Relaxin Concentrations in Serum and Urine of Endangered Species

Correlations with Physiologic Events and Use As a Marker of Pregnancy

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ABSTRACT: Many mammalian species are facing extinction due to problems created by human encroachment, agriculture, pollution, and willful slaughter. Among those at risk are the Asian and African elephant, Sumatran rhinoceros, and giant panda. Conservation groups try to save species in the wild by preserving habitat and limiting animal-human conflicts, often with limited success. Another alternative is to preserve the extant gene pool through captive breeding as a hedge against extinction. Measurement of circulating reproductive hormones is impractical for most wildlife species; determination of urinary or fecal hormone metabolites provides a more viable approach. To aid breeding management, one important tool is the ability to diagnose and monitor pregnancy, especially in species with long gestations (e.g., rhinos over 15 mo and elephants over 20 mo). Unfortunately, measuring progestins often is not useful diagnostically, because concentrations are similar during at least part of the pregnancy and the nonpregnant luteal phase in some species (e.g., elephants, rhinoceroses, and giant pandas). As serum relaxin reliably distinguishes between pregnancy and pseudopregnancy in bitches, relaxin measurement might also provide a method for detecting a successful pregnancy in endangered species. Appropriate immunoassay reagents have enabled the estimation of relaxin concentrations in the serum of elephants and rhinos and the determination of pregnancy establishment and the outcome. Relaxin was also detected in panda serum and urine. However, the extreme variability of the time between observed mating and parturition and the confounding factors of

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delayed implantation, pseudopregnancy, and frequent fetal resorptions made it impossible to use the panda relaxin data as a specific marker of pregnancy.

KEYWORDS: relaxin; endangered species; elephant; rhinoceros; giant panda; pregnancy

INTRODUCTION

The human population explosion, while rapidly depleting the world's natural resources, straining the food supply, and polluting the environment (land, sea, and air), is also exacting a terrible toll on the earth's other creatures, pushing many towards extinction. This is being accomplished by encroachment and/or pollution of their habitats as well as by wanton killing for food, hides, fur, traditional medicines, or trophies.

The current communication is one aspect of the researchers' attempts to conserve four endangered mammalian species by providing a better understanding of their reproductive processes and, specifically, by testing the utility of a relaxin immunoassay to detect viable pregnancies. The reasons for choosing relaxin as a potential marker of pregnancy are as follows: Elephants and rhinos have extremely long pregnancies, and giant pandas are monestrus, have periods of pseudopregnancy, and exhibit variably delayed implantation. Thus, conservationists urgently need a reliable tool to determine if mating or insemination has resulted in a true pregnancy in these species, so that years are not wasted on false premises. We previously discovered that relaxin is a marker of pregnancy in the labrador retriever bitch[1] and can discriminate between true pregnancy (elevated relaxin) and pseudopregnancy (no detectable relaxin). By contrast, progesterone is elevated during both true and false pregnancies at concentrations that are indistiguishable.[1]

The species of this study are the giant panda (*Ailuropoda melanoleuca*), Sumatran rhinoceros (*Dicerorhinus sumatrensis*), African elephant (*Loxodonta Africana*), and Asian elephant (*Elephas maximus*). At this writing, approximately 1,000 giant pandas remain in the bamboo forests of China, with an additional 100 or so in zoos and refuges around the world.[2] The Sumatran rhino population is even closer to extinction, with about 300 remaining in the wild and only 9 in captivity.[3] The Asian and African elephants are the only surviving species of the family Elephantidae, the two having diverged into separate species five million years ago. While the threat to the elephants is not as dire as that to the pandas and Sumatran rhinos, nonetheless the populations of each species have been steadily diminishing. There are now in the wild only 300–500,000 African elephants and about 35–50,000 Asian elephants, with an additional 16,000 Asian elephants in "domestication" (in circuses, zoos, timber camps, etc.).[4,5]

REPRODUCTIVE TRAITS

Giant Panda

The giant panda is endemic to the mountains of Sichuan, Gansu, and Shanxi Provinces in China. The species is now found in only six mountain ranges at the eastern edge of the Tibetan plateau, distributed in as many as 30–40 distinctive populations. More than half of their habitat was destroyed in the 1970s and 1980s, resulting in a reduction in the giant panda ecospace to about 5,000 square miles, which is less than *25%* of the size of the Greater Yellowstone Ecosystem.[6] While there is currently a ban on hunting giant pandas or capturing them for export to zoos, the officially protected areas (currently approximately 40 reserves) are severely under-resourced, lacking the personnel and equipment to properly attend to daily and routine activities, let alone conservation priorities. Moreover, not all giant pandas live in protected areas. There is also a growing concern over panda-human competition for wild bamboo, including shoots (a dietary favorite of both species) and stems that have many uses by people ranging from basket weaving to tools to fencing. A final threat to giant pandas in nature is the lack of broad-based knowledge about their complex reproductive biology and numbers in nature. Rather, most of what we know about the giant panda comes from studies done on captive individuals.

Unlike other bears, giant pandas are monestrus, exhibiting sexual receptivity only once per year for only 2–3 consecutive days in the late winter/early spring.[7–9] Male pandas produce copious numbers of motile spermatozoa during this period to facilitate successful breeding as soon as the female becomes receptive. If conception occurs, the embryos remain free-floating in the uterine horns for an indeterminate interval until implantation occurs (delayed implantation). Successful fertilization results in the birth of one or two small, comparatively immature cubs, one of which is usually rejected by the mother and dies soon after birth.

Reproductive events in the giant female panda can be monitored using non-invasive hormone analysis techniques. Urinary estrogen concentrations in the female increase approximately 8 d before mating, reach a maximum during the proceptive period, and then decrease during the period of receptivity.[10,11] Pheromones produced by the anogenital glands of both sexes are detectable in urine and appear to play an important role in coordinating mating behavior.[12] In captivity, successful reproduction depends on the ability of animal managers to detect estrous for the accurate timing of pairing with a male or attempting artificial insemination.

Sumatran Rhinoceros

The Sumatran rhinooceros is one of the most endangered mammals on earth, with probably fewer than 300 currently extant in the wilds of Sumatra, peninsular Malasia, and Borneo.[13] Because Sumatran rhinos are close to extinction, largely due to poaching and, to a lesser extent, habitat destruction, a formal captive breeding program was established in the mid-1980s. Forty animals were brought into zoos and captive breeding centers in the United States, Europe, Malaysia, and Indonesia.[13,14] Unfortunately, illness and breeding difficulties reduced the captive population by more than 50% by the late 1990s.[13] Recently, a tragic disease outbreak in Malaysia reduced the captive population to fewer than 10 animals.[15]

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Sumatran rhinos are difficult to breed because of aggressiveness and hostility towards prospective mates. Females do not exhibit reliable behavioral signs of estrus, and introduction of males when the female is not receptive sometimes results in serious injuries.[13] Recent studies involving measurement of reproductive hormones together with ultrasound examinations have increased the understanding of Sumatran rhino reproductive physiology. The discovery that the female rhinoceros appears to be an induced ovulator with a unique progesterone secretion pattern was instrumental in the development of a reliable method for determining the optimal time for mating.[16] However, repeated early pregnancy losses hindered achieving a term live birth, despite many successful matings.[16] In some cases, progesterone levels remained elevated several weeks after embryonic loss was confirmed by ultrasound, suggesting that elevated progesterone levels are not always indicative of a healthy, progressing pregnancy.[16] In 2001, a female successfully carried to term and delivered a healthy calf after receiving a synthetic progestin supplement (altrenogest) throughout gestation.[3] That same female produced her second calf in 2004, this time after a pregnancy carried to term naturally without supplemental progesterone.[17]

Asian and African Elephants

Asian (*Elephas maximas*) and African (*Loxodonta africana*) elephants are the only surviving species of the family Elephantidae, the two having diverged into separate species 5 million years ago. Both species inhabited Africa during the Pleistocene age; African elephants were located in the forests of Central Africa, while Asian elephants lived in the more arid regions. *Loxodonta* became the dominant elephant of Africa only after *Elephas* migrated to Eurasia.[4] Today, the few remaining *E. maximus* exist among the dwindling forests of Southeast Asia, a result of massive habitat destruction. *L. africana* is only found south of the Sahara Desert, and much of the population is fragmented because of human encroachment.

Both species of elephant have a 13–16-wk ovarian cycle[18–20] with two luteinizing hormone surges that occur 3 wk apart during the interluteal phase.[21,22] Gestation lasts 20–22 mo[19,23-25] and is characterized by increases in prolactin[25,26] after the first third of gestation. The primary luteal steroid in circulation is not progesterone, but 5α-reduced pregnanes.[27–30] However, many progesterone assays are capable of measuring this immunoactivity.[31]

Because neither progestins nor prolactin serve as reliable markers of early pregnancy in these four species (e.g., prolactin is diagnostic in the elephant, but not until after 7 mo. of gestation), the goal of the present work was to examine the utility of radioimmunoassays (RIAs) for relaxin to diagnose early pregnancy status through measurements of circulating and/or excreted hormone.

MATERIAL AND METHODS

Radioimmunoassays

Porcine Relaxin Radioimmunoassay.[32] The porcine relaxin RIA was used for measurement of immunoactive relaxin in 425 giant panda urine samples, 36 panda serum samples, and 33 Sumatran rhino serum samples. The panda samples were from collections at the Smithsonian National Zoological Park and the Zoological Society of San Diego. The Sumatran rhino samples were provided by the Cincinnati Zoo. The RIA employed rabbit anti-porcine relaxin antiserum (R6), ^{125}I-labeled mono-tyrosyl porcine relaxin (a gift from Dr. C. Schwabe, Medical University of South Carolina) as tracer, and purified porcine relaxin as standards. The RIA was conducted according to the method of O'Byrne and Steinetz,[32] except that incubation was shortened to 24 h and the antigen-antibody complex was precipitated by the addition of ice-cold goat anti-rabbit antiserum in 5% polyethylene glycol, as described by Roth *et al.*[3] Displacement of ^{125}I-labeled porcine relaxin was observed with milk and serum from pregnant rhinos and serum and urine from pregnant pandas, but not from male or nonpregnant female rhinos or pandas. Dilutions of the immunoactive substances in panda and rhino body fluids were parallel to porcine relaxin standards in the RIA, and these substances were additive with the porcine relaxin standards.

Equine Relaxin Radioimmunoassay.[33] The equine relaxin RIA was used to measure relaxin in the serum of Asian and African elephants. A total of 538 Asian and African elephant samples were made available from collections at the Smithsonian National Zoo (Meyer *et al.*[34]). The equine relaxin RIA had previously been validated for Asian elephants by Niemuller *et al.*[35] The reagents for the current study were kindly provided by Dr. Dennis Stewart. These included rabbit anti-equine relaxin antiserum (#3150) and highly purified equine relaxin for standards and ^{125}I labeling. Displacement of radiolabeled equine relaxin by serial dilutions of pregnant Asian and African elephant serum samples was parallel to and additive with equine relaxin standards. Limited displacement was observed with serum of males or nonpregnant females. The RIA was conducted according to the original protocol except that incubation was shortened to 24 h and the antigen-antibody complex was precipitated by the addition of ice-cold goat anti-rabbit antiserum in 5% polyethylene glycol, as described by Meyer *et al.*[34]

Other Hormones

Data for prolactin, progesterone/progestin, and estrogen concentrations in body fluids are taken from the original papers and are cited in the figures.

RESULTS AND DISCUSSION

Giant Panda

FIGURE 1 describes the reproductive cycle of the female panda. FIGURE 1A illustrates the urinary/fecal estrogen and progestin pattern during the normal estrus cycle with an obligatory pseudopregnancy. FIGURE 1B illustrates the steroid pattern during the variably delayed implantation in a successful pregnancy. Samples of urine and serum were obtained from female and male giant pandas during the mating season in the spring. Significant relaxin immunoactivity was detected in the few available serum samples from a pregnant panda (especially those obtained 3 wk or less before delivery). In eight of these samples, immunoactivity ranged from 7–12 ng porcine relaxin equivalents per mL. By contrast, no relaxin activity was found in the serum of nonpregnant females ($n = 8$) or in five of six samples from male pandas. The finding of immunoactive relaxin in one serum sample from a male panda may be significant given that in other species, like the boar and the male bonnethead shark, circulating relaxin is present during the reproductive season. This finding requires further investigation.

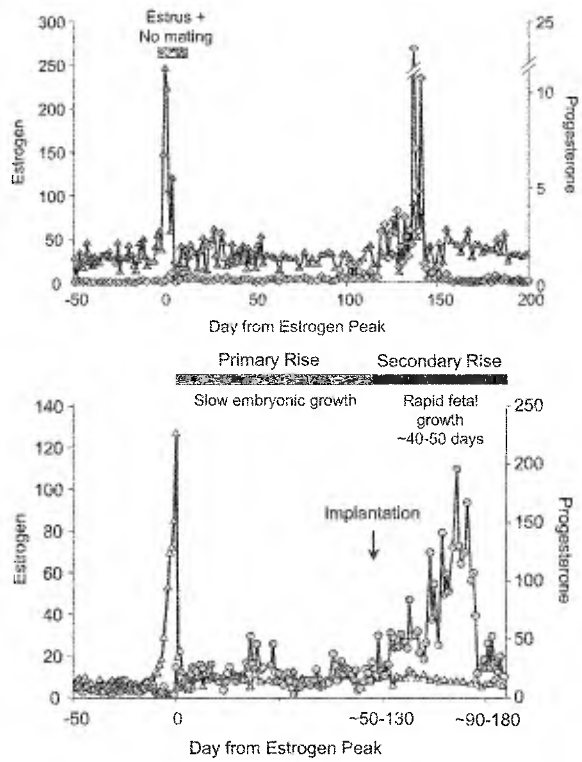

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FIGURE 1. Patterns of fecal estrogen and progesterone excretion in a giant panda. (**A**) The single yearly estrous cycle with its obligate pseudopregnancy. (**B**) The phenomenon of delayed implantation. Implantation can occur anywhere from 50–130 d after mating, resulting in a highly variable time of parturition (90–180 d postmating). (*Triangles*, fecal estrogens; *diamonds* or *circles*, fecal progestins.) Data from Kersey *et al.*[36]

A total of 450 urine samples were analyzed in the porcine relaxin RIA and represented 5 pregnancies, 5 pseudopregnancies, and 1 nonovulatory cycle (i.e., no pseudopregnancies). In general, concentrations of relaxin in most of the urine samples were low or undetectable, and because of the small volumes, it was not possible to concentrate samples by acetone precipitation. It was possible, however, to assay relaxin immunoactivity directly in urine volumes up to 300 mL without matrix interference problems, and added porcine standards were linear.

Concentrations of relaxin in urine samples during pregnancy spiked at around 42 d before delivery (i.e., around the time of implantation), with additional spikes observed during the last 10 d of pregnancy (FIG. 1B, for orientation). These spikes reached concentrations of 20–30 ng porcine relaxin equivalents/mL, although most samples were in the 0.5–5 ng/mL range. However, because many of these samples had to be pooled to provide sufficient volume, precise patterns were difficult to interpret. Furthermore, the relaxin increases invariably occurred in the form of spikes, not as consistently prolonged elevations during these periods. Therefore, for relaxin to be used as a diagnostic tool for pregnancy, longitudinal samples need to be collected and at a nearly daily sampling regimen to pick up these spikes. Single point in time sampling would not be effective. Data on the mated pandas that did not give birth (i.e., pseudopregnant) were even more difficult to interpret, because there also were a few random relaxin spikes, although not as many as those observed in the pregnant females. For example, in one of the pseudopregnant pandas there was a relaxin spike 46 d before the end of "pseudopregnancy," but no relaxin near term. Another mated pseudopregnant panda exhibited spikes of relaxin about 40 d before anticipated delivery, but none thereafter. In some analyses of pooled pregnancy urine from 3 wk prior to delivery to term, large increases in relaxin concentration were found. Thus, it is not clear if these were true nonpregnancies or pregnancies that failed at an unknown stage. In the one unmated panda, the relaxin concentrations remained consistently low, and only a few elevations could be considered spikes. It is difficult to obtain urine sets from unmated pandas to study this further, because most facilities are actively trying to breed all captive pandas (in both the U.S. and China). It would be of interest, however, to determine the role of the corpus luteum (pregnant or nonpregnant) in the production of immunoactive relaxin in both mated and nonmated pandas. The final problem relates to the phenomenon of variably delayed implantation (FIG. 1B). Currently, there is no dependable way to estimate the time of implantation, and we lack reliable evidence to suggest that the implanting embryo actually initiates relaxin secretion (as it does in the dog, for example).

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In conclusion, it may be possible to use serum relaxin as a pregnancy diagnostic tool, although more samples need to be analyzed to confirm this, and there are few facilities that collect blood from pandas. A urinary test would certainly be more useful for pandas, but it does not appear at the present time that urine relaxin assays can be used as reliable markers of pregnancy in this species.

Sumatran Rhinoceros

The porcine relaxin RIA worked well with rhino serum, and the assay validation for pregnant rhino serum was straightforward. The data for pregnant rhino "Emi" are shown in FIGURE 2. Although relaxin inmmunoactivity clearly increased during pregnancy, a significant increase over baseline was not detected until after 6 mo of gestation. Thereafter, relaxin concentrations plateaued in the range of 2–5 ng/mL for an additional 8 mo before rising to a large peak near 1 µg/mL porcine equivalents 2 wk prior to parturition. Serum immunoactive relaxin was still elevated 4 d postpartum and was also detected in the milk (about 6 ng/mL).

Clearly, relaxin is a marker of pregnancy after midgestation in the Sumatran rhinoceros, and it appears to be much more useful than serum prolactin, which was not elevated until just before parturition (FIG. 2).

Asian and African Elephants

Niemuller *et al.*[35] originally reported that the rabbit anti-equine relaxin antibody (#3150 of Dr. Dennis Stewart) was capable of diagnosing pregnancy in the Asian elephant. Our results confirmed that finding and also showed that the same antibody was capable of monitoring increases in relaxin in pregnant African elephants.

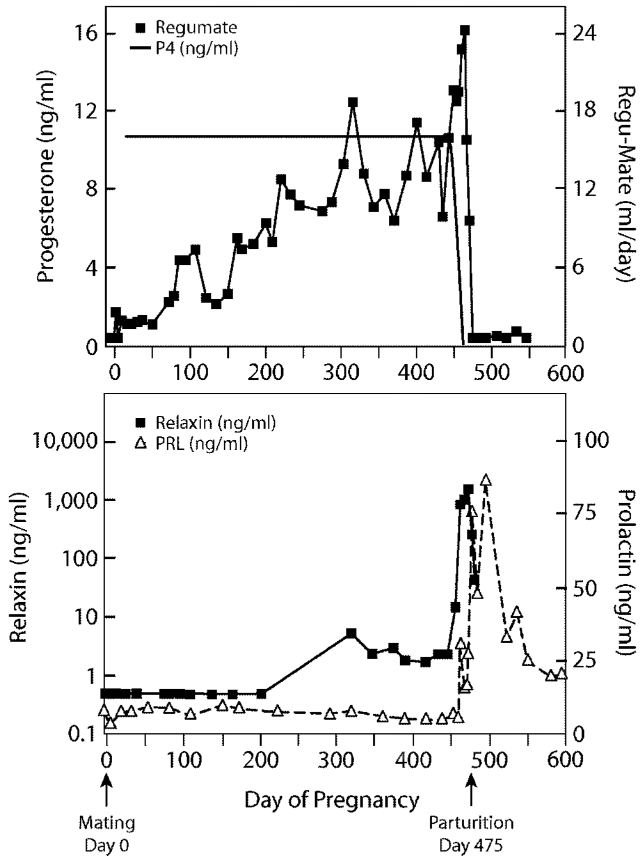

FIGURE 2. Patterns of progesterone, prolactin, and relaxin during Regumate®–maintained pregnancy in a Sumatran rhinoceros. *Top panel* illustrates the concentration of serum progesterone (P4) throughout pregnancy. Regumate, a synthetic progestin (altrenogest, Intervet, Inc, Millsboro, DE) was administered as a precautionary measure because this female had a history of spontaneous abortions. *Bottom panel* illustrates concentrations of immunoactive relaxin and prolactin (PRL) during pregnancy and after parturition. Relaxin concentrations are in equivalents of porcine relaxin. From Roth *et al.*[3]

Recently, Meyer *et al.*[34] compared concentrations of progesterone, prolactin, and relaxin during gestation in Asian and African elephants. Although there was no difference (P >0.05) in overall mean progestin concentrations between Asian and African elephants, a significant difference was noted in the temporal profiles when data were compared during months 5–12 (P <0.05) and months 16–22 (P <0.05) (FIG. 3A). Serum progestins were higher in African elephants during the first half of pregnancy, whereas progestin levels were higher in Asian elephants during the latter half. Profiles for Asian elephants tended to be biphasic, with a rise followed by a plateau and then a decline in progestin concentrations (FIG. 3A). By contrast, in African elephants, after the initial rise in progestins, serum levels declined in mid-

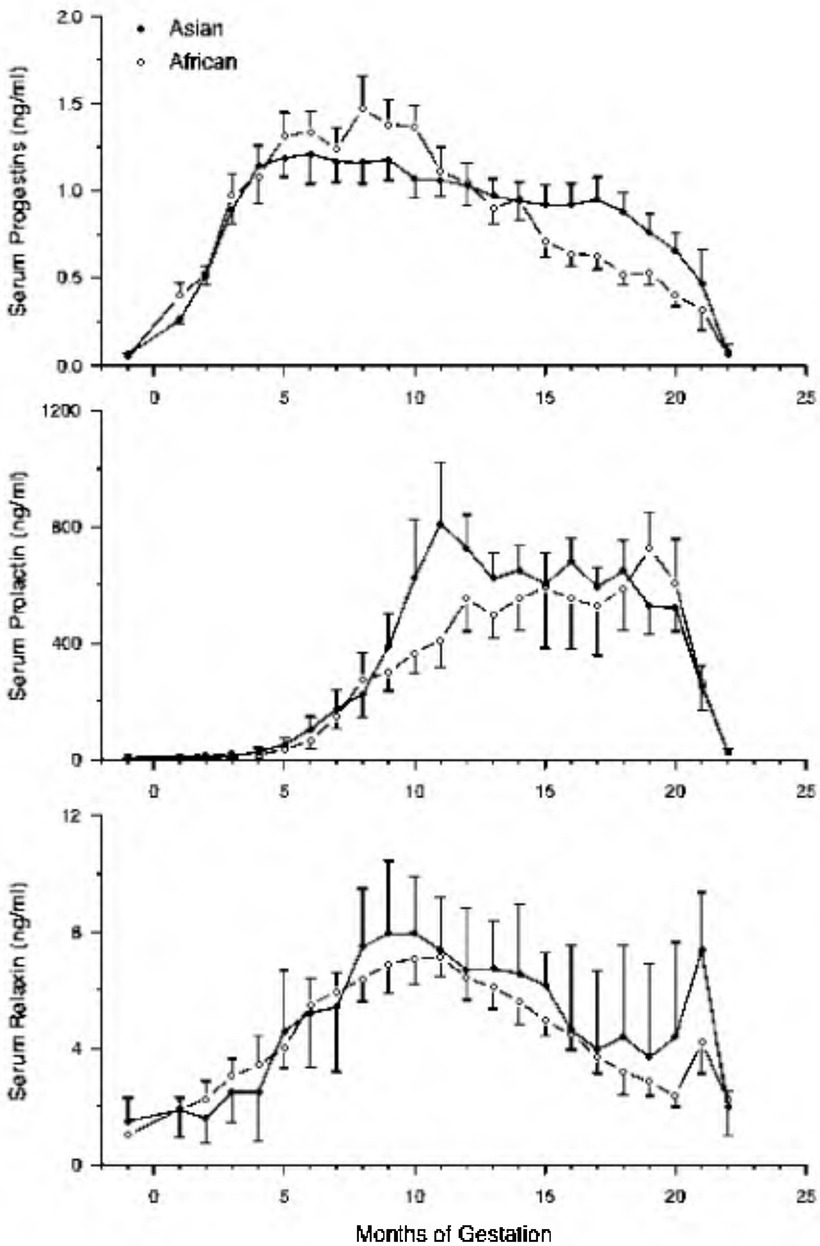

FIGURE 3. Mean (± SEM) profiles of serum progestins (**A**), prolactin (**B**), and relaxin (**C**) in Asian (n = 19) and African (n = 8) elephants throughout gestation. Relaxin concentrations are in equivalents of equine relaxin. From Meyer *et al.*[34]

pregnancy and were lower at the end of pregnancy than at the beginning. In both species of elephant, prolactin concentrations remained low for the first 5 mo of pregnancy and then began to rise between months 6 and 7 (FIG. 3B). After 8 mo, serum prolactin levels were higher in Asian than in African elephants ($P < 0.05$) until approximately 15 mo of gestation (FIG. 3B).

Serum relaxin patterns were similar during pregnancy in Asian and African elephants (FIG. 3C). Relaxin increased within the first 3–5 mo, peaked after about 10 mo of pregnancy, and then declined through the twentieth month. A final increase in serum relaxin occurred in the month preceding parturition. The prolonged elevated secretion of relaxin in these elephant species is consistent with that observed in the horse, dog, and cat.[37] Thus, RIA of serum relaxin offers a reliable means of diagnosing pregnancy after mating in both species of elephants, its rise being detectable well before any increase in serum prolactin concentration.

CONCLUSIONS

In conclusion, measurement of serum relaxin provides a reliable "pregnancy test" for the Sumatran rhinoceros, and the Asian and African elephant after 7–10 mo and 3–5 mo of gestation, respectively. Attempts to use relaxin radioimmunoassays on serum or urine to determine pregnancy in giant pandas were not successful, largely because of the peculiar reproductive cycle of this animal. However, the fact that relaxin can be measured at all in body fluids of pandas lends encouragement for further work to combine relaxin determination with that of other pregnancy-associated hormones to eventually provide a reliable pregnancy test for this species.

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