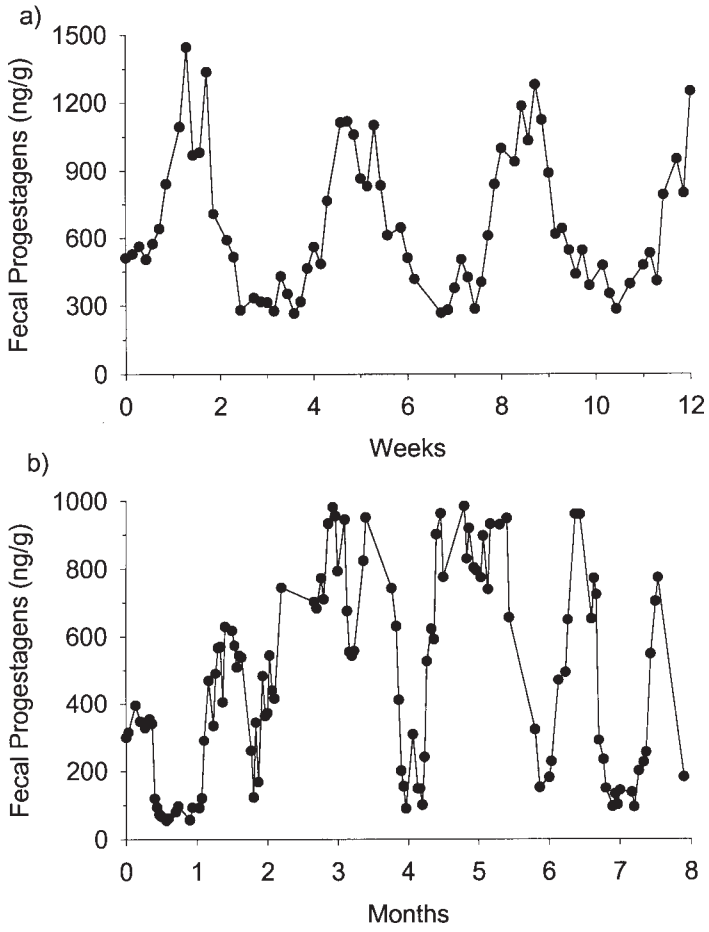

Fig. 2. Fecal progestogens in **(a)** a black rhinoceros and **(b)** a white rhinoceros.

al., 1982; Schwarzenberger et al., 1993, 1999]. Fecal progestogen analysis in the white rhinoceros indicated two different types of estrous cycles: those of approximately 1 month and those of approximately 2 months. This is similar to the patterns observed in white rhinoceros in a previous investigation [Patton et al., 1999]. Changes in fecal progestogen concentrations mirrored changes in serum progestogens in the African elephant, confirming previous investigations [Wasser et al., 1996; Fiess et al., 1999]. Finally, fecal progestogen analysis indicated a non-conceptive cycle length of approximately 30 days in the Nile hippopotamus, and after conception, fecal progestogen concentrations remained elevated until parturition. Although the endocrine patterns associated with estrous cyclicity have not previously been characterized for this species, an estrous cycle of approximately 4 weeks is similar to that observed in other large mammals.

The EIA system chosen for this study used the second antibody technique [Meyer and Guven, 1986], where the second antibody is an antibody to the primary antibody and is used to coat the microtiter plate. In the described EIA using this technique, goat anti-mouse immunoglobulin G is coated to the microtiter plate and then samples

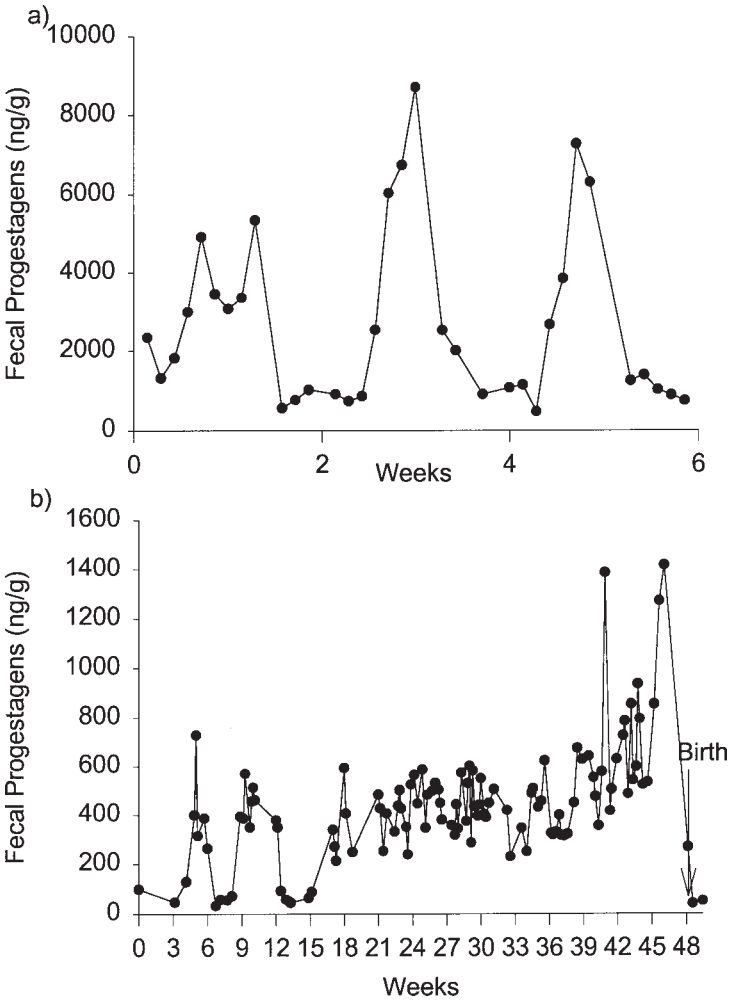

Fig. 3. Fecal progestogens in **(a)** an okapi and **(b)** a hippopotamus.

and label are added. The primary (progestogen-specific) antibody is dispensed last and is immobilized to the plate by being bound by the second antibody. This technique has several advantages over currently popular EIAs, where the microtiter plate is coated with the primary antibody itself. First, when coating the plate with the primary antibody, the readings of the wells on the outer rim of the plate often deviate from those of the inner wells. As a result, the outside wells are often not used, which reduces the number of samples that can be run in a single plate. Coating the plate with the second antibody reduces this assay variability associated with uneven binding of the primary antibody to the wells [Meyer, 1986], which allows more samples to be run in a single plate, reducing the cost of analysis. Second, it has been observed that the second antibody technique can drastically reduce the amount of primary antibody needed [Mutayoba et al., 1990], further reducing the cost of analysis. Finally, in EIAs where plates are coated with the primary antibody, there is often

time-dependent drift of the results [Munro and Stabenfeldt, 1984]. This is caused by different incubation times within the plate because of the time required for pipetting different samples/standards into 96 wells. The second antibody technique allows the primary antibody to be dispensed after samples and label have been added, resulting in an almost simultaneous initiation of the immune reaction across the plate, eliminating plate drift [Meyer and Hoffman, 1987].

The other important aspect to the progestogen EIA used here was the biotinylated progesterone used as the label. There are advantages to labeling the antigenic hormone with biotin instead of labeling it directly with the enzyme. The biotin label is stable, and its small size gives it nearly an equal chance of binding in an immunological reaction as the unlabeled hormone. This is in contrast to labels with directly coupled enzymes, which are at least 100 times larger than the parent steroid [Palme and Mostl, 1993]. Furthermore, mediation via the avidin–biotin interaction serves to amplify the sensitivity of the assay [Strasburger et al., 1988; Mutayoba et al., 1990].

The dynamics of the EIA in the present study are very similar to a previously developed EIA using a progestogen antibody specific for 20-oxo-progestogens that has been successfully used to measure progestogens in the serum of elephants [Schwarzenberger et al., 1997] and feces of black and white rhinoceros [Schwarzenberger et al., 1996b, 1998]. However, in the EIA of the present study, much less of the primary antibody and biotin label is needed to achieve an adequate signal, which is an important consideration when considering the cost of doing serum/fecal progestogen analysis.

Until recently, fecal progestogen analysis in non-domestic species was performed primarily with RIAs, which limited their use to institutions with facilities for the storage and disposal of radioactive materials. Few zoological institutions are equipped with RIA laboratories, and those that are equipped are often overwhelmed with requests for endocrine analysis from other institutions, which often results in delays in receiving endocrine data. An alternative is to pay suitably equipped hospital or university laboratories to conduct RIA analysis. However, this can prove to be very costly. The results presented here suggest that the described EIA could be a practical alternative to RIAs for non-invasively monitoring ovarian function via progestogen analysis of fecal samples from non-domestic species in captivity and in the wild. EIAs can be performed with a minimum amount of equipment, and performing the analysis on-site could reduce the cost of analysis as well as the time from collection to results.

CONCLUSION

The clear progestogen profiles generated in a variety of species in this study suggest that the described EIA could be a practical and economical alternative to RIAs for monitoring ovarian function via progestogen analysis of feces in non-domestic species.

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