Urinary Steroid Evaluations to Monitor Ovarian Function in Exotic Ungulates: X. Pregnancy Diagnosis in Perissodactyla

Edward C. Ramsay, Francisco Moran, Janet F. Roser, and Bill L. Lasley

Departments of Medicine (E.C.R.) and Reproduction (F.M., B.L.L.), School of Veterinary Medicine, and the Department of Animal Science (J.F.R.), University of California, Davis, California

Enzymeimmunoassays (EIAs) for estrone conjugates (EC), pregnanediol-3-glu-curonide (PDG), and C-19 and C-21 progesterone metabolites (C-19/C-21) were used to analyze urine samples from four nondomestic equid species, four tapir species, and two rhinoceros species in an attempt to identify if these assays could be used for diagnosing and monitoring pregnancy. The same urine samples were also analyzed for the presence of equine chorionic gonadotropin (eCG) activity, using a field dipstick test and a radioimmunoassay (RIA). The EC EIA was validated for three equid species and the Malayan tapir. Neither the PDG nor the C-19/C-21 EIAs were validated in any species evaluated. In equid species, the EC EIA demonstrated a specificity (the percentage of nonpregnant samples identified correctly) of 100% and a sensitivity (the percentage of pregnant samples identified correctly) of ≥88%. With the exception of the Grevy's zebra, the C-19/C-21 EIA showed a similar accuracy in identifying pregnant and nonpregnant equids. The PDG EIA was not sufficiently accurate to merit its use in equids or tapirs for pregnancy diagnosis. From the data collected, it appears analysis of a single urine by both the EC EIA and the C-19/C-21 EIA would be the best method of pregnancy detection during the last 2 trimesters of gestation, in equid species. In tapirs, the C-19/C-21 EIA was slightly more accurate for pregnancy diagnosis than the EC EIA. The C-19/C-21 EIA had a specificity of 93%, but a sensitivity of only 73% in tapir species. None of the EIAs evaluated demonstrated a sufficient specificity or sensitivity to be useful, as presently performed, for pregnancy diagnosis from a single sample in the black rhinoceros. The eCG dipstick used in this study did not prove a sufficiently reliable test for routine pregnancy in nondomestic equids. The eCG RIA results in the Przewalski's horses and the Hartman's mountain zebra were positive early in gestation, and indicate that gonadotropin analysis may be useful for pregnancy detection in these species. Only very low amounts of eCG activity was measured by the eCG RIA in the tapir and rhinoceros urine samples.   © 1994 Wiley-Liss, Inc.

INTRODUCTION

The diagnosis and monitoring of pregnancy are two of the most important reproductive evaluations that can be made for the management of animals in breeding programs. Pregnancy detection permits initiation of monitoring and health care for the pregnant individual. Knowledge of the reproductive status of each female allows animal managers to optimize production of a colony.

Urinary steroid analysis has been demonstrated to be a noninvasive method for monitoring reproductive events in a wide variety of mammals [Ensley et al., 1982; Loskutoff et al., 1982; Shideler et al., 1983; Walker et al., 1988; Mainka et al., 1990; Masters et al., 1991; Paul-Murphy et al., 1991]. Members of the order Perissodactyla, many of whom are endangered species, have been a particular focus of urinary reproductive monitoring research. Pregnancy diagnosis via estrogen conjugate (EC) evaluation has been demonstrated in domestic and wild equids [Evans et al., 1984; Kirkpatrick et al., 1988; Czekala et al., 1990] and in the tapir by Kasman et al. [1985]. Monitoring the estrous cycle and diagnosis of pregnancy in the greater one-horned (Indian) rhinoceros has been described by Kassam and Lasley [1981], Kasman et al. [1986], and Hodges and Green [1989]. Estrogen and progesterone metabolite excretion has been studied in the white and black rhinoceros [Hindle et al., 1992]. Diagnosis of pregnancy using urinary pregnanediol-3-glucuronide (PDG) was reported in the black rhinoceros by Ramsay et al. [1987] and in the white and black rhinoceros by Hodges and Green [1989]. All but two of these studies [Hodges and Green, 1989; Hindle et al., 1992] have measured urinary hormone metabolites by direct radioimmunoassays (RIAs).

Radioimmunoassays have the disadvantages of expense, requiring sophisticated analysis equipment and the handling of radioisotopes. Recently, enzymeimmunoassays (EIAs) have been developed for evaluation of urinary hormone metabolites in nondomestic species [Czekala et al., 1986; Hodges and Green, 1989; Shideler et al., 1993]. These EIAs have the advantages of being less expensive to perform and do not require the use of controlled radioisotopes.

Over the past 10 years, it has been recognized that progesterone metabolism and excretion can vary between closely related species [Loskutoff et al., 1983]. Pregnanediol-3-glucuronide concentration in urine is a reliable indicator of circulating progesterone in hominoids [Mitchell et al., 1982], New World primates [Hodges et al., 1981], okapi [Loskutoff et al., 1986], and greater one-horned rhinoceros [Kasman et al., 1986], but has limited use for monitoring luteal phases or pregnancy in Old World primates [Shideler et al., 1985], the killer whale [Walker et al., 1988], the black rhinoceros [Ramsay et al., 1987], and feral horses [Kirkpatrick et al., 1993]. A nonspecific EIA, using antisera to PDG and related progesterone metabolites, has recently been developed and demonstrated to be useful in monitoring luteal activity in the horse [Kirkpatrick et al., 1990]. A similar assay has been used to describe luteal activity in the white rhinoceros [Hindle et al., 1992]. Thus a broad-spectrum assay for progesterone metabolites seems appropriate for the evaluation of progesterone excretion in most perissodactyl species.

Analyses of chorionic gonadotropins in urine have been used for the diagnosis

TABLE 1. Species of Perissodactyla from which urine samples were evaluated

Common name	Scientific name	Numbers of animals	Numbers of samples analyzed
Przewalski's horse	*Equus przewalskii*	7	27
Grevy's zebra	*Equus grevyi*	16	29
Grant's zebra	*Equus burchelli*	4	4
Hartman's mountain zebra	*Equus zebra*	9	29
Malayan tapir	*Tapirus indicus*	7	24
Mountain tapir	*Tapirus pinchaque*	2	5
Common tapir	*Tapirus terrestris*	4	5
Baird's tapir	*Tapirus bairdii*	1	3
Black rhinoceros	*Diceros bicornis*	11	83[a]
White rhinoceros	*Ceratotherium simum*	10	27

[a]Samples from one black rhinoceros pregnancy were evaluated but discarded due to the animal's death during midgestation and lack of knowledge regarding date of fetal death.

of pregnancy in humans [Josimovich, 1980], nonhuman primates [Woodward, et al., 1976], and horses [Roser and Lofstedt, 1989]. Urinary human chorionic gonadotropin (hCG) can be detected between days 30–40 of pregnancy [Josimovich, 1980]. Similarly, urinary equine chorionic gonadotropin (eCG) is observed about days 35–40 of pregnancy [Roser and Lofstedt, 1989]. Urinary levels of eCG peak during the first trimester of pregnancy and then decline shortly thereafter [Roser and Lofstedt, 1989]. McFarlane and coworkers [1991a] have reported the presence of equine chorionic gonadotropin in the serum of zebras during the first trimester of pregnancy. Although pituitary gonadotropins have been partially characterized in the rhinoceros [McFarlane et al., 1991b], chorionic gonadotropins have yet to be isolated or characterized in this species. Previous studies on the urinary excretion patterns of these gonadotropins in nondomestic perissodactyl species have not been performed.

In the present study, an estrone conjugate and two progesterone metabolite EIAs were used to analyze urine samples from a wide range of perissodactyl species in an attempt to identify if these assays could be used for diagnosing and monitoring pregnancy. In addition, the possibility that members of the order Perissodactyla, other than the domestic horse, excretes a chorionic gonadotropin in urine that can be detected using antibodies to eCG was explored. The specific objective was to identify if currently available EIAs could be used both for the detection and monitoring of pregnancy in a number of perissodactyl species.

MATERIALS AND METHODS
Animals

Urines samples from perissodactyl species were solicited from U.S. zoos during 1989–1990. Additional samples, which had been stored frozen at −4°C, from previous endocrine studies, were analyzed. Table 1 shows the species and the number of animals evaluated and number of samples analyzed. All animals included in the study were captive, sexually mature females. All had access to a male of their species prior to the collection of the urine samples and were potentially pregnant.

Determination of pregnancy status was based upon subsequent parturition or indentified loss of a fetus. All pregnancy samples were indexed to the day of partu-

rition (described as days prior to parturition) due to the lack of known breeding dates on most animals and absence of recognized gestation lengths for most species. Gestations were estimated as 330–350 days for equids, 390 days for tapirs, and 450–500 days for rhino species [Grzimek, 1964; Lang, 1964; Volf, 1964; Ramsay et al., 1987]. Samples collected less than 330 days, 360 days, and 450 days prepartum were considered pregnancy samples for equids, tapir, and rhinoceros, respectively. Urine samples collected more than 360 days, 400 days, and 500 days prepartum were considered nonpregnant urines.

Most zebra and Przewalski's horse urine samples were collected by catheterization, while the animal was immobilized for elective procedures such as hoof trimming or shipment. The remainder of the equid samples, and the tapir and rhinoceros urine samples were collected by either midstream catch or aspirated from the floor of enclosures, as quickly as feasible, following urination. Samples were placed in glass or plastic tubes and frozen, at $-4°C$, until being aliquoted for analysis. Samples were frozen from <1 to 10 years (mean = 4.01 years) prior to analyses. All samples underwent several freeze-thaw cycles prior to analysis in this study.

Sample Analysis

All urine samples were allowed to thaw, mixed thoroughly, and diluted to 1:10, 1:50, and 1:100 concentrations using distilled water. Each sample was analyzed for creatinine (Cr) content, using a microtiter plate technique, based on the method of Taussky [1954]. All hormone conjugate values are expressed as mass/mg Cr, to adjust for variation in urine concentration.

The estrone conjugate EIA employed was performed as described by Munro et al. [1991]. The cross-reactivity for this assay is: estrone-3-glucuronide 100%, estrone-3-sulfate 66.6%, estrone 236%, estradiol-17 7.8%, estradiol-3-glucuronide 3.8%, estradiol-3-sulfate 3.3%, other steroid metabolites ≤0.1%. Separate EIAs were used to detect either specific PDG-like, C-21 urinary metabolites or the combination of both C-21 and C-19 urinary metabolites of progesterone. The first system (PDG) utilized a specific polyclonal antibody to pregnanediol-3-glucuronide and was employed as reported by Munro et al. [1991]. The cross-reactivity of this assay is: pregnanediol-3-glucuronide 100%, 20α-hydroxyprogesterone 60.7%, 20α-hydroxyprogesterone 2.5%, pregnanediol 7.3%, all other steroid metabolites <0.1%. The second assay system (C-19/C-21) utilized a monoclonal antibody to PDG that demonstrated cross reactivity to the C-19 and C-21 conjugated metabolites of progesterone [Bahar, 1988; Shideler, et al., 1993].

Equine chorionic gonadotropin was measured in urine by two different methods. The first method employed an enzyme-immunoassay using a dipstick field test for eCG as described by Roser and Lofstedt [1989]. The urinary CG molecule was captured on a dipstick coated with a monoclonal antibody against bovine luteinizing hormone (518B7). A second monoclonal antibody, 759G10, an antiequine luteinizing hormone antibody, which was conjugated to alkaline phosphatase, recognized the chorionic gonadotropin molecule at a different epitope to form a sandwich complex. The dipstick turned blue in a substrate solution of bromo-chloro-indolyl phosphate. In the absence of CG, the dipstick remained white since the sandwich complex was not formed. The assay was carried out at room temperature and took 75 min. A pregnant and cycling mare urine was run during each assay as a positive and negative control. The samples were scored as either positive (blue) or negative (white). The second

method employed a radioimmunoassay for urinary eCG as described by Roser and Lofstedt [1989]. A purified preparation of eCG (PM230GB) was used as the tracer and standard. The monoclonal antibody against bovine luteinizing hormone (518B7) was used as the first antibody. The sensitivity of the assay was 4.5 ng/ml. All samples were run in one assay. Intra-assay coefficient of variation was 6.0% using a pregnant mare plasma pool. Concentrations of CG in samples were expressed as ng/mg of creatinine.

Assay Validation

Urinary EC, PDG, and C-19/C-21 were measured in individual, serially diluted (1:10, 1:50 and 1:100 in distilled water) urine samples to determine parallelism of each metabolite as well as to determine the most appropriate dilution for final analysis. When more than one dilution fell within the range of the standard curve and the dose-response dynamics indicated parallelism of the metabolite to the standard curve, the assay was considered validated for that species. This was demonstrated for EC in at least 10 samples from Przewalski's horse, Grevy's zebra, Hartman's mountain zebra, and the Malayan tapir. An insufficient number of dilutions tandemly fell within the standard curve to evaluate parallelism for the EC EIA and the PDG and C-19/C-21 EIAs in other species. Thus, for EC assay measurements in non-Malayan tapir species and rhinoceros species and both progesterone metabolite assays measurements in all species, comparisons are made of quantity of substance measured and not quantity of specific metabolite. For non-validated assays, hormone metabolite amounts are expressed as equivalents of the hormone metabolite.

RESULTS

The results of the EC, PDG, and C-19/C-21 EIA analyses of Przewalski horse, the Grevy's and Grant's zebra, and Hartman's mountain zebra urine samples are depicted in Figures 1, 2, and 3, respectively. Due to the large number of samples that contained either non-measurable amounts (below the sensitivity of the assay) of these hormones or quantities of hormone that were greater than the assays could measure in a 1:100 dilution, statistical analyses of the data could not be performed.

Based on visual inspection of the data, 1,000 ng EC/mg Cr or the displacement equivalents of 1,000 ng EC/mg Cr, 100 ng PDG/mg Cr, and 100 ng C-19/C-21/mg Cr were selected as pregnancy discrimination levels for the equid species, with one exception. For the Grevy's zebra, an equivalent of 500 ng C-19/C-21/mg Cr was used as the discrimination criterion. Urine samples from nonpregnant animals would be expected to contain less hormone conjugate than the discrimination amount. Samples from pregnant animals would be expected to contain equal or more hormone conjugate than the discrimination criteria. Table 2 summarizes the abilities of the EC, PDG, and C-19/C-21 EIAs to identify pregnancy in these species, using the described criteria.

Figure 4 shows the CG dipstick assay and eCG RIA results for the Przewalski's horse, Grevy's zebra, and Hartman's Mountain zebra. In all of the samples, a positive dipstick was associated with CG levels that were positive by the RIA, i.e., levels greater than the sensitivity of the assay. In 39% of the samples, the RIA detected CG in the samples that tested negative with the dipstick, indicating the dipstick assay was not a reliable indicator of pregnancy. All dipstick tests performed on tapir and rhi-
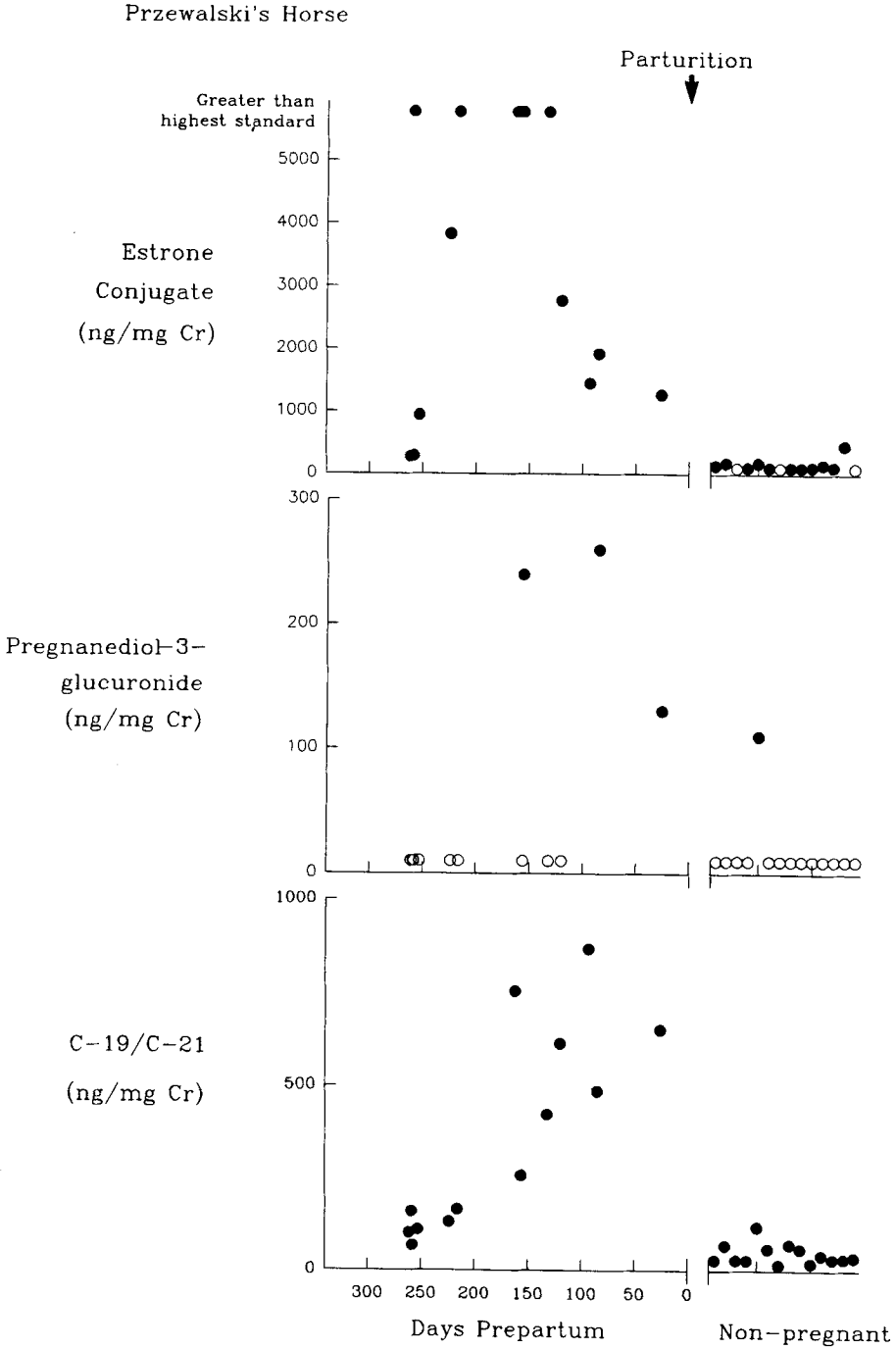
Przewalski's Horse

Fig. 1. Urinary estrone conjugate values and pregnanediol-3-glucuronide and C-19/C-21 progesterone metabolite equivalent values in pregnant and non-pregnant Przewalski's horses as measured by enzyme-immunoassays. All pregnancies are normalized to the date of parturition (day 0). Open circles (O) represent values below the sensitivity of the assays.
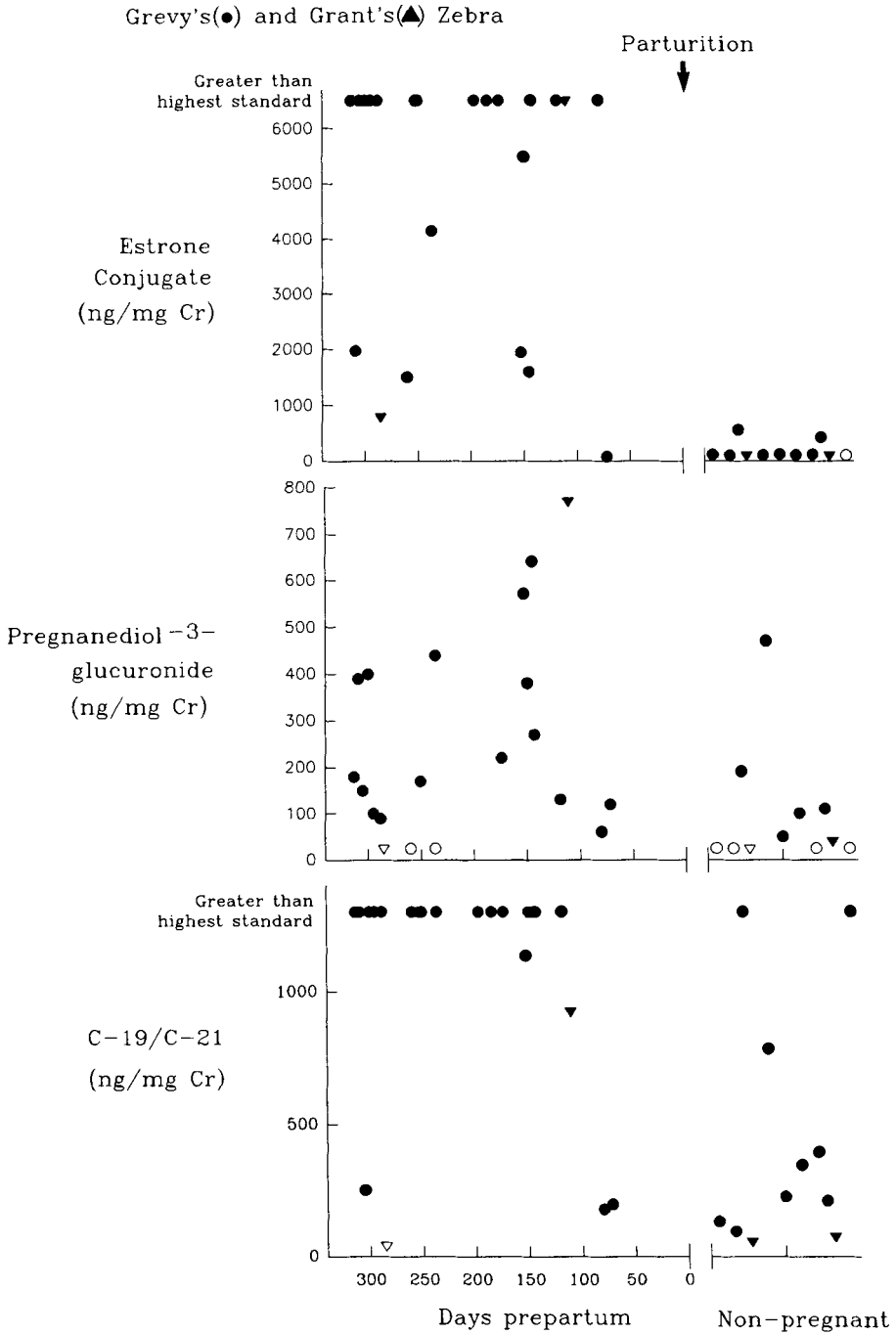
Fig. 2. Urinary estrone conjugate values (Grevy's zebra) and estrone conjugate (Grant's zebra), pregnanediol-3-glucuronide and C-19/C-21 progesterone metabolite equivalent values in pregnant and nonpregnant Grevy's (●) and Grant's (▲) zebra as measured by enzymeimmunoassays. All pregnancies are normalized to the date of parturition (day 0). Open circles (○) and triangles (△) represent values below the sensitivity of the assays.
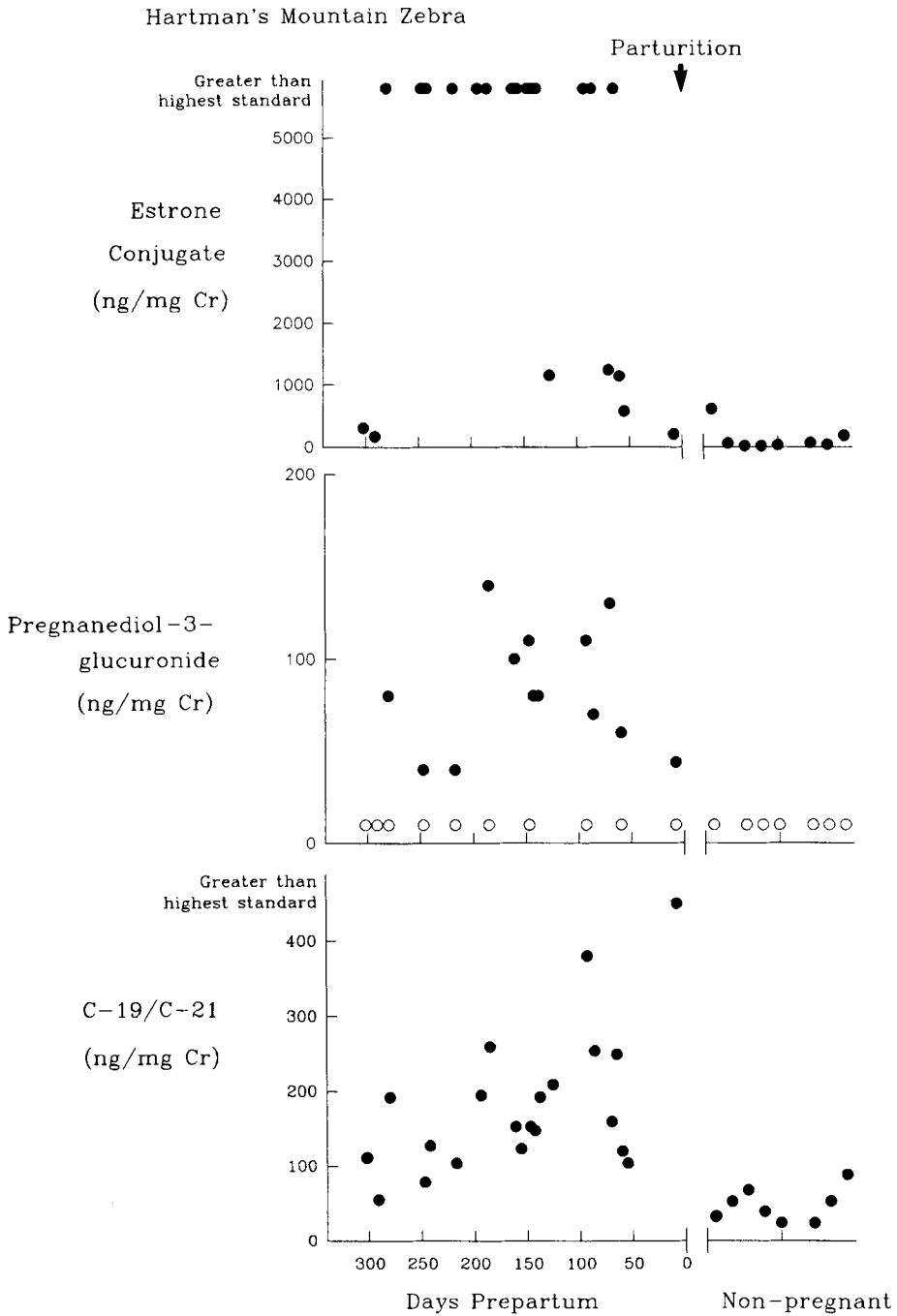
Hartman's Mountain Zebra

Fig. 3.   Urinary estrone conjugate, pregnanediol-3-glucuronide and C-19/C-21 progesterone metabolite equivalent values in pregnant and non-pregnant Hartman's mountain zebra as measured by enzymeimmunoassays. All pregnancies are normalized to the date of parturition (day 0). Open circles (O) represent values below the sensitivity of the assays.

TABLE 2. Summary of the ability of an estrone conjugate (EC), a pregnanediol-3-glucuronide (PDG), and a nonspecific progesterone metabolite (C-19/C-21) enzymeimmunoassay to identify nonpregnant and pregnant urine samples from equid species.*

Assay/species	Nonpregnant[a]	Pregnant[b]	
		At any time during gestation	Within 240 days of parturition
Estrone conjugates			
Przewalski's horse	9/12 (75%)	12/12 (100%)	9/9 (100%)
Grevy's zebra	9/9 (100%)	19/20 (95%)	10/11 (91%)
Grant's zebra	2/2 (100%)	1/2 (50%)	1/1 (100%)
Hartman's mountain zebra	8 (100%)	17/21 (81%)	14/16 (88%)
Pregnandiol-3-glucuronide			
Przewalski's horse	13/14 (93%)	4/13 (31%)	4/9 (44%)
Grevy's zebra	5/9 (56%)	16/20 (80%)	9/11 (82%)
Grant's zebra	2/2 (100%)	1/2 (50%)	1/1 (100%)
Hartman's mountain zebra	8/8 (100%)	6/21 (29%)	6/16 (38%)
C-19/C-21			
Przewalski's horse	13/14 (93%)	12/13 (92%)	9/9 (100%)
Grevy's zebra	6/9 (67%)	17/20 (85%)	9/11 (82%)
Grant's zebra	2/2 (100%)	1/2 (50%)	1/1 (100%)
Hartman's mountain zebra	8/8 (100%)	19/21 (90%)	16/16 (100%)

*Using the equivalent of 1,000 ng EC/mg Cr, 100 ng PDG/mg Cr, and 100 ng C-19/C-21/mg Cr as discrimination criteria, except in Grevy's zebra, where the equivalent of 500 ng C-19/C-21/mg Cr was used as the discrimination criterion.

[a]Number of urine samples containing less hormone metabolites than the discrimination criteria/the number of samples analyzed (specificity of the assay).

[b]Number of urine samples containing amounts of hormone metabolites greater than or equal to the discrimination criteria/the number of samples analyzed (sensitivity of the assay).

noceros were negative (data not shown). Only extremely low amounts of CG, if any, could be detected in tapir urine by RIA. The levels ranged from 1.85 to 4.69 ng/ml, close to or below the sensitivity of the assay. Similarly, very low levels of CG were detected in the rhinoceros urine by RIA, with concentrations ranging from 1.18 to 8.65 ng/ml.

Figure 5 depicts the results of EC, PDG, and C-19/C-21 EIA analyses for tapir urine samples. Many nonpregnant and some pregnant urines contained less hormone conjugate than could be measured by the EC and PDG EIAs. By visual inspection of the data, the discrimination criteria for EC and PDG were set at any measurable amount of hormone metabolite equivalents. Discrimination criterion for the C-19/C-21 EIA was set at the equivalent of 500 ng/mg Cr. Table 3 summarizes the accuracy of these EIAs in identifying pregnancy in members of the family Tapiridae, using the described criteria.

Figure 6 shows the results of the EC, PDG and C-19/C-21 EIA analyses of black rhinoceros urine samples. Only 6 of 83 pregnant and nonpregnant black rhinoceros urine samples contained measurable amounts of EC equivalents. Based on visual evaluation of the data, two pregnancy discrimination criteria were evaluated for the PDG (any measurable amount metabolite equivalent and the equivalent of 500 ng/ml Cr) and C-19/C-21 (the equivalent of 200 and 500 ng/mg Cr). Only nonpregnant white rhinoceros urines were available for analysis. Values determined by EC, PDG,
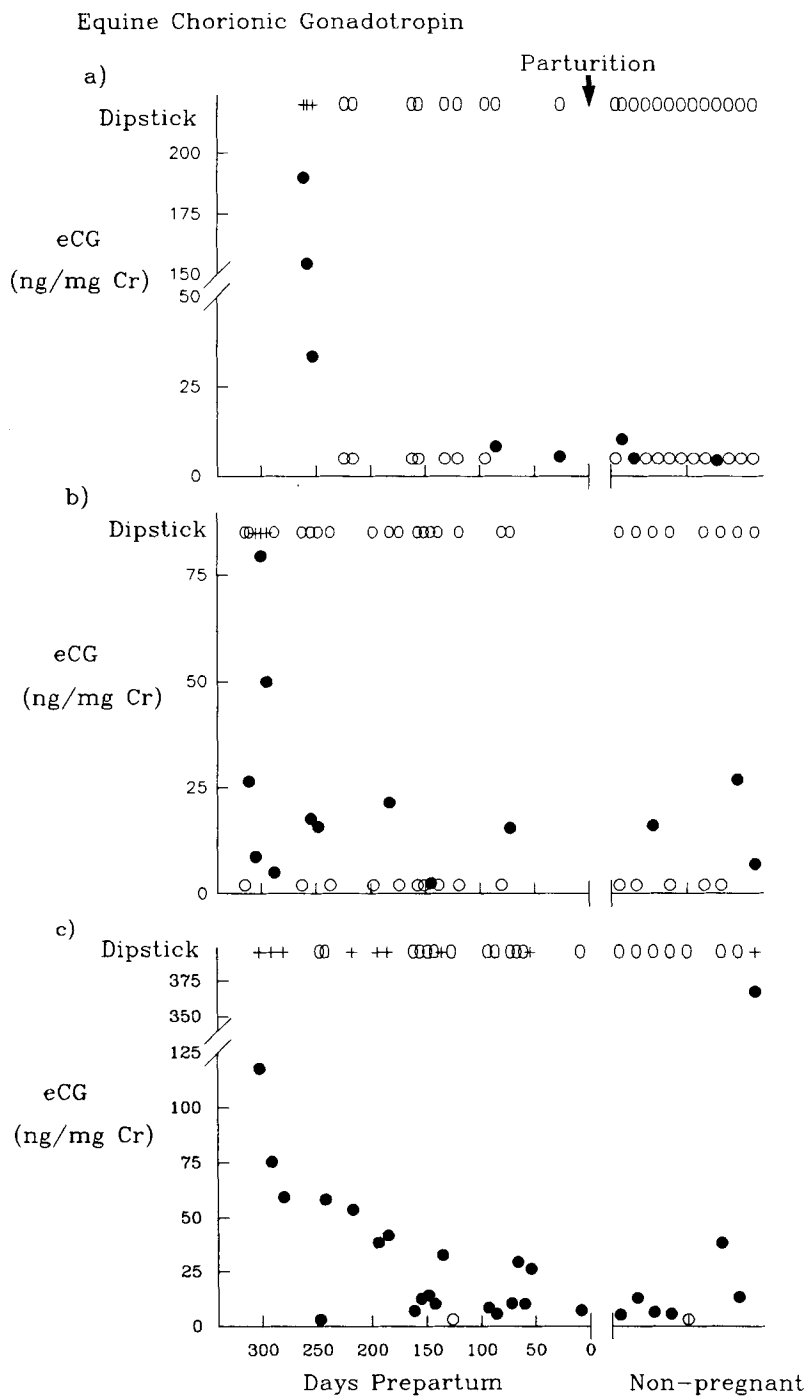
Fig. 4. Urinary chorionic gonadotropin values in (a) Przewalski's horses, (b) Grevy's zebra, and (c) Hartman's mountain zebra, measured by a dipstick technique (+ /O) and a radioimmunoassay (●). All pregnancies are normalized to the date of parturition (day 0). Open small circles (O) represent values below the sensitivity of the radioimmunoassay.

TABLE 3. Summary of the ability of an estrone conjugate
(EC), a pregnanediol-3-glucuronide (PDG), and a nonspecific
progesterone metabolite (C-19/C-21) enzymeimmunoassay
to identify nonpregnant and pregnant urine samples from
tapir species*

Assay/species	Nonpregnant[a]	Pregnant[b]
Estrone conjugates		
Malayan tapir	6/8 (75%)	11/16 (69%)
Mountain tapir	NS	2/5 (40%)
Common tapir	4/5 (80%)	NS
Baird's tapir	2/2 (100%)	1/1 (100%)
Total all species	12/15 (80%)	14/22 (64%)
Pregnanediol-3-glucuronide		
Malayan tapir	7/8 (88%)	10/16 (63%)
Mountain tapir	NS	3/5 (60%)
Common tapir	3/5 (80%)	NS
Baird's tapir	2/2 (100%)	1/1 (100%)
Total- all tapir	12/15 (80%)	14/22 (64%)
C-19/C-21		
Malayan tapir	7/8 (88%)	12/16 (75%)
Mountain tapir	NS	4/5 (80%)
Common tapir	5/5 (100%)	NS
Baird's tapir	2/2 (100%)	0/1 (0%)
Total all tapir	14/15 (93%)	16/22 (73%)

*Using any measurable amount of EC and PDG equivalents and the
equivalent of 500 ng C-19/C-21/mg Cr as the discrimination criteria
for determination of pregnancy.
[a]Number of urine samples containing less hormone metabolites than
the discrimination criteria/the number of samples analyzed (specificity
of the assay).
[b]Number of urine samples containing amounts of hormone metabolites
greater than or equal to the discrimination criteria/the number of sam-
ples analyzed (sensitivity of the assay).
NS = no samples available.

and C19/C-21 EIAs were similar to those found in nonpregnant black rhino urine
samples. Table 4 summarizes the ability of the EC, PDG and C-19/C-21 EIA to
identify pregnancy in the black rhinoceros.

DISCUSSION

Urinary steroid metabolite analyses have been demonstrated to be an accurate
method for monitoring reproductive function in captive animals, but have yet to be
widely utilized. The major limitation to the broad application of urinary steroid
analysis has been the availability of the required RIAs. Enzyme immunoassays offer
an attractive alternative to RIAs, in that EIAs do not require the use of radioisotopes
and utilize much less expensive analysis equipment. These allow EIAs to be per-
formed outside of a traditional endocrine laboratory [Czekala et al., 1986; Hodges
and Green, 1989]. Previous comparisons of EIAs and RIAs in analyzing urines from
the greater one-horned rhinoceros and certain primate species indicate good correla-

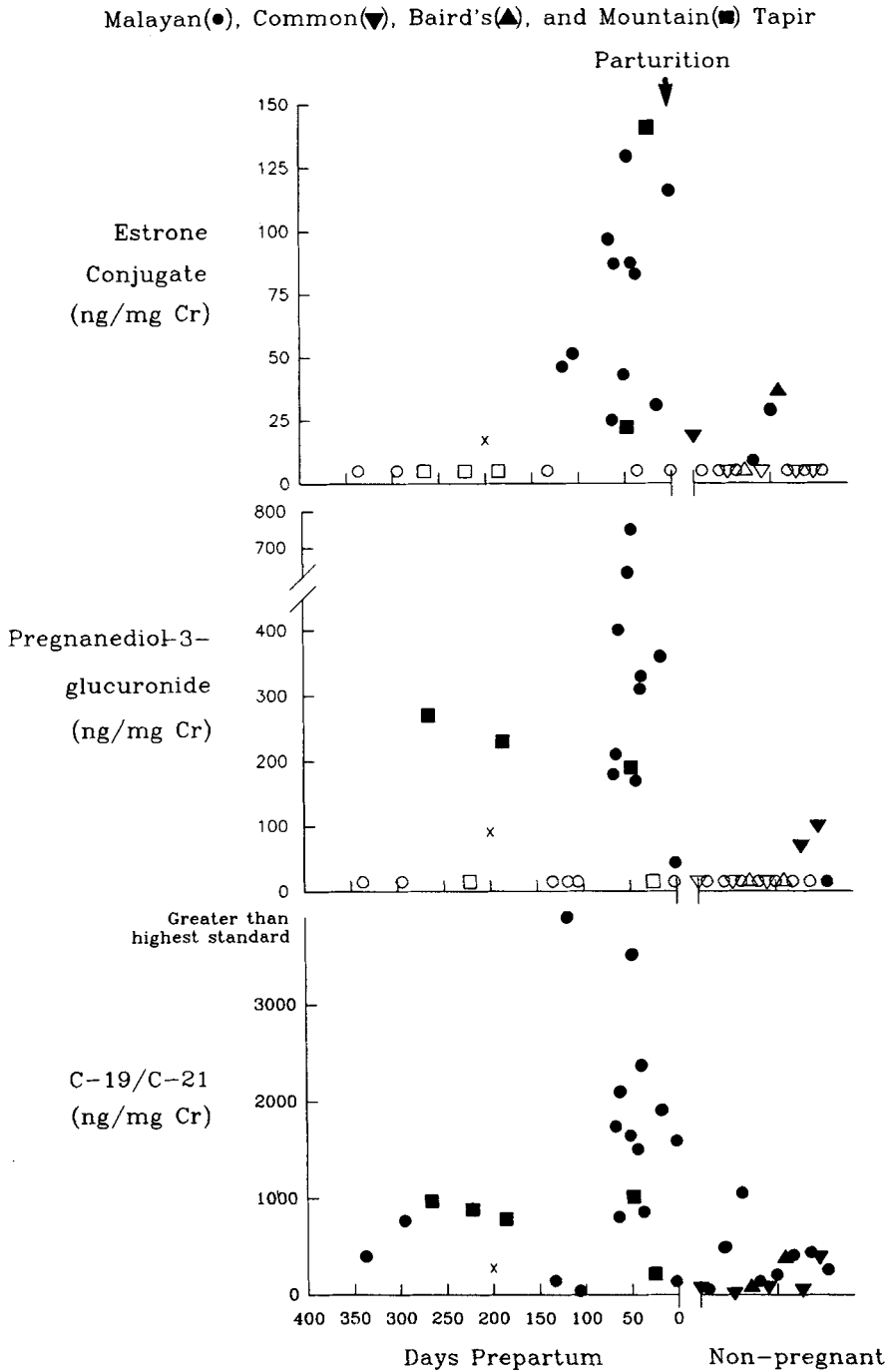Malayan(●), Common(▼), Baird's(▲), and Mountain(■) Tapir

Fig. 5. Urinary estrone conjugate (Malay tapir) and estrone conjugate (other species), pregnanediol-3-glucuronide and C-19/C-21 progesterone metabolite equivalent values in pregnant and non-pregnant Malayan tapir (●), mountain tapir (■) common tapir (▼), and Baird's tapir (▲) as measured by enzyme-immunoassays. The date for one Baird's tapir sample (x) is estimated based on date of abortion and maturity of an aborted fetus. All pregnancies are normalized to the date of parturition (day 0). Open symbols represent values below the sensitivity of the assays.
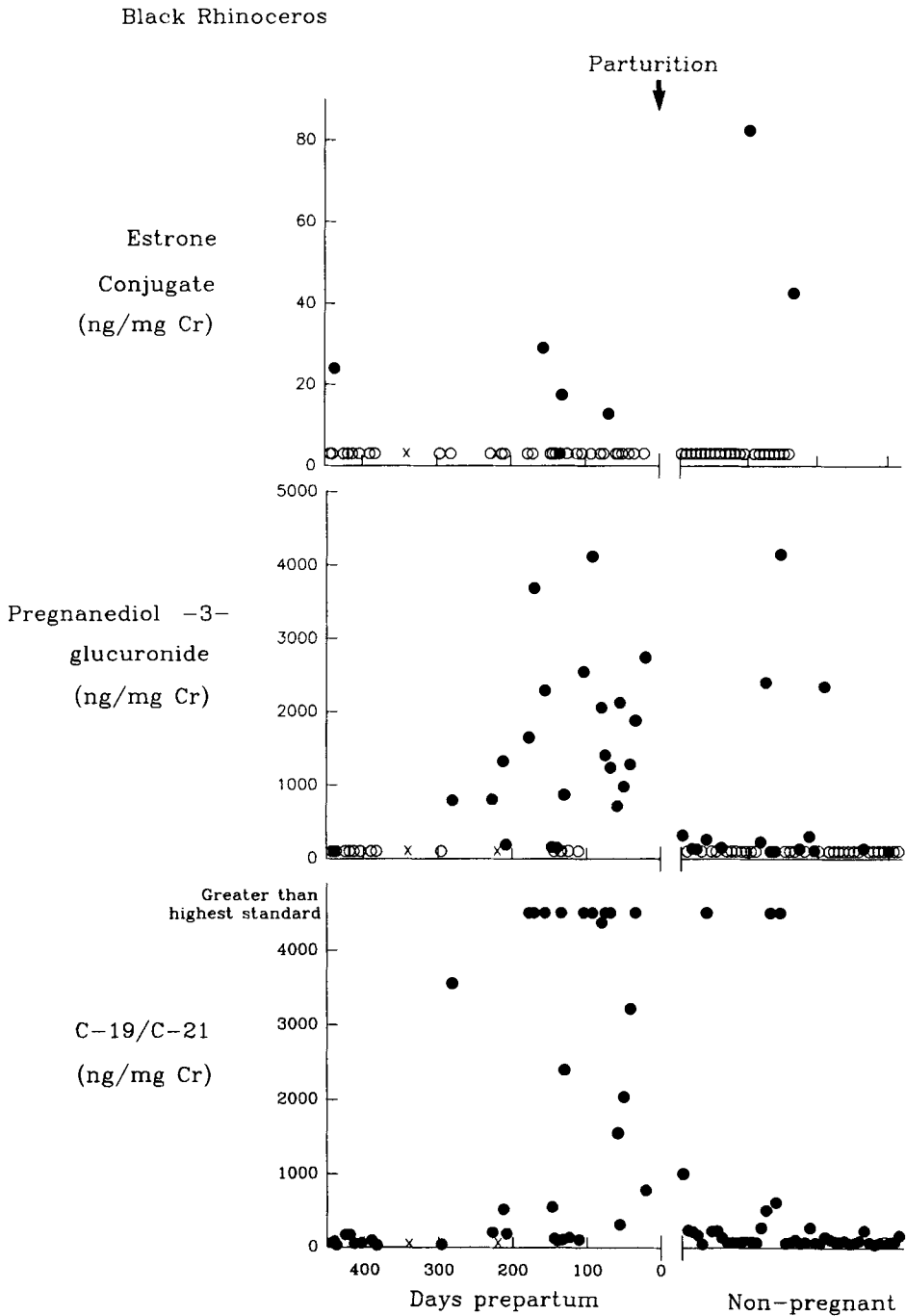
Fig. 6. Urinary estrone conjugate, pregnanediol-3-glucuronide, and C-19/C-21 progesterone metabolite equivalent values in pregnant and non-pregnant black rhinoceros as measured by enzymeimmunoassays. All pregnancies are normalized to the date of parturition (day 0). The dates for 2 samples (x) are estimated based on date of abortion and maturity of aborted fetus. Open circles (O) represent values below the sensitivity of the assays.

TABLE 4. Summary of the ability of an estrone conjugate (EC), a pregnanediol-3-glucuronide (PDG), and a non-specific progesterone metabolite (C-19/C-21) enzymeimmunoassay to identify nonpregnant and pregnant urine samples from black rhinoceros*

Species/hormone EIA (discrimination criteria)	Nonpregnant[a]	Pregnant[b]	
		At any time during pregnancy	Within 300 days of parturition
Black rhinoceros			
Estrone conjugates (any measurable amt of EC equivalents)	44/46 (96%)	4/37 (11%)	4/28 (14%)
PDG (any measurable amt of PDG equivalents)	32/46 (78%)	22/37 (59%)	22/28 (75%)
(equivalent to 500 ng/mg Cr)	43/46 (93%)	20/37 (53%)	20/28 (71%)
C-19/C-21 (equivalent to 200 ng/mg Cr)	33/46 (72%)	21/37 (57%)	21/28 (75%)
(equivalent to 500 ng/mg Cr)	40/46 (87%)	19/37 (51%)	18/28 (64%)

*Using several discrimination criteria.
[a]Number of urine samples containing less hormone metabolites than the discrimination criteria/the number of samples analyzed (specificity of the assay).
[b]Number of urine samples containing amounts of hormone metabolites greater than or equal to the discrimination criteria/the number of samples analyzed (sensitivity of the assay).

tion between values produced by these two techniques [Czekala et al., 1986; Hodges and Green, 1989; Shideler et al., 1993].

The EC EIA results of the present study for members of the family Equidae generally agree with previous studies of urinary EC excretion using RIA in these species [Kirkpatrick et al., 1988; Czekala et al., 1990]. These studies indicated low urinary estrone sulfate concentrations in nonpregnant mares and elevated levels in urines from females that foaled. In the equid species evaluated in the present report, the EC EIA had a specificity (the percentage of nonpregnant samples identified correctly) of 100%. The EC EIA demonstrated a sensitivity (the number of pregnant samples identified correctly) of ≥88% for identifying urines from equids in the second and third trimester of pregnancy.

Given the variation in progesterone metabolism among species, it is not surprising that the C-19/C-21 EIA was both more specific and sensitive in identifying nonpregnant and pregnant nondomestic equids than the PDG EIA. Loskutoff et al. [1983] measured low concentrations of PDG in a zebra species, compared to a variety of artiodactyls and primates. Kirkpatrick et al. [1990] showed that neither the horse nor zebra produce significant quantities of PDG, as measured by EIA, during luteal phases, but ovarian progesterone production could be monitored with a nonspecific progesterone metabolite EIA. The ability of the C-19/C-21 EIA to identify all pregnant Przewalski's horses, Grant's zebras, and Hartman's mountain zebras in the second and third trimester is encouraging and only slightly mitigated by the one nonpregnant Przewalski's horse sample with an elevated amount of C-19/C-21 me-

tabolites. This sample may represent luteal activity in this animal. The C-19/C-21 values for the Przewalski's horse, Grant's zebra, and Hartman's mountain zebra were generally similar. The Grevy's zebra was slightly different. Despite the use of a different discrimination criterion for the C-19/C-21 EIA, the sensitivity of this assay in the Grevy's zebra was only 82%, and most likely reflects subtle differences in progesterone metabolism in that species. From the data collected, it appears that evaluation of single urine samples by both the EC and C-19/C-21 EIAs would be the best method for diagnosis of pregnancy in female equids during the latter two thirds of gestation. Serial urine analyses using the C-19/C-21 may also be effective.

Results of the gonadotropin dipstick test in the Przewalski's horse and Grevy's zebra are similar to expected results in the domestic horse, where chorionic gonadotropin is measurable in the urine between days 40–120 of gestation (or days 215–295 prepartum) [Roberts, 1986]. Positive results were obtained from samples 253, 258, and 261 days preparturition in Przewalski's horses and days 300 and 311 preparturition in Grevy's zebras. Dipstick results were more variable in the Hartman's mountain zebra, with positives occurring throughout pregnancy and one positive observed in a nonpregnant sample. The latter may be the result of an ovulatory gonadotropin surge, but there is no clear explanation for the pattern of positive dipstick tests seen during pregnancy in the Hartman's mountain zebra. McFarlane and coworkers [1991c] have reported that chorionic gonadotropin-like material can be found in full-term placenta of horse and zebra, suggesting that there are cell types in the placenta that may be secreting CG throughout gestation. This CG may have been picked up by the dipstick assay throughout pregnancy in the Hartman's mountain zebra.

The positive dipstick results correlated 100% with elevated RIA values. However, in 39% of the samples, the RIA concentrations were elevated, whereas the dipstick tested negative, indicating that the dipstick EIA used in this study is not a reliable pregnancy test for these species. When using the dipstick assay for the horse, Roser and Lofstedt [1989] found that the test appeared more reliable when testing plasma than urine. They suggested two reasons for this phenomenon: (1) the assay is insensitive to low concentrations in very dilute urines, and (2) changes in the pH of the urine from day to day may interfere with the kinetics of the enzyme assay. In the present study, the gonadotropin-like molecule in the urine of these horse-related species is most likely slightly different from the eCG. Consequently, the double antibody sandwich EIA developed specifically for the eCG would not detect small amounts of a gonadotropin-like substance present in the urine of these species. Very little eCG, if any, could be detected in the urine of pregnant tapirs or rhinoceroses by EIA or RIA. Further testing is required on the plasma and urine of these animals, using more specific reagents, in order to fully evaluate the use of gonadotropin analysis for pregnancy diagnosis in these species.

Results from the present study differ slightly from a previous report on pregnancy monitoring in tapirs. Kasman et al. [1985] indicated estrone conjugates rose above baseline (20–25 ng/mg Cr) during the second and third trimesters of pregnancy in the Malayan and common tapir, with all pregnancy values remaining over 50 ng/mg Cr until parturition. Tapir urinary estrone conjugate concentrations determined by the EIA were generally lower than those measured by RIA and both the specificity and sensitivity of the EC EIA was much lower than expectations based on the results of the RIA study. In the Malayan tapir, the EC EIA identified 75% of the nonpregnant

animals and only 69% of the urine samples from pregnant animals. The overall sensitivity of the assay was severely affected by samples from one individual, which represented all low EC values found during pregnancy. The cause of this discrepancy is unclear, as other samples from the same individual, during the same gestation, contained amounts of EC above the discrimination criteria. Pooling data from all tapir species showed EC EIA to be a slightly more specific, but less sensitive, test for the family Tapiridae than for the Malayan species. The estrone conjugate EIA identified 80% of the nonpregnant animal urine samples but only 64% of the samples from pregnant females.

In tapirs, the profiles of urinary PDG and C-19/C-21 EIA data were similar. In the Malayan tapir, both the PDG and C-19/C-21 EIAs had a specificity of 88% but were only 63% and 75% sensitive, respectively, in accurately identifying pregnant females, using the chosen discrimination criteria. For all tapir species the C-19/C-21 EIA was 93% specific in identifying nonpregnant animals and 73% sensitive at identifying urine samples from pregnant individuals, using the equivalent of 500 ng/mg Cr as a discrimination criterion. The use of both the EC and C-19/C-21 EIAs on individual samples, or either assay on serial samples, may improve accuracy of pregnancy diagnosis. These should be evaluated on a greater number of tapir samples.

Estrone conjugate levels in black rhinoceros urines, as measured by EIA, showed similar, noninformative results as EC RIA analyses in that species [Ramsay et al., 1987]. Recently, Hindle et al. [1992] demonstrated that conjugated estrone was the major urinary estrogen in the nonpregnant black rhinoceros and that measurement of estrone in hydrolyzed urine samples best reflected ovarian follicular activity. As suggested by Hindle et al. [1992], it is possible the black rhino excretes a form of conjugated estrone not recognized by the EC EIA or RIA used in the present and previous studies. Evaluation of pregnant black rhinoceros urine samples for estrone may prove more informative and useful than analyses using the EC EIA.

In one previous study, a PDG RIA was shown to clearly indicate pregnancy in the black rhinoceros in the second and third trimesters of pregnancy [Ramsay et al., 1987]. Another study, using the PDG EIA in evaluating a single pregnancy each in a black and a white rhinoceros showed that PDG levels were elevated during mid to late pregnancy and dropped sharply at parturition or loss of the fetus [Hodges and Green, 1989]. Both studies validated their assays' identification of PDG in the urine of pregnant black rhinoceros. The results of the present study show the PDG EIA to be considerably less sensitive than the PDG RIA. Only 59% of the urine samples from pregnant animals contained measurable amounts of PDG. The accuracy of the PDG EIA was slightly better in identifying urines from females during the second and third trimester (75% sensitivity, using any measurable amount of PDG equivalents as the discrimination criteria), but was still much lower than the reported RIA sensitivity. The EIA demonstrated that once PDG equivalent levels rose, they remained elevated in each pregnant female, although values did not consistently rise throughout gestation in all animals. The reason for the discrepancy between the PDG EIA results and previous studies of PDG excretion during pregnancy in the black rhinoceros remains unclear, but it may reflect the difference in the functional sensitivity of the two assay systems.

The C-19/C-21 EIA demonstrated slightly less specificity and essentially the same sensitivity in distinguishing nonpregnant and pregnant black rhinoceros as the PDG EIA. Hindle et al. [1992] showed that a 20 alpha-hydroxyprogesterone EIA,

similar to the C-19/C-21 EIA, was useful in monitoring luteal activity in black and white rhinoceros. It would appear from that study and the present work that, similar to the domestic horse, progesterone metabolism differs considerably during luteal activity and pregnancy in the black rhinoceros. Only nonpregnant white rhinoceros samples were available for the present study, but EC, PDG, and C-19/C-21 equivalent concentrations in nonpregnant white rhinoceros urines are similar to metabolite equivalent concentrations seen in nonpregnant black rhinoceros. In summary, data suggest the C-19/C-21 and PDG EIA assays used in the present report do not appear accurate enough to be of use to managers of captive black rhinoceros for the diagnosis of pregnancy.

The effect on the samples of long-term storage and multiple freeze-thaw cycles may have affected the hormone measurements, especially for eCG. There are no reports however that storage or freeze-thaw cycles cause degradation of steroid conjugates or creatinine and previous studies, in the senior author's (B.L.L.) laboratory, using samples that have been repeatedly frozen and thawed do not indicate degradation of steroid conjugates. A study of the effects of handling on the eCG assays' accuracy would be useful.

The use of visually determined discrimination criteria was necessary in the present study due to the number of samples for which no numerical hormone metabolite concentration was determined. This is disturbing, given the small sample size and the lack of a clear bimodal distribution of pregnant and nonpregnant values for most EIAs and species. The number of samples with values that fell near the discrimination criteria reinforces observations made previously in evaluation of estrous cycle and pregnancy urinary hormone excretion in Indian (greater one-horned) and black rhinoceros, that because of individual animal variation, serial evaluations are essential for accurate assessment of reproductive status in some species [Kasman et al. 1985; Ramsay et al. 1986].

The present data demonstrate that, although it is not possible to generalize regarding hormone metabolism or excretion across species in the order Perissodactyla, specific simplified steroid metabolite urinalyses are of use in detecting and monitoring pregnancy in selected species. The EIAs were validated in only a few species, but certain patterns were easily discernible. Estrone appears to be a consistent urinary metabolite excreted in equids and tapirs, although their hormone excretion during pregnancy varies widely. Use of the EC EIA alone only appears useful for pregnancy diagnosis for the members of the family Equidae. The finding of chorionic gonadotropin activity, which can be monitored by eCG assays, in the urine of Przewalski's horses and zebra species is intriguing and invites further investigation of gonadotropin analyses as an alternate area for pregnancy detection in nondomestic equids.

The apparent differences in the metabolism and excretion of progesterone among the perissodactyl species, previously identified by others, is confirmed by the present study. The PDG EIA did not appear sufficiently accurate for use in pregnancy detection in any species. The C-19/C-21 EIA appears of value in pregnancy detection and monitoring in the equids, but its use is more limited in other perissodactyls. In tapir species, the C-19/C-21 EIA showed good specificity but poor sensitivity. Restructuring the C-19/C-21 EIA and evaluation of more samples from individual tapir species should be undertaken. Unfortunately, none of the EIA's used in this study proved sufficiently sensitive or specific for use in black rhinoceros species. The

present data underscore previous suggestions [Loskutoff et al., 1982] that caution should be exercised in applying progesterone metabolite assays to previously undefined species before they are used for management purposes.

ACKNOWLEDGMENTS

REFERENCES

Bahar, I. The development of over-the-counter(OTC) assays for pregnanediol-3-glucuronide and estrone -B,D glucuronide. Pp. 139–151 in NON-RADIOMETRIC ASSAYS: TECHNOLOGY AND APPLICATIONS IN POLYPEPTIDE AND STEROID HORMONE DETECTION. F. Haseltine; B.D. Albertson, eds. New York, Alan R. Liss, 1988.

Czekala, N.M.; Gallusser, S.; Meier, M.E.; Lasley, B.L. The development and application of an enzyme immunoassay for urinary estrone conjugates. ZOO BIOLOGY 5:1–16, 1986.

Czekala, N.M.; Kasman, L.H.; Allen, J.; Oosterhuis, J.; Lasley, B.L. Urinary steroid evaluations to monitor ovarian function in exotic ungulates: VI. Pregnancy detection in exotic Equidae. ZOO BIOLOGY 9:43–48, 1990.

Ensley, P.K.; Wing, A.E.; Gosink, B.L.; Lasley, B.L.; Durrant, B. Application of noninvasive techniques to monitor reproductive function in a brown hyena (*Hyena brunnea*). ZOO BIOLOGY 1:333–343, 1982.

Evans, K.L.; Kasman, L.H.; Hughes, J.P.; Couto, M.; Lasley, B.L. Pregnancy diagnosis in the domestic horse through direct urinary estrone conjugate analysis. THERIOGENOLOGY 22:615–620, 1984.

Grzimek, B. Tapir, P. 17 in GRZIMEK'S ANIMAL LIFE ENCYCLOPEDIA, Vol. 13. B. Grzimek, ed. New York, Van Nostrand, 1964.

Hindle, J.E.; Hodges, J.K. Metabolism of oestradiol-17 and progesterone in the white rhinoceros (*Ceratotherium simum simum*). JOURNAL OF REPRODUCTION AND FERTILITY 90:571–580, 1990.

Hindle, J.E.,; Mostl, E.; Hodges, J.K. Measurement of urinary oestrogens and 20-dihydroxyprogesterone during the ovarian cycle of black

(*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros. JOURNAL OF REPRODUCTION AND FERTILITY 94:237–249, 1992.

Hodges, J.K.; Green, D.I. The development of an enzyme-immunoassay for urinary pregnanediol-3-glucuronide and its application to reproductive assessment in exotic mammals. JOURNAL OF THE ZOOLOGICAL SOCIETY OF LONDON 219:89–99, 1989.

Hodges, J.K.; Gulick, B.A.; Czekala, N.M.; Lasley, B.L. Comparison of estrogen excretion in South American primates. JOURNAL OF REPRODUCTION AND FERTILITY 61:83–90, 1981.

Josimovich, J.B. Hormonal physiology of pregnancy: Steroid hormones of the placenta and polypeptide hormones of the pituitary. Pp. 147–149 in GYNOCOLOGIC ENDOCRINOLOGY. J.J. Gold; J.B. Josimovich, eds. New York, Harper & Row, 1980.

Kasman, L.H.; McCowan, B.; Lasley, B.L. Pregnancy detection in tapirs by direct urinary estrone sulfate analysis. ZOO BIOLOGY 4:301–306, 1985.

Kasman, L.H.; Ramsay, E.C.; Lasley, B.L. Urinary steroid evaluations to monitor ovarian function in exotic ungulates: III. Estrone sulfate and pregnanediol-3-glucuronide excretion in the Indian rhinoceros (*Rhinoceros unicornis*). ZOO BIOLOGY 5:355–361, 1986.

Kassam, A.A.H.; Lasley, B.L. Estrogen excretory patterns in the Indian rhinoceros (*Rhinoceros unicornis*), determined by simplified urinary analysis. AMERICAN JOURNAL OF VETERINARY RESEARCH 2:251–255, 1981.

Kirkpatrick, J.F.; Lasley, B.L.; Shideler, S.E. Urinary steroid evaluations to monitor ovarian function in exotic ungulates: VII. Urinary pro-

gesterone metabolites in the equidae assessed by immunoassay. ZOO BIOLOGY 9:341–348, 1990.

Kirkpatrick, J.F.; Kasman, L.H.; Lasley, B.L.; Turner, J.W.Jr. Pregnancy determined in uncaptured feral horses. JOURNAL OF WILDLIFE MANAGEMENT 52:305–308, 1988.

Kirkpatrick, J.F.; Lasley, B.L.; Shideler, S.E.; Roser, J.F.; Turner, J.W. Noninstrumented immunoassay field test for pregnancy detection in free roaming horses. JOURNAL OF MANAGEMENT 57:168–173, 1993.

Lang, E.M. Rhinoceros. P. 36 in GRZIMEK'S ANIMAL LIFE ENCYCLOPEDIA, Vol. 13. B. Grzimek, ed. New York, Van Nostrand, 1964.

Loskutoff, N.M.; Ott, J.E.; Lasley, B.L. Urinary steroid evaluations to monitor ovarian function in exotic ungulates. I. Pregnanediol-3-glucuronide immunoreactivity in the okapi (*Okapia johnstoni*). ZOO BIOLOGY 1:45–53, 1982.

Loskutoff, N.M.; Ott, J.E.; Lasley, B.L. Strategies for assessing ovarian function in exotic species. JOURNAL OF ZOO ANIMAL MEDICINE 14:3–12, 1983.

Loskutoff, N.M.; Walker, J.E.; Ott-Joslin, J.E.; Raphael, B.L.; Lasley, B.L. Urinary steroid evaluations to monitor ovarian function in exotic ungulates: II. Comparison between the giraffe (*Giraffe camelopardalis*) and the okapi (*Okapia johnstoni*). ZOO BIOLOGY 5:331–338, 1986.

McFarlane, J.R.; Czekala, N.M.; Papkoff, H. Zebra chorionic gonadotropin: Partial purification and characterization. BIOLOGY OF REPRODUCTION 44:827–833, 1991a.

McFarlane, J.R., Coulson, S.A. and Papkoff, H. Biological and immunoactive substances resembling chorionic gonadotropin are present in fullterm horse and zebra placentas. BIOLOGY OF REPRODUCTION 45:353–349, 1991c.

McFarlane, J.R.; Cabrera, C.M.; Coulson, S.A.; Papkoff, H. Partial purification and characterization of rhinoceros gonadotropins, growth hormone and prolactin: comparison with the horse and sheep. BIOLOGY OF REPRODUCTION 44:94–101, 1991b.

Mainka, A.A.; Cooper, R.M.; Mao, L.; Guanlu, Z. Urinary hormones in two juvenile female giant pandas (*Ailuropoda melanoleuca*). JOURNAL OF ZOO AND WILDLIFE MEDICINE 21:334–341, 1990.

Masters, A.M.; Markham, R.J. Assessing reproductive status in orang-utans by urinary estrone. ZOO BIOLOGY 10:197–207, 1991.

Mitchell, W.R.; Presley, S.; Czekala, N.M.; Lasley, B.L. Urinary immunoreactive estrogen and PdG during the normal menstrual cycle of the female gorilla (*Gorilla gorilla*). AMERICAN JOURNAL OF PRIMATOLOGY 2:167–175, 1982.

Munro, C.J.; Stabenfeldt, G.H.; Cragen, J.R.; Addiego, L.A.; Overstreet, J.W., Lasley, B.L. Re-

lationship of serum estradiol and progesterone concentrations to the excretion profiles of their major metabolites as measured by enzyme immunoassay and radioimmunoassay. CLINICAL CHEMISTRY 37:838–844, 1991.

Paul-Murphy, J.; Tell, L.A.; Bravo, W.; Fowler, M.E.; Lasley, B.L. Urinary steroid evaluations to monitor ovarian function in exotic ungulates: VIII. Correspondence of urinary and plasma steroids in the llama (*Lama gama*) during non-conceptive cycles and conceptive cycles. ZOO BIOLOGY 10:225–236, 1991.

Ramsay, E.C.; Kasman, L.H.; Lasley, B.L. Urinary steroid evaluations to monitor ovarian function in exotic ungulates: V. Estrogen and pregnanediol-3-glucuronide excretion in the black rhinoceros (*Diceros bicornis*). ZOO BIOLOGY 6:275–282, 1987.

Roberts, S.J. Gestation and pregnancy diagnosis in the mare. Pp. 670–678 in CURRENT THERAPY IN THERIOGENOLOGY 2. D.A. Morrow, ed. Philadelphia, W.B. Saunders, 1986.

Roser, J.F.; Lofstedt, R.M. Urinary eCG patterns in the mare during pregnancy. THERIOGENOLOGY 607–622, 1989.

Shideler, S.E.; Mitchell, W.R.; Lindburg, D.G.; Lasley, B.L. Monitoring luteal function in the lion-tailed macaque (*Macaca silenus*) through urinary progesterone metabolite measurements. ZOO BIOLOGY 4:65–73, 1985.

Shideler, S.E.; Moran, F.M.; Lohstrah, P.N.; Lasley, B.L. Enzyme immunoassays for ovarian steroid metabolites in urine of laboratory macaques. JOURNAL OF MEDICAL PRIMATOLOGY, 22:301–312, 1993.

Shideler, S.E.; Czekala, N.M; Kasman, L.H.; Lindburg, D.G.; Lasley, B.L. Monitoring ovulation and implantation in the lion-tailed macaque (*Macaca silenus*) through urinary estrone conjugate evaluations. BIOLOGY OF REPRODUCTION 29:905–911, 1983.

Taussky, A.H. A microcolorimetric determination of creatinine in urine by the Jaffe reaction. JOURNAL OF BIOLOGY AND CHEMISTRY 208:853–861, 1954.

Volf, J. Equines. P. 539 in GRZIMEK'S ANIMAL LIFE ENCYCLOPEDIA, Vol. 12. B. Grzimek, ed. New York, Van Nostrand, 1964.

Walker, L.A.; Cornell, L.; Dahl, K.D.; Czekala, N.M.; Dargen, C.M.; Joseph, B.; Hsueh, A.J.; Lasley, B.L. Urinary concentrations of ovarian steroid hormone metabolites and bioactive follicle-stimulating hormone in killer whales (*Orcinus orchus*) during ovarian cycles and pregnancy. BIOLOGY OF REPRODUCTION 39:1013–1020, 1988.

Woodard, D.K.; Graham, C.E.; McClure, H.M. Comparison of hemagglutination inhibition pregnancy tests in the chimpanzee and the orangutan. LABORATORY ANIMAL SCIENCE 26:922–927, 1976.