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RESEARCH ARTICLE

Sexual Maturation in the Sumatran Rhinoceros (*Dicerorhinus sumatrensis*)

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To help save the Sumatran rhino from extinction, the captive breeding program must capitalize on each rhino's reproductive lifespan. Doing so requires knowing when calves are sexually mature. The goal of this study was to monitor physiological changes associated with sexual maturation in two captive born calves (one male and one female) to determine the approximate age of maturity for both sexes of this species. Fecal testosterone metabolites were monitored in the male calf from 6 months to 7 years of age, and fecal pregnane metabolites were measured in the female calf from 6 months to 5.5 years of age. In addition, rectal ultrasonography was employed to monitor changes in ovarian activity from 2 to 5.5 years of age. The male calf's fecal testosterone concentrations reached levels comparable to those detected in samples from adult males when he was 6–6.5 years of age. The first pre-ovulatory sized follicle was observed on the ovaries of the female calf when she was 4.75 years old, but fecal pregnane metabolite concentrations only reached maximum mean concentrations and variability when she was 5–5.5 years of age. Results from this study indicate that male and female Sumatran rhino calves are sexually mature at 6–6.5 and 5–5.5 years of age, respectively. Zoo Biol. 32:549–555, 2013.

Keywords: testosterone; progesterone; pregnane; ultrasonography; follicle

INTRODUCTION

The critically endangered Sumatran rhino's future is questionable with a shrinking wild population currently estimated at fewer than estimated at 100 rhinos [http://www.rhinos.org/rhinos/sumatran-rhino]. Given the crisis facing the wild population and the recent success of the captive breeding effort (three calves produced at the Cincinnati Zoo and one calf produced in Sumatra), more conservationists are acknowledging that captive breeding may become a necessity to save this species. However, for captive breeding to be effective in reversing current population trends, it must be conducted aggressively to ensure that every rhino reproduces to capacity during its tenure in captivity. Knowing the age at which Sumatran rhinos achieve sexual maturation is essential for capitalizing on the reproductive potential of any calves produced in the breeding program.

Due to the challenges of studying this solitary, forest-dwelling species in the wild, there are no data available regarding age at first reproduction for wild Sumatran rhinos. However, the birth of male and female calves at the Cincinnati Zoo in 2001 and 2004, respectively, provided the perfect opportunity to study the physiological progression of sexual maturation in this species.

In most species, estrogen is the hormone of choice for studying the onset of follicular development on the ovaries of a young female. However, attempts to correlate fluctuations in Sumatran rhino fecal estrogen metabolite concentrations or

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serum estrogen with ovarian activity in a mature, naturally breeding female have failed to produce meaningful data [Roth et al., 2001; Roth, 2006]. In contrast, fecal progesterone metabolite analysis and serial rectal ultrasonography have been established as valuable methodologies for monitoring reproductive activity in this rhino species [Roth et al., 2001]. Although fecal testosterone metabolite analysis in the Sumatran rhino has not previously been reported, it has proven valuable in studying other rhino species [Brown et al., 2001] and therefore seemed likely to yield useful information. Fortunately, samples from two mature male Sumatran rhinos, one of which was a proven sire [Roth et al., 2004], were available to serve as controls.

The objective of this study was to determine the age at which male and female Sumatran rhinos become sexually mature by monitoring fecal testosterone metabolites and pregnane metabolites, respectively. Serial rectal ultrasonography was employed in studying the female rhino to help validate the fecal hormone data with visual observations of ovarian activity.

MATERIALS AND METHODS

Animal Care

Two Sumatran rhino calves, one male (SB#42) and one female (SB#43), born at the Cincinnati Zoo and Botanical Garden, were the subjects of this study. Both calves were produced by the same dam and sire following natural mating. The male calf was born on Sept. 13, 2001 and the female on July 29, 2004. Both calves were housed with the dam continuously for at least the first 12 months of their lives during which they were nursing while being fed a diet consisting primarily of fresh ficus (30–50 kg per day of up to 10 types of ficus and occasional Kaffir plum) supplemented with 1–2 flakes of hay (40% alfalfa, 60% orchard grass) and 1.8 kg of grain (ADF 16; Mazuri, St. Louis, MO). They also received a variety of fresh fruits (apples, bananas) and vegetables (sweet potatoes, carrots) daily and had unlimited access to fresh water and iodized salt blocks. The dam was supplemented with 48 g/day Osteo-form (Vet-A-Mix, Shenandoah, IA) from the day following parturition until the calves were weaned.

The male calf was weaned at 14 months of age and the female was weaned at 15 months of age. The female calf remained at the Cincinnati Zoo after weaning and continued to receive a similar diet. The male calf was moved to the Los Angeles Zoo at 21 months of age. There his diet consisted of up to four varieties of freshly harvested, locally grown browse daily (i.e., ficus, mulberry, kaffir plum, Chinese elm; 32–42 kg), 3.6 kg of 3-way hay, 1.4 kg ADF 16 grain supplemented with beet pulp and 20–25 fresh fruits and vegetables (papaya, carrots, yams, apples, bananas). At 5.5 years of age, the male calf was sent to the Sumatran Rhino Sanctuary (SRS) in Way Kambas National Park on Sumatra. At the SRS, he was offered 25–43 kg of freshly collected browse each day consisting of 8–10 varieties. In addition, he received 7–10 kg of fruit and vegetables (watermelon, papaya, jackfruit, apple, banana, carrots, yam bean, and sweet potato). He also had the opportunity to browse naturally in his 10 ha forested enclosure and had unlimited access to water and a mineral block which was changed to an iodized salt block in August 2007. He did not receive any hay or grain at the SRS.

Body Weights

Both calves were weighed daily starting the day after they were born, and the first year of weight data for both calves has been published in detail [Plair et al., 2011]. Daily body weights continued to be recorded for the female calf throughout the study. Daily body weight was recorded for the male calf until he left the Cincinnati Zoo. After a 10-month interval without being weighed, daily weights were recorded at the Los Angeles Zoo, with a few exceptions of several days or weeks, until the male was moved to Sumatra at 5.5 years of age. At the SRS, staff weighed the male calf weekly.

Ultrasonography

Rectal ultrasonography was employed to assess the ovaries and reproductive tract of the young female calf beginning at 2 years of age. A Sonosite Titan ultrasound machine with a variable 4–2 mHz C15 curved array transducer was used for most of the exams, but an Aloka 500 machine (Aloka, Wallingford, CT) with a 5 mHz linear array probe was used when the Sonosite was not available. Monthly exams were conducted until the first pre-ovulatory sized follicle was observed and then weekly exams were conducted until the calf was 5.5 years old. During each exam, the largest follicle on the two ovaries was measured and recorded (diameter, area, and circumference).

Fecal Sample Collections and Preparations

Several fecal samples from two adult male Sumatran rhinos, one a proven sire (Ipuh, SB#28, approximately 25 years of age) at the Cincinnati Zoo and one unproven (Torgamba, SB#4; approximately 30 years of age) at the SRS, were collected to serve as controls. Both adult male rhinos copulated with females during the years their fecal samples were collected. Fecal samples were collected once monthly from the male calf from 6 months to 7 years of age with the exception of one 11-month period during which no samples were collected. Fecal samples were collected monthly from the female calf from 6 to 21 months of age. From 22 months to 5 years 7 months of age, fecal samples were collected thrice weekly. Samples were stored in plastic bags in a freezer (−20°C) and transported to CREW after several days to several months where they were stored frozen (−20°C) until they were processed for hormone analyses.

Wet fecal samples were thawed, transferred to a 15 ml polystyrene conical tube (Fisher Scientific, Pittsburgh, PA)

and freeze-dried for 24 hr to account for variation in fecal water content between samples. Fecal samples collected in Sumatra were stored frozen at −20°C for at least a month before being thawed, placed in a 5 cm diameter aluminum weigh dish (VWR, West Chester, PA), and dried in an oven (60°C, 24 hr) before shipment to CREW for steroid extraction. Consistency in assay results using the two drying methods was confirmed with five wet fecal samples ($n = 5$) that were divided into two aliquots, dried by the two methods and analyzed. Variation in testosterone metabolite concentrations between the matched samples was <5%. All lyophilized/dried fecal matter was transferred to a plastic bag and crushed to a powder. Because a relatively large volume (100 μl) of sample at a relatively low dilution (1:8) was added to each well for the testosterone assay and there was concern of a potential matrix effect, steroids were extracted from 0.05 g of male feces by mechanically shaking samples overnight in 5 ml 50% v:v ethanol (ETOH): phosphate buffered saline (PBS), whereas for the pregnane assay 0.1 g of feces was extracted in 3 ml v:v 90% ETOH: ddH_2O. Samples were centrifuged 1,500*g* for 15 min, the supernatant recovered, and stored at −20°C until analysis. All assays were run within 1 month of extraction.

Enzyme Immunoassays

Pregnane metabolites

The general assay protocol and the preparation of buffers followed the methods previously described by Schwarzenberger et al., [1991]. The antiserum was raised against 5β-pregnane-3α, 20α-diol 3HS:BSA (E Mostl, University of Veterinary Medicine, Vienna, Austria). Briefly, plates (Nunc Maxisorp, VWR) were coated with Protein A (Sigma–Aldrich, St. Louis, MO) at a concentration of 0.52 μg/well and incubated overnight at room temperature. Plates were washed and blotted dry before standards (5.12–500 pg/well) and samples (1:30 dilution), steroid antibody (1:30,000 dilution) and biotinylated label (1:750,000 dilution) were added and incubated overnight at 4°C. Plates were emptied, washed and blotted dry prior to adding strepavidin peroxidase conjugate (Roche Applied Science, Indianapolis, IN). After a 45 min incubation at 4°C, plates were emptied, washed and blotted dry. Substrate (3,3',5,5'-tetramethylbenzidine; Sigma–Aldrich) was added and incubated for 45 min at room temperature before the enzymatic reaction was stopped with 2 mol/L sulfuric acid and read at 450 nm. The sensitivity range for the assay was 5.12–500 pg/well.

Testosterone metabolites

Concentrations of fecal testosterone were measured using EIA methods described by Munro and Lasley [1988]. The antiserum was raised against testosterone-6-carboxymethyl oxime (R156/7) (C Munro, University of California, Davis, CA). In brief, microtiter plates (Nunc Maxisorp, VWR) were coated with antiserum (1:10,000 dilution) in coating buffer and incubated overnight at 4°C. Unbound antiserum was washed away and plates blotted dry before samples (1:8 to 1:64 dilution) and standards were added in duplicate followed by horseradish peroxidase conjugate diluted (1:60,000) in EIA buffer. Plates were incubated for 2 hr at room temperature. Following incubation, plates were washed, blotted dry and substrate solution (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Sigma–Aldrich) was added. Absorbance was measured at 405 nm. The sensitivity range for the assay was 3.125–200 pg/well.

Both EIA's were validated by examining parallelism of serially diluted samples (neat to 1:256) to the standard curve. Based on those results, fecal extracts were diluted in EIA buffer at a ratio that was expected to result in approximately 50% displacement. If sample results indicated binding was not within the linear portion of the standard curve (<20% or >80% binding), samples were diluted and re-analyzed accordingly. Extraction efficiencies were >90% for both assays when wet fecal samples were spiked with standard prior to processing and analysis. Standards and samples were analyzed in duplicate in both assays. Fecal hormone concentrations are presented as mass/gram dry weight.

RESULTS

Growth Rates

After the first year of rapid growth, the male calf's body weight gradually increased until he reached a maximum weight of ~780 kg at 6–6.5 years of age (Fig. 1A). Although the data in the graph ends about the time he reached this weight his weekly weight continued to fluctuate between 780 and 800 kg in the years that followed. The female calf exhibited a similar rapid growth rate during her first year followed by a slower rate of weight gain until she achieved her adult weight of 680–700 kg at 4 years of age (Fig. 1B). In contrast to the male rhino, the female's weight fluctuated seasonally with a decrease in body weight recorded each summer.

Enzyme Immunoassays

Serial dilutions of fecal extracts produced displacement curves parallel to the standard curve for each of the assays used to document testicular and ovarian activity (testosterone, $r = 0.987$; pregnane, $r = 0.995$). The CV's among both assays for the various standard concentrations were all below 15%. CV's were 5% and 6% for internal controls at 79% and 43% binding in the pregnane EIA, and 10% and 12% for internal controls at 64% and 24% binding in the testosterone EIA.

Male Calf Fecal Testosterone

Fecal testosterone metabolites increased in two different phases as the male calf achieved sexual maturity. The first

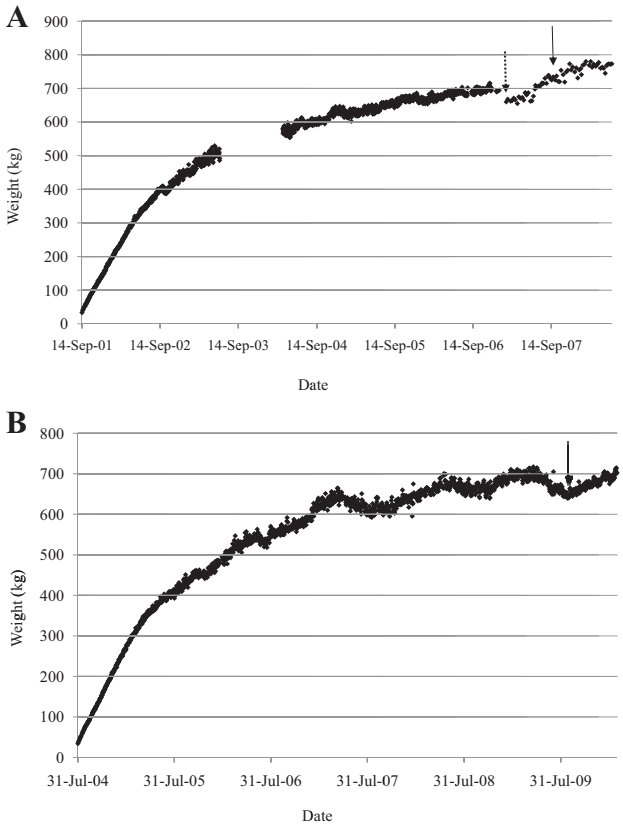

Fig. 1. Body weights for male (**A**) and female (**B**) Sumatran rhino calves from birth until sexual maturation was achieved. The dashed arrow in (A) denotes when the male rhino was moved to Sumatra, and the solid arrows denote when sexual maturation was confirmed based on fecal hormone metabolites.

increase above neonatal baseline values occurred when the calf was about 3.5 years of age (Fig. 2) and coincided with the first observed penile erection exhibited by the calf. Testosterone concentrations remained at this modest level

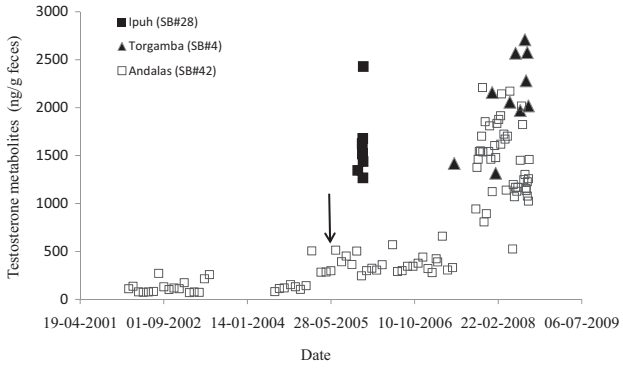

Fig. 2. Fecal testosterone metabolite concentrations in a male Sumatran rhino calf (□) from 5 months to 7 years of age, and two mature male Sumatran rhinos approximately 25–30 years of age (■,▲). The solid arrow denotes first observed penile erection in the male calf and the dashed arrow denotes when the young male rhino was moved to Sumatra.

until the calf reached 6 years of age when a substantial increase in testosterone metabolites was measured (Table 1) with concentrations reaching a level similar to that of two adult male rhinos (Fig. 2).

Female Calf Fecal Pregnane

The female calf appeared to experience a slight increase in mean pregnane metabolite excretion above neonatal baseline values when she reached 2 years of age (Fig. 3), but both mean pregnane concentration and the variation in concentrations continued to increase each year until the female reached 5.5 years of age (Table 1 and Fig. 3).

Female Calf Follicle Size

Ultrasonography was successfully used to visualize the ovaries of the calf beginning at 2 years of age. However, the reproductive tract was immature with very small, undeveloped uterine horns making it difficult to find the ovaries during some exams. During most of these early exams, the ovaries were relatively small and inactive containing only a few small follicles, typically no >10 mm in diameter or 3.0–4.0 cm in circumference (Fig. 4). A mature pre-ovulatory follicle (18 mm $\times$ 20 mm in diameter; 5.8 cm in circumference) was first observed during an exam when the female was 4 years and 9 months of age. From that point on, the size of the largest follicle on the ovaries during any given exam often was 5.0–8.0 cm in circumference (16–27 mm in diameter), and the uterine horns became more prominent and easier to track. Follicles often were not perfect spheres so the two measurements of follicle diameter could be fairly disparate. Therefore, circumference was the measurement of choice as previously reported by Roth et al., [2001].

DISCUSSION

The male and female Sumatran rhino calves were a little slower to reach sexual maturation than calves of other rhino species maintained in captivity. The male calf's testosterone concentrations only achieved adult male levels during the 6 months following his sixth birthday. In contrast, the youngest male black, white, and Indian rhino sires on record are 3.2, 3.8, and 4.7 years of age [Christman, 2011] and these could not be considered outliers because several additional males of each species sired calves following a mating that occurred before the male reached 6 years of age. Although the male Sumatran rhino calf did not succeed in mating with a female until he was 8.25 years old, that result was likely due to management decisions that limited his opportunities to mate when he was younger. Sumatran rhinos are solitary and are known to be aggressive especially when introduced to anestrual females. Therefore, male introductions to females are rare and carefully managed [Khan et al., 1999; Roth et al., 2001; Roth et al., 2004]. Because the male calf in this study was placed in close proximity to adult females a few months prior to this testosterone increase, it is

TABLE 1. Mean (±SD) fecal testosterone and pregnane metabolite concentrations in a male and a female Sumatran rhino calf, respectively, throughout sexual maturation

Male calf SB#42		Female calf SB#43	
Age	Testosterone (ng/g feces)	Age	Pregnanes (ng/g feces)
6–12 months	120 ± 78	6–12 months	0.82 ± 0.34
1–2 year	133 ± 63	1–2 year	0.76 ± 0.74
2–3 year	105 ± 21	2–3 year	1.26 ± 0.68
3–3.5 year	208 ± 168	3–4 year	1.57 ± 0.78
3.5–4 year	371 ± 97	4–4.5 year	1.81 ± 1.49
4–5 year	356 ± 97	4.5–5 year	3.58 ± 2.34
5–6 year	388 ± 108	5–5.5 year	4.21 ± 3.84
6–6.5 year	1,506 ± 362		
6.5–7 year	1,405 ± 396		

possible the introduction to females had a stimulatory effect on testosterone production.

The female Sumatran rhino is an induced ovulator that develops follicles which often grow beyond pre-ovulatory size and luteinize when the female is not mating [Roth et al., 2001]. Therefore, a consistent, cyclic pattern of progesterone production cannot be expected in solitary females. Instead, fecal progestin concentrations fluctuate unpredictably in mature, non-mating females depending on the extent and duration of luteinization that follows pre-ovulatory follicle formation [Roth et al., 2001].

Although the female Sumatran rhino produced her first pre-ovulatory follicle at 4.75 years of age, her progesterone concentrations and fluctuations continued to increase when she was 5–5.5 years of age, suggesting that she was not fully mature at 4.75 years. It is possible that the female undergoes a transitional period as her ovaries begin to exhibit follicular activity, much like a mare does as she enters the breeding season [Ginther, 1990] or a girl as she reaches puberty [Apter, 1980]. Therefore, sexual maturation may coincide with the increased mean pregnane concentrations a few months after the first pre-ovulatory follicle has developed. The apparent decrease in follicle size observed towards the end of the 2009 summer coincided with the period during which the highest concentrations of fecal pregnanes were measured. Therefore, it is likely the lack of follicular development during this interval was due to an extended period of follicular luteinization. Alternatively, the absence of mature follicles during that period may have simply represented individual variability since reproductive activity in non-mating Sumatran rhinos can be erratic [Roth et al., 2001]. The female was managed without access to a mate during this study, so the age of first sexual receptivity was not documented. However, when the female was first introduced to a male for breeding at 6 years 2 months of age, she was receptive allowing the male to mount her [personal observation]. White, black, and Indian rhino females have conceived at 2.7, 3.8, and 2.7 years of age, respectively [Christman, 2011]. In fact, there are 10 female white rhinos in the North American studbook that have conceived before the age of 4 indicating that age of sexual maturation occurs quite early in this rhino species. The female Sumatran rhino appears to mature later than the other three rhino species. Furthermore, this study documented physiological maturation and it is possible that behavioral maturation occurs even later. However, given that this study included just a single calf of each sex, we cannot rule out the possibility that these two individuals were slow developers and other

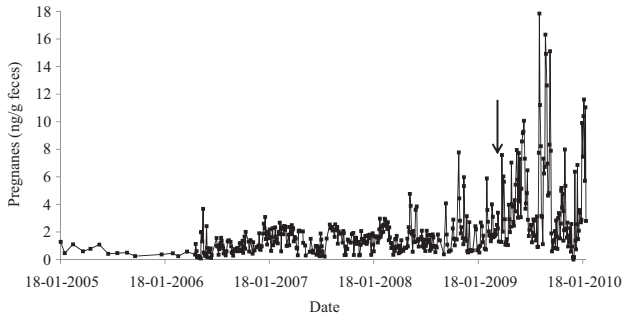

Fig. 3. Fecal pregnane metabolite concentrations in a female Sumatran rhino calf from 6 months to 5.5 years of age. Arrow denotes when the first pre-ovulatory sized follicle was observed by ultrasound.

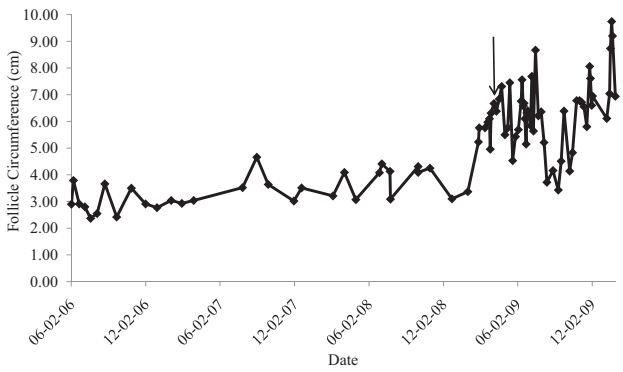

Fig. 4. Circumference of the largest follicle observed by ultrasound on the ovaries of a female Sumatran rhino calf from 2 to 5.5 years of age. Arrow denotes when the first pre-ovulatory sized follicle was recorded.

calves of this species could achieve sexual maturation at slightly younger ages.

It is broadly known that in many species, sexual maturation is linked with body weight and nutritional status [Widdowson, 1981]. Therefore, animals in zoos have been known to achieve sexual maturation at younger ages than what has been reported for their wild counterparts [Wheaton et al., 2006]. However, there did not appear to be a close correlation between the onset of sexual maturation and a particular weight threshold in these two rhinos. Although the male rhino continued to gain weight incrementally as he reached sexual maturity, the female calf reached her maximum weight a year prior to developing her first pre-ovulatory follicle. Because this species is an obligate browser that requires a large percentage of its diet to consist of fresh, diverse browse species [Dierenfeld et al., 2000], it is challenging to maintain in zoos and is less likely to receive a higher plane of nutrition than its wild counterparts browsing in their native forests of Sumatra. Therefore, it is unlikely that the captive environment will promote sexual maturation at a younger age in this rhino species, whereas in grazing rhinos that receive abundant, high quality hay year-round in zoos, such a shift is more likely.

Fecal progestin metabolite analysis via radioimmunoassay has proven valuable for monitoring mature female Sumatran rhino reproductive activity [Roth et al., 2001]. However, in this study an enzyme immunoassay was employed, and initial attempts to monitor fecal progesterone metabolites failed to produce meaningful data. Two of the three primary progesterone metabolites excreted by the female Sumatran rhino are 5β-pregnane-3α, 20 α-diol and 5β-pregnane-3α-ol-20-one [Heistermann et al., 1998] aligning it with its Asian cousin the Indian rhinoceros (*Rhinoceros unicornis*) which also excretes appreciable amounts of 5β-pregnanes [Schwarzenberger et al., 2000]. In contrast, the dominant fecal metabolites excreted by the African black and white rhino species are 5α-reduced pregnanes [Schwarzenberger et al., 1996, 1998]. In fact, 5β-pregnane-3α, 20 α-diol and 5β-pregnane-3α-ol-20-one represent just 4.57% and 0.54% of excreted progesterone in the Southern black rhino [Lance et al., 2001]. After acquiring an antibody for 5β-pregnane-3α, 20 α-diol, the EIA data generated from the fecal extracts of the female calf proved valuable and correlated with the ultrasonographic changes observed on the ovaries throughout the study. Prior to producing pre-ovulatory sized follicles, the female rhino's pregnane concentrations were low, but following the formation of pre-ovulatory sized follicles, fecal pregnane concentrations rose and became more variable, similar to what was previously reported for a mature, non-mating female Sumatran rhino [Roth et al., 2001].

The specific androgen metabolites excreted in Sumatran rhino fecal samples have not been characterized. Regardless, the EIA and antibody used in this study revealed unambiguous differences in testosterone metabolite concentrations of immature and mature male Sumatran rhinos. Furthermore, the testosterone metabolite concentrations measured in adult male Sumatran rhinos in this study (~1,500 ng/g) were significantly greater than mean values reported by Brown et al., [2001] for the adult male black rhino (27.6 ng/g) and white rhino (16.8 ng/g), suggesting that the assay and antibody employed herein were relatively effective since systemic testosterone concentrations do not differ appreciably among adult males of these rhino species. Serum testosterone in Sumatran rhinos has been measured at 1.9 ng/ml [unpublished data] which is comparable to concentrations measured in serum from black rhinos (0.6–1.8 ng/ml) and white rhinos (0.9–2.2 ng/ml) [Christensen et al., 2009].

Although some individual variability would be expected, male and female Sumatran rhino calves should be physiologically ready to breed when they reach approximately 6.5 and 5.5 years of age, respectively. This information is important given the urgency of the captive breeding effort to produce calves as quickly as possible from all potentially reproductive individuals. Furthermore, uterine masses and cysts have been documented in several older female Sumatran rhinos [Schaffer et al., 1994; Khan et al., 1999; Roth et al., 2001] indicating that this species, like many others in captivity, can lose its fertility to reproductive pathology that develops in females maintained for an extended period in a non-pregnant state [Hermes et al., 2004; Roth, 2006]. Therefore, to prolong their fertility and maximize their reproductive life, female Sumatran rhinos should be bred soon after their 5th birthday.

CONCLUSIONS

1. Male Sumatran rhino calves achieve sexual maturation at 6–6.5 years of age.
2. Female Sumatran rhino calves achieve sexual maturation at approximately 5–5.5 years of age.
3. Sexual maturation can be monitored in male Sumatran rhinos by measuring fecal testosterone metabolite concentrations.
4. Sexual maturation can be monitored in female Sumatran rhinos by evaluating the fecal pregnane metabolite profile.
5. Sexual maturation in female Sumatran rhinos can be confirmed by ultrasonographic characterization of ovarian follicular size and growth.

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REFERENCES

Apter D. 1980. Serum steroids and pituitary hormones in female puberty: a partly longitudinal study. Clin Endo (Oxf) 12:107–120.

Brown JL, Bellem AC, Fouraker M, Wildt DE, Roth TL. 2001. Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. Zoo Biol 20: 463–486.

Christman J. 2011. North American Regional Black Rhino, White Rhino and Indian Rhino Studbooks. Orlando, FL: Disney's Animal Kingdom.

Christensen BW, Troedsson MHT, Young LJ, Oliva M, Penfold LM. 2009. Effects of sociosexual environment on serum testosterone in captive male African rhinoceros. Theriogenology 71:1105–1111.

Dierenfeld ES, Wildman REC, Romo S. 2000. Feed intake, diet utilization and composition of browses consumed by the Sumatran rhino (*Dicerorhinus sumatrensis*) in a North American Zoo. Zoo Biol 19:169–180.

Ginther OJ. 1990. Folliculogenesis during the transitional period and early ovulatory season in mares. J Reprod Fert 90:311–320.

Heistermann M, Agil M, Hodges JK. 1998. Metabolism and excretion of oestradiol-17β and progesterone in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*). Anim Reprod Sci 53:157–172.

Hermes R, Hildebrandt TB, Göritz F. 2004. Reproductive problems directly attributable to long-term captivity-asymmetric reproductive aging. Anim Repro Sci 82–83:49–60.

Khan MKM, Roth TL, Foose TJ. 1999. In situ and ex situ efforts to save the Sumatran rhinoceros (*Dicerorhinus sumatrensis*). Proc 7th World Conf on Breeding Endangered Species. Ohio: Cincinnati Zoo. p 163–174.

Lance VA, Patton ML, Hagey LR. 2001. Identification of a series of $C_{21}O_2$ pregnanes from fecal extracts of a pregnant black rhinoceros (*Diceros bicornis minor*). Steroids 66:875–881.

Munro CJ, Lasley BL. 1988. Non-radiometric methods for immunoassay of steroid hormones. In: Albertson BD, Haseltime FP, editors. Nonradiometric assays: technology and application in polypeptide and steroid hormone detection. New York: Alan R Liss, Inc. p 289–329.

Plair BL, Reinhart PR, Roth TL. 2011. Neonatal milestones, behavior and growth rate of Sumatran rhinoceros calves (*Dicerorhinus sumatrensis*) bred and born in captivity. Zoo Biol 30:1–15.

Roth TL. 2006. A review of the reproductive physiology of rhinoceros species in captivity. Int Zoo Yrbk 40:130–143.

Roth TL, O'Brien JK, McRae MA, et al. 2001. Ultrasound and endocrine evaluation of ovarian cyclicity and early pregnancy in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*). Reproduction 121:139–149.

Roth TL, Bateman HL, Kroll JK, et al. 2004. Endocrine and ultrasonographic characterization of a successful pregnancy in a Sumatran rhinoceros (*Dicerorhinus sumatrensis*) supplemented with progesterone. Zoo Biol 23:219–238.

Schaffer NE, Zainal-Zahari Z, Suri MSM, Janudeen MR, Jeyendran RS. 1994. Ultrasonography of the reproductive anatomy in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*). J Zoo Wildl Med 25:337–348.

Schwarzenberger F, Mostl E, Bamber E, Pammer J, Schmehlik O. 1991. Concentrations of progestagens and oestrogens in the faeces of pregnant Lipizzan, Trotter and Thoroughbred mares. J Reprod Fertil Suppl 44: 489–499.

Schwarzenberger F, Tomasova K, Holeckova D, Matern B, Möstle E. 1996. Measurement of fecal steroids in the black rhinoceros (*Diceros bicornis*) using group-specific enzyme-immunoassays for 20-oxo-pregnanes. Zoo Biol 15:159–171.

Schwarzenberger F, Walzer C, Tomasova K, et al. 1998. Faecal progesterone metabolite analysis for non-invasive monitoring of function in the white rhinoceros (*Ceratotherium simum*). Anim Repro Sci 53: 173–190.

Schwarzenberger F, Rietschel W, Vahala J, et al. 2000. Fecal progesterone, estrogen and androgen metabolites for noninvasive monitoring of reproductive function in the female Indian rhinoceros, *Rhinoceros unicornis*. Gen Comp Endo 119:300–307.

Wheaton CJ, Joseph S, Reid K, et al. 2006. Body weight as an effective tool for determination of onset of puberty in captive female Nile hippopotami (*Hippopotamus amphibious*). Zoo Biol 25:59–71.

Widdowson EM. 1981. The role of nutrition in mammalian reproduction. In: Gilmore D, Cook B, editors. Environmental factors in mammal reproduction. Baltimore: University Park Press. p 145–159.