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Monitoring and controlling ovarian function in the rhinoceros

T.L. Roth [a, *], M.W. Schook [b], M.A. Stoops [a]

[a] Center for Conservation and Research of Endangered Wildlife (CREW), Cincinnati Zoo & Botanical Garden, 3400 Vine Street, Cincinnati, OH 45220, USA
[b] Disney's Animal Kingdom, 1200 North Savannah Circle East Bay Lake, FL 32830, USA

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ABSTRACT

Despite their size and potentially dangerous demeanor, the rhinoceros has been a preferred subject of wildlife reproductive scientists. Several factors contribute to this taxon's popularity including the ability to utilize insightful tools like non-invasive hormone metabolite monitoring and transrectal ultrasonography, the necessity for mate introductions to coincide with the female's estrus when breeding certain species or individuals, and the desire to develop assisted reproductive technologies to facilitate the genetic management and ultimate sustainability of small, managed populations in human care. The resulting profusion of rhinoceros reproductive studies has revealed significant species-specific characteristics and exposed the prevalence of aberrant reproductive activity within this taxon. Of equal importance, it has guided necessary intervention and enhanced our success in overcoming challenges associated with breeding rhinoceroses.

1. Introduction

Most advances in our knowledge of female rhinoceros reproductive physiology can be attributed to three prodigious discoveries. The advent of noninvasive hormone metabolite monitoring gave scientists their first glimpse, albeit indirect, of ovarian activity in the rhinoceros [1] which catalyzed a series of studies detailing endocrine dynamics of reproductive cycles and pregnancy in all four managed rhinoceros species [2–11] and free-ranging African black and white rhinoceroses [12–15]. A decade later, the feasibility of using transrectal ultrasonography to view the reproductive tracts of the rhinoceros was revealed [16,17], thereby empowering researchers with a mechanism for directly observing ovarian activity and pregnancy in this taxon. The third critical innovation evolved from scientists, veterinarians and animal care staff working together to integrate operant conditioning into the basic animal husbandry routine, eventually proving that rhinoceroses of all species could be trained for voluntary blood collection, rectal and transabdominal ultrasonography and even transcervical artificial insemination (AI). This animal management advancement made possible performing serial and/or critically timed procedures to study reproductive function in real time [6,7,10,18–22]. The combined use of these invaluable tools is allowing the collection of more detailed and accurate data on rhinoceros ovarian function, which in turn is helping to explain earlier findings, revealing new facts and dispelling erroneous theories of the past.

2. Endocrine monitoring of ovarian function

2.1. Importance of antibody specificity

Non-invasive endocrine studies relying on hormones and their metabolites found in feces, urine and saliva have provided valuable insight into ovarian function, estrous cycle dynamics and pregnancy in all managed rhinoceros species [for review, 23]. However, differences in the major metabolites excreted in biological material from the African and Asian species highlight the importance of hormone extraction techniques, cross-reactivity of antibodies used and the assay validation procedures employed during novel studies in each species. For example, although the same antibody and radioimmunoassay could be used to monitor fecal progestogens in both African white and black rhinoceroses [3], a different antibody directed specifically against the 5βpregnane-3α, 20 α-diol metabolite was required to accurately document fecal progestogen patterns associated with progression to sexual maturation in the female Sumatran rhinoceros [21]. Additionally, during a study examining ovarian function in response to GnRF vaccination in African white and Indian rhinoceroses, conflicting data from ultrasound and endocrine monitoring led to the discovery that non-

* Corresponding author.
E-mail addresses: terri.roth@cincinnatizoo.org (T.L. Roth), Mandi.Schook@disney.com (M.W. Schook), monica.stoops@cincinnatizoo.org (M.A. Stoops).

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specified phytochemicals in the diet fed several Indian rhinoceroses cross-reacted to the 20α-OH-pregnane assay [24]. Therefore, assay results did not accurately reflect systemic progestogen concentrations in these individuals.

2.2. Estrous cycle irregularity

Whereas, each species has an established estrous cycle length, non-invasive hormone metabolite monitoring has also revealed that it is relatively common for individuals to vary from this standard. Population level hormone assessments can reveal important insight into a particular rhinoceros species and what the implications may be for deviations from the standard. When hormone data of estrous cycle lengths are cross referenced to breeding management notes, correlates of fertility can be established. To date, no pregnancies have been associated with estrous cycle lengths of <19 or >40 d in the African black rhinoceros [4] and higher rates of pregnancy follow a 30 versus 70 d estrous cycle in the African white rhinoceros [5]. It is interesting to note that similar measures of ovarian function and dysfunction have been observed on two different continents in separately managed populations of the same species [3,4].

For African rhinoceros species, longer cycle lengths in non-mated individuals have been associated with prolonged progesterone production resulting from an extended luteal phase [3–5,8,13]. The same cannot be said for the variable estrous cycle lengths displayed by their Asian counterparts. To-date, extended luteal phases have not been observed in non-pregnant female Indian rhinoceroses exhibiting extended cycles. Instead, these longer cycles appear to be due to a delay in the emergence of the next cycle's dominant follicle or the development of an ovarian cyst [10]. The variable estrous cycle lengths associated with the Sumatran rhinoceros can be attributed to its status as an induced ovulator. When not mated, the female fails to ovulate, and the anovulatory follicle often luteinizes [7]. The timing and extent of luteinization then dictates the length of the cycle which can be shorter or longer than normal. It is only when kept in a repetitive pattern of mating that the female Sumatran rhinoceros exhibits a normative cycle length of 21 d [7].

The African white rhinoceros is the only species to exhibit extended periods of anestrous as reflected by baseline progesterone concentrations [3,5,8]. Whereas the causative factor(s) for this phenomenon is not yet known, several avenues have been explored or are currently under investigation. Fecal corticosterone concentrations do not differ between acyclic and cyclic, or nulliparous and parous females, so it does not appear social stress is contributing to the former's overall poor reproductive success [25]. It was recently demonstrated that phytoestrogens can bind to and activate recombinant white rhinoceros estrogen receptors *in vitro* [26]. As constituents of captive herbivore diets contain compounds relatively high in phytoestrogen content, it has been speculated they may be contributing to the reproductive failure observed in this species [27].

2.3. Application of endocrine monitoring to rhinoceros breeding

As a plethora of hormone data for this taxon has emerged over the past three decades, the utility of endocrine analyses in guiding management decisions for both natural and assisted breeding efforts of captive rhinoceros populations has similarly increased. Beyond confirming estrous cyclicity and diagnosing pregnancy, endocrine studies have been used to monitor ovarian response to exogenous hormone treatments to improve [6,10,11,22,28–30] maintain [2,11,22,23,31] or reduce fertility [24]. In addition, hormone monitoring can provide insight regarding the effects on ovarian function of pharmacological drugs [32] or changes in management techniques over an extended period of time [33,34]. Furthermore, non-invasive hormone metabolite monitoring can be used to both evaluate the progression of and confirm sexual maturation in young rhinos. To-date, the use of fecal progestogen concentrations as evidence of sexual maturation has only been reported in the Sumatran rhinoceros [21], but it likely could be used similarly in females of all rhinoceros species.

The different endocrine matrices used to monitor ovarian activity have varied by species and management scenario, and their specific use can depend on their efficacy in the species and the outcome required for analysis. The hormone matrix of choice for assisted reproductive technologies (ARTs) [10,22,28,29,31] or natural breeding introductions [7,11] are those that more closely reflect systemic concentrations at the time of collection and analysis, such as serum or urine. As several urinary and behavioral biomarkers of ovarian function have been established in the Indian rhinoceros, they provide the primary data used to time AI procedures and/or the administration of ovulation inducing agents in this species [10,22]. In contrast, most studies in black and white rhinoceroses have utilized serum or fecal analysis. However, when timing ARTs, fecal analyses are not ideal due to prolonged gut passage time and processing requirements that result in a delay between physiological events, and resulting fecal hormone changes [3,33].

2.4. Estrogen

The Indian rhinoceros is the only rhinoceros species in which estrogen metabolites can be reliably measured and accurately reflect follicular dynamics [10]. Androgen metabolites have been similarly quantified in this species and shown to correlate with estrogen excretion and follicular activity (Fig. 1) [9,35,36]. Therefore, hormone monitoring in Indian rhinoceroses is very informative and paints a more complete picture regarding ovarian function compared to that in the other rhinoceros species (Fig. 2). To date, estrogen measures in biological samples obtained from the other three rhinoceros species have failed to produce meaningful data. Tandom gas chromatography/mass spectrometry analysis has revealed that estrous levels of urinary estrone and estradiol are excreted in at least 10-fold higher concentrations in the Indian rhinoceros compared to both the Sumatran rhinoceros and domestic horse (Schook, unpublished results) which allows for reliable estrus detection in this species. Furthermore, attempts to validate testosterone as an alternative method for monitoring

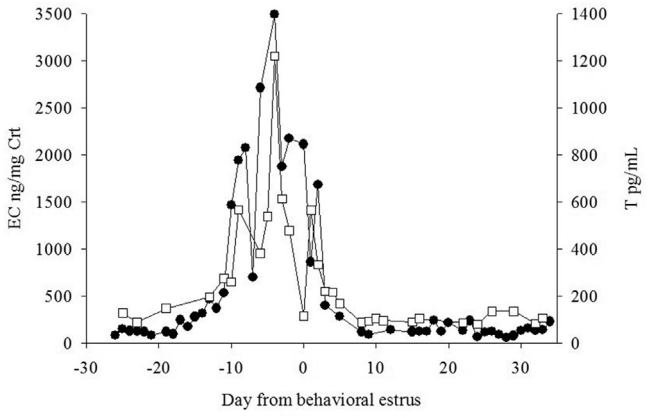

Fig. 1. Correlation (r = 0.81) between urinary estrogen conjugate (EC) and salivary testosterone (T) during an ovulatory estrous cycle in a female Indian rhinoceros [36] aligned by day from behavioral estrus.

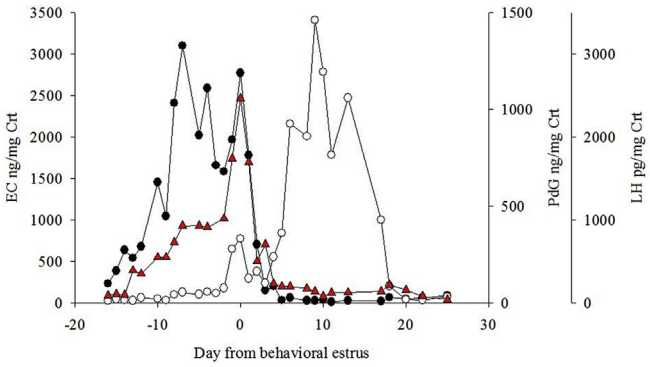

Fig. 2. Urinary estrogen conjugate (EC; solid circles), progesterone metabolite (PdG; open circles) and luteinizing hormone (LH; triangles) concentrations and pattern of excretion following a natural estrous cycle associated with successful ovulation in an Indian rhinoceros. Note the LH surge on day of estrus and the transient spike in urinary PdG that follows. Ultrasonographic evidence of ovulation was noted Day 2 after estrus.

follicular growth in the Sumatran rhinoceros failed, with values so low they fell outside the linear portion of the assay's standard curve (<80% binding; Stoops, unpublished results). Therefore, fecal and/or serum progesterone alone must be used to hormonally monitor estrous cycle dynamics for both African rhinoceros species and the Sumatran rhinoceros. However, LH can be monitored in urine of both Asian rhinoceros species and has been valuable for evaluating pituitary function relating to ovulation (Figs. 2 and 4, see ovulation induction section) [10,31].

3. Ultrasound monitoring of ovarian function

Whereas, hormone monitoring clearly has been an important tool for understanding rhinoceros reproductive physiology, the more recent, concurrent use of ultrasonography has revealed aspects of ovarian function previously unknown for each species. The different rhinoceros species vary in the size pre-ovulatory follicles attain during an estrous cycle with the smallest and largest sizes produced by the Sumatran rhinoceros (20–22 mm) and Indian rhinoceros (150–200 mm), respectively [for review [23]]. Unlike those of the domestic mare, rhinoceros ovaries do not contain an ovulation fossa, and therefore ovum release can occur on any part of the ovary. The reliability of utilizing follicle size to guide reproductive management decisions has facilitated breeding and ART procedures in Sumatran, African black and African white rhinoceroses, but has proven less informative in Indian rhinoceroses. In fact, the pre-ovulatory follicle of both African rhinoceros species displays similar morphological changes to those observed in the mare. A shift from spherical to guitar-pick shape signals impending ovulation [19]. The most definitive ultrasonographic feature of ovulation common to all species of rhinoceros is the collapse and disappearance of a pre-ovulatory sized follicle where one previously existed [7,10,19,20]. With the exception of the Sumatran rhinoceros, in which a functional corpus luteum (CL) may go undetected [20], the other rhinoceros species produce ultrasonographically detectable luteal glands that can take two morphologically different forms (corpus luteum or corpus hemorrhagicum) [10,19,20].

3.1. Application of ovarian ultrasound monitoring to rhinoceros breeding

Ultrasonographic assessment of follicular maturity is key to accurately timing ART procedures in African and Sumatran rhinoceroses [28,29,31,37] and is paramount for timing natural breeding introductions in the Sumatran rhinoceros [6,7]. In contrast, the substantial size and growth dynamics displayed by the pre-ovulatory follicle of the Indian rhinoceros make similar ultrasound metrics for timing exogenous hormone administration infeasible [10,22]. Therefore, endocrine and behavioral data dictate administration of ovulation inducing agents in this species [22] or assist in timing male-female introductions for natural mating [11]. Fortunately, females of this species often do express obvious signs of behavioral estrus and estrogen can be measured reliably; however, inter- and intra-individual variation in urinary estrone conjugate (EC) concentrations throughout the follicular phase prohibit a consistently reliable diagnostic threshold for follicular maturation in this species. Aside from baseline progesterone concentrations, signaling a female is in the follicular phase of an estrous cycle, no endocrine evidence of follicular maturity exists in the Sumatran or African rhinoceros species, making ultrasound measures of follicle size crucial to timing ovulation induction and AI.

Given the Indian rhinoceros's unique and variable follicular growth patterns, ultrasound data were analyzed for their utility as indicators of ovulatory versus anovulatory cycles and conceptive versus non-conceptive cycles. Mean follicle length, ellipse and circumference aligned by day from AI procedures were compared, but no differences were observed on Day 0 from AI for the length ($P = 0.415$), ellipse ($P = 0.145$) or circumference ($P = 0.13$) of follicles associated with anvoulatory (n = 6), ovulatory, non-conceptive (n = 12) and ovulatory, conceptive (n = 4) estrous cycles (Stoops, unpublished results).

3.2. Doppler ultrasound

Because initial ultrasound data in Indian rhinoceroses did not appear promising for accurately timing AI or GnRH injections, color Doppler ultrasound technology was tested during estrous cycles of the Indian (n = 2 females) and Sumatran (n = 2 females) rhinoceros to determine if follicular blood flow patterns could be used as accurate indicators of follicle maturation and pre-ovulatory status in Asian rhinoceroses. Color Doppler ultrasound changes have been documented during follicle selection, impending ovulation and anovulation in the mare [54]. Furthermore, a significant relationship has been established between blood flow characteristics of the wall of both equine and bovine pre-ovulatory follicles and resulting pregnancy rates [54]. The first evidence of blood flow to the base of a follicle in the Indian rhinoceros correlates with urinary EC concentrations rising above baseline and hence the start of the follicular phase of the estrous cycle (Figs. 1 and 3A). Similar visual presence of blood flow was noted once pre-ovulatory follicle size (20–22 mm) was attained in the Sumatran rhinoceros (Fig. 3E). Doppler spectral traces of Indian and Sumatran follicular blood flow were difficult to obtain due to movement of animals during examinations. However, during three estrous cycles in two female Indian rhinoceroses, spectral traces of blood flow to the pre-ovulatory follicle were successfully captured at 48, 24 and 12 h prior to ovulation. The low number of replicates prohibited statistical comparisons, but in all three cycles, resistance index decreased (<0.38) substantially in the 12 h prior to ovulation (Fig. 3D). Although it appears Doppler technology can confirm presence or absence of blood flow to developing follicles of Asian rhinoceroses, the difficulty in obtaining spectral trace data in non-sedated animals prohibits its widespread utility. However, when sedation is needed to facilitate ART procedures, Doppler ultrasonographic data may prove useful in tracking follicular characteristics and outcomes following ovulation induction trials or those associated with oocyte quality following ovum pick-up procedures.

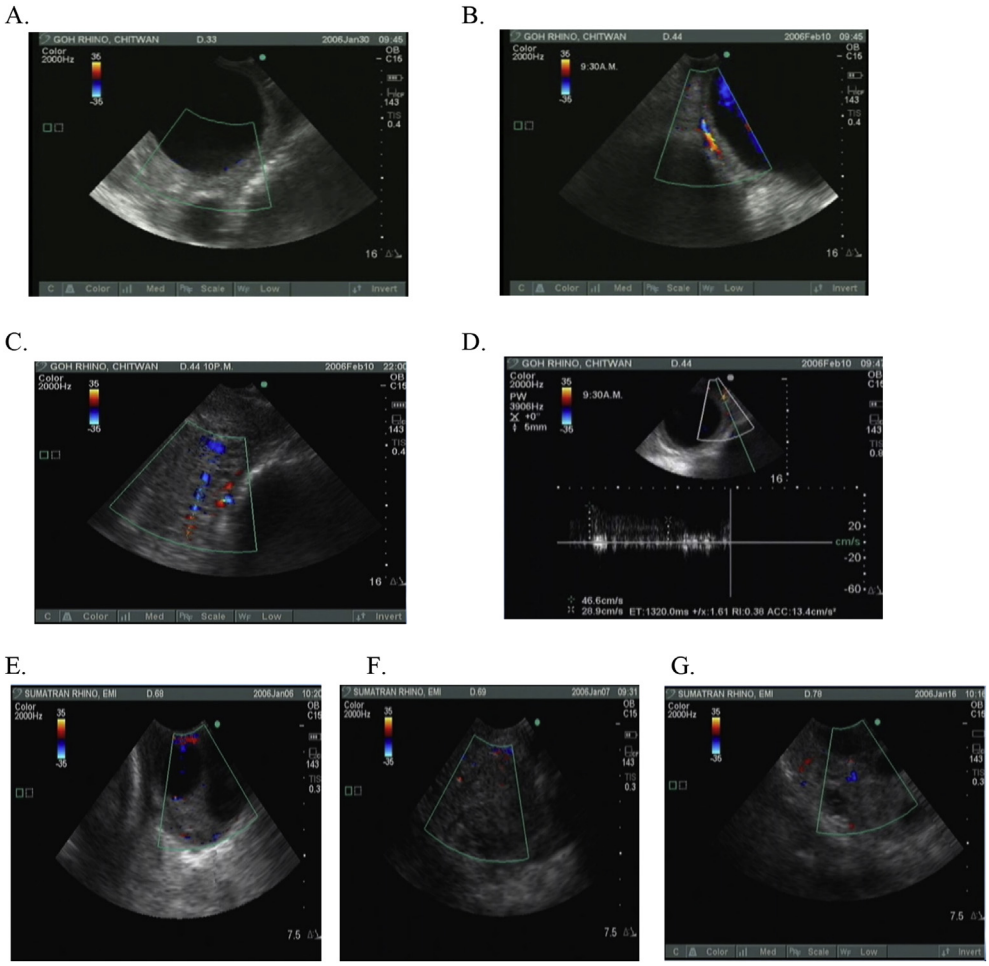

Fig. 3. Color Doppler ovarian ultrasound images from Indian (A–D) and Sumatran (E–G) rhinoceroses during the peri- and post-ovulatory period. A) Presence of blood flow to the base of a 10.5 cm follicle in the Indian rhinoceros 9 d prior to behavioral estrus, B) blood flow in the pre-ovulatory follicle wall, C) CL (fresh ovulation) and D) spectral trace data obtained from a 13.7 cm follicle <12 h prior to ovulation and approximately 30 h after the LH surge was detected in urine. E) Pre-ovulatory follicle of a Sumatran rhinoceros showing presence of blood flow to the base of a follicle concomitant with mature follicle size (21 mm) 24 h post-breeding and LH surge of a conceptive estrous cycle, F) Day 0 and G) Day 9 post-ovulation color Doppler detection of possible CL. Distance between graduation marks is 1 cm (right margin).

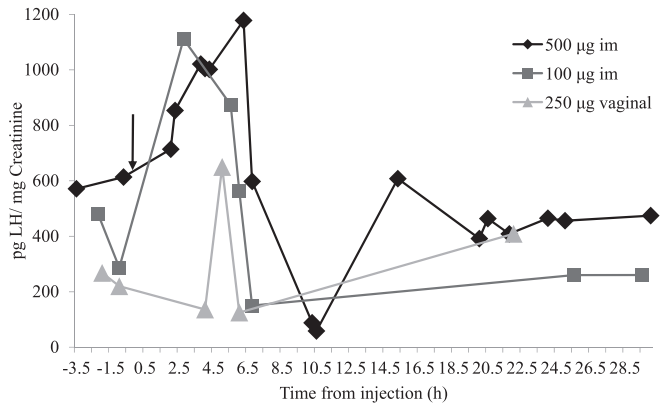

Fig. 4. Sumatran rhinoceros urinary LH concentrations following administration (arrow) of high (diamonds) and low (squares) dose im injections and vaginal deposition (triangles) of gonadorelin. Ovulation occurred by 48 h following both high and low dose injections but not following vaginal or rectal deposition, even when a higher dosage (500 µg) was inserted.

3.3. Anovulation

It is now well established that endocrine profiles alone cannot distinguish ovulatory versus anovulatory estrous cycles in the rhinoceros. Therefore, a cyclic rise and fall in progesterone concentrations, while suggestive, is not confirmation that successful ovulation has occurred. Increasingly, research involving serial ultrasound exams has demonstrated the frequency with which rhinoceros of all species produce anovulatory follicles. These persistent anovulatory follicles (PAFs), can luteinize and produce progesterone concentrations on par with and of similar duration to that of a CL during a normal post-ovulatory luteal phase [7,10,19,20,22]. Traditional endocrine analysis alone would not detect this seemingly common form of ovarian dysfunction displayed by all rhinoceros species. Similarly, the absence of measurable or cyclic progesterone concentrations in the African white rhinoceros may not reflect a lack of ovarian follicular activity or exhausted source of oocytes as previously proposed [38]. Early on it was discovered that many captive African white rhinoceroses exhibit flat-line progesterone concentrations indicative of an anestrous state [3,5,8]. Likewise, it was assumed the ovaries of these individuals were devoid of follicular activity during extended periods of acyclicity. Ultrasonographic studies have revealed that in

fact continuous waves of follicles emerge on the ovaries but fail to reach pre-ovulatory size [30]. The lack of entrainment to a regular cyclic pattern of follicle growth and ovulation appears to be a unique ovarian phenomenon limited to this rhinoceros species. Fortunately, this problem can be remedied with exogenous hormone administration (for review, see section on controlling ovarian function) [28,30].

3.4. Persistent anovulatory follicles

The occurrence of PAFs has been reported in African black, African white, Sumatran and Indian rhinoceroses (for review [23]). Also called hemorrhagic anovulatory follicles, hemorrhagic follicles or luteinized follicles, PAF's are dominant follicles that fail to ovulate, and in most cases fill with echogenic spots, strands of clotted blood and progesterone producing luteal tissue [39]. They are a commonly described phenomenon in several domestic species including the closest living domestic relative of the rhinoceros (the horse), and contribute to decreased fertility and breeding success [40]. The incidence of PAF's in horses is reported to range from 4 to 13% of cycles and the incidence increases with age in mares [40].

Although the incidence of PAFs has not been documented at a population level in any rhinoceros species, the described occurrence of PAFs in almost every case study involving ultrasonographic monitoring of the ovaries suggests it may be quite common. Low overall breeding success is reported for all managed rhinoceros species, and multiple matings often are required prior to successful conception. PAFs hinder the success of AI in Indian rhinoceroses [22] and may be one relatively under investigated reason for reduced fertility during natural breeding [41]. Because PAFs can produce aberrant and prolonged progesterone increases [7,10,20,22], ovulatory failure can go unrecognized in rhinoceroses not trained for serial ultrasound exams. Furthermore, old PAFs look similar to old CL and sometimes persist for several cycles so randomly timed ultrasound exams can lead to the erroneous conclusion that the female is ovulating. Therefore, the importance of operant conditioning for serial transrectal ultrasound monitoring should be stressed for those females that mate but do not conceive, or are suspected to experience early embryo loss due to prolonged luteal phases, since anovulation could be a contributing factor.

The exact cause of PAFs has not been elucidated. In domestic horses, an increase in serum estradiol was observed preceding anovulatory compared to ovulatory cycles 3–4 d prior to the day of expected ovulation [39]. However, this difference was not observed in urinary estrogen metabolites monitored in Indian rhinoceroses [10]. Similar to domestic horses [40], rhinoceroses may be responding to seasonal or management related photoperiod changes as anovulatory follicles appear to be more commonly observed in the winter and early spring in African white, African black and Indian rhinoceroses [10,19,20]. In the Sumatran rhinoceroses, PAFs simply coincide with most estrous cycles during which the female is not stimulated to ovulate by the male, regardless of season or age of female [7]. However, PAFs in African white [19], African black [20] and Sumatran rhinoceroses (Roth, unpublished results) have developed even when females do have access to males for mating, so their formation is not a function of reduced male exposure.

As in domestic horses [39,40], the majority of PAF's seem to develop progesterone producing luteal tissue [7,10,19,20], whereas a small minority of reported anovulations appear to be follicular cysts and not associated with increased progesterone production [10]. Prostaglandin administration has been used successfully in African white [42] and black rhinoceroses (Schook, unpublished results) to lyse and regress the former type of PAF involving luteinized tissue. Typically, administration of prostaglandin 7 d following initial PAF development is effective, although two doses 5 d apart are sometimes required (Schook, unpublished results).

4. Controlling ovarian function

There are a variety of applications for controlling ovarian function in rhinoceroses, from timed breeding and ART procedures to down-regulating ovarian activity in individuals with extensive reproductive pathology. Most reports have focused on various methods employed to synchronize estrus or to stimulate ovarian function in post-partum, anestrous or abnormally cycling African white and black rhinoceroses (Table 1). Substantially less research in this area has been conducted in Asian rhinoceroses, in part, because much can be achieved using natural cycles, and Asian rhinoceroses are not prone to acyclicity. However, the success of AI in Indian rhinoceroses timed to a natural estrus warrants the establishment of a synchronization protocol to improve logistical feasibility and expand its application and reach. The most common form of controlling ovarian function in rhinoceroses has mirrored that proven successful in domestic livestock species; the administration and subsequent withdrawal of exogenous progesterone to prompt follicular growth.

4.1. Estrus induction and synchronization

Much has been accomplished in terms of stimulating follicle development and maturation in both cycling and non-cycling female rhinoceroses. Similar to domestic and non-domestic equids [43,44], long and variable follicular phase lengths make consistent timing of ovulation a challenge. Therefore, an important aspect of exogenous progestin protocols in rhinoceros species involves validating via ultrasonography that follicle growth is halted and existing follicles become atretic during the timeframe of progestin administration. This has been shown to narrow the window of ovulation in equids [43–45], Indian (Stoops, unpublished results), and African white (Stoops, unpublished results) rhinoceroses.

4.1.1. Altrenogest

Multiple attempts have been made in African rhinoceros species to induce estrus and breeding using the synthetic progestin (altrenogest) for 12–23 d, sometimes preceded by administration of a synthetic prostaglandin (cloprostenol sodium) [46]. Eight of eleven trials were unsuccessful in stimulating breeding activity. However, in three eastern black rhinoceroses, 12–14 d trials of altrenogest, two of which were initiated during the luteal phase of a cycle, resulted in conception via natural breeding and birth of a calf [46,47]. In three trials involving white rhinoceroses, additional ovarian stimulation was included in the days immediately prior to and following altrenogest withdrawal via administration of FSH or PMSG. Estrous behavior and/or large follicles were observed in three of four animals but ovulation was not confirmed in any of the individuals [46]. Without serial ultrasound data to document ovarian activity during altrenogest administration, it is difficult to accurately assess the effectiveness of its down-regulatory role in these early trials. Follicle development has been observed with altrenogest-only treatment (0.044 mg/kg body wt) in Indian rhinoceroses (Stoops, unpublished results) and Sumatran rhinoceroses (Dr. Dedi Candra, personal communication).

4.1.2. Chlormadinone acetate (CMA)

Hermes et al. [30] conducted more than 30 trials involving an alternative progestin (chlormadinone acetate) coupled with one of three ovulation inducing agents and demonstrated variable success at stimulating ovarian and luteal activity in both anestrous and

Table 1
Review of attempted protocols for control of ovarian function in black and white rhinoceroses.

Species	Repro status	Trial	Prostaglandin (days, dose)	Progestin (days, dose)	Ovarian stimulation (day, dose)	Ovulation induction (day, dose)	Result	Reference
Black Rhinoceros	1 ♀ nulliparous	1	Fluprostenol (unk)		PMSG (unk)		no estrus	[46]
Diceros bicornis		2		altrenogest (0–12; 82.5 mg)			no estrus	[46]
	3 ♀, 2 nulliparous, 1 parous	3		altrenogest (0–12; 110 mg)			estrus, breeding, calf	[46]
White Rhinoceros	1 ♀ nulliparous, anestrous	1		altrenogest (0–23; 110 mg)			no change	[46]
Ceratotherium simum	1 ♀ nulliparous, anestrous	1	cloprostenol (0,12; 75 mg)	altrenogest (53–66; 92.4 mg)			no change	[46]
	2 ♀ nulliparous, anestrous	1	cloprostenol (0,13; 75 mg)	altrenogest (57–65; 92.4 mg)			attemtped 2x/no change	[46]
		2	cloprostenol (0,3; 75 mg)	altrenogest (39–45; 92.4 mg)			no change	
		3	cloprostenol (0–2; 75 mg)	altrenogest (3–17; 92.4 mg)			attempted 2x/no change	
	2 ♀ unk	1		altrenogest (0–23; ~40 mg)		deslorelin (21; 2.5 μg/kg)	no mating or estrus	[53]
	1 ♀ unk, prior to euthanasia	1	cloprostenol (0,23; 500 μg)	altrenogest (0–21; 2.2 mg/kg)	PMSG (19, 22; 5000, 2500 IU)	cystorelin (26, 500 μg)	estrus, antral follicles 28 d prior to euthanasia	[46]
	nulliparous, anestrous	1	dinoprost (0,32; 50 mg)	altrenogest (15–30; 0.044 mg/kg)	FSH-P (29; 20 units)	hCG (38; 5000 IU)	large follicles 39 d prior to euthanasia	[46]
	1 ♀ anestrous	1		CMA (0–45; 35 mg/36 h)		hCG (50; 8500 IU)	attempted 2x, P4 increase 17–18 d post hCG	[46]
	2 ♀, nulliparous, anestrous			altrenogest (0–21; 132 mg)	FSH-P (20–23; 40,30,20,10 units)	hCG (25; 8000 IU)	no response in one ♀; etrus + breeding in second ♀ on d 24, 26, 27	[46]
	12 ♀, anestrous	1		CMA (0–45, 35 mg)		hCG (25; 10,000 IU)	4 ♀, no change; 8 ♀, luteal level P4; 4 ♀, reduced luteal progestins	[30]
	8 ♀, anestrous	2		CMA (0–45, 35 mg)		deslorelin biorelease (55; 3 mg)	1 ♀ pregnant, 3 ♀ no change, 3 ♀ prolonged luteal P4, 1 ♀ 30-d luteal P4	[30]
	5 ♀, anestrous; 1–5 replicates/trial/♀	1		P4LA (0; 3000 mg or 4500 mg)		gonadorelin (~23; 250 μg)	all ♀ ovulated (12 ov total), 6 ♀ 30-d and 6 ♀ 70-d luteal phases	[49]
		2		P4LA (0; 3000 mg)	estradiol (~28; 100 mg)		3 ♀ grew large follicles and 2 ♀ bred, both 30- and 70-d luteal phases observed	[49]
	15 ♀, 12 anestrous and 3 cycling.	3		CMA (0–45, 35 mg)		deslorelin implant (55; 4.2 mg)	1 ♀ pregnant, 1 ♀ no change, 10 ♀ prolonged luteal P4, 3 ♀ 30-d luteal P4	[30]

unk, unknown; CMA, chlormadinone acetate; PMSG, pregnant mare serum gonadotropin; FSH-P, Follicle stimulating hormone -porcine; hCG, hunan chorionic gonadotropin; P4, progesterone; P4LA, biorelease progesterone.

cycling white rhinoceroses. One day after ovulation induction, 82.8% of those examined by ultrasound contained a pre-ovulatory size follicle, but 10% already exhibited large, established CLs suggesting a portion of these rhinoceroses may have experienced follicle development, and perhaps even ovulation, while under the influence of exogenous progestins, similar to what has been reported in domestic horses [45,48]. Such activity can impede timed ovulation protocols that rely on ovarian down regulation and recruitment of a new cohort of follicles following progestin removal, because existing follicles may proceed to ovulate earlier than expected once progestin administration halts [45,48]. In addition, several females exhibited prolonged luteal increases in progesterone (70 d) [30]. Given that ovulation was not confirmed by ultrasound in most trials, it is possible that the progesterone producing structure was a PAF in some cases rather than a post-ovulatory CL.

4.1.3. Long acting progesterone

New, long-acting formulations of progesterone and estrogen make application to non-domestic species more viable [44]. A recent study of anestrous white rhinoceroses in North America was carried out using a single injection of long-acting progesterone in combination with estradiol or GnRH [49]. Regardless of treatment, all females (n = 5) responded with growth of a pre-ovulatory follicle after progesterone decline and ovulated in response to GnRH (Table 1). Two of three females treated with estradiol instead of GnRH at late follicular phase exhibited estrus, mated, ovulated and resumed spontaneous reproductive activity. Subsequent estrous cycles occurred at either 30 or 70 d post-GnRH or estradiol. Interestingly, without GnRH or estradiol, follicles continued to grow and did not ovulate.

4.2. Ovarian superstimulation

Ovarian superstimulation has been attempted in African black and white rhinoceroses with some success. Treatment consisted of deslorelin administration three times at 48 h intervals, and follicle number was significantly higher than that observed during unstimulated cycles [37]. An average of 4.8 (range, 2 to 9) cumulus oocyte complexes (COCs) per ovum pick up attempt were aspirated 48–72 h following the final deslorelin treatment in both species, and one oocyte recovered from a black rhinoceros cleaved to four cells following *in vitro* fertilization [37]. Similar attempts in two Sumatran rhinoceroses in Sabah, Malaysia, and more recent attempts in the African white rhinoceros have been less encouraging with just 1.7 and 2.1 COCs on average collected during seven and eight collection attempts, respectively [50]. Regardless, two of the oocytes harvested from African white rhinoceroses cleaved to the

5-cell stage following intracytoplasmic sperm injections [50]. However, the details of the superstimulation protocols were not reported, and because Sumatran rhinos naturally develop multiple follicles during a cycle [6,7], it would be possible to harvest ≥ 2.0 COCs/cycle without exogenous stimulatory treatment.

4.3. Ovarian down regulation

In contrast to the above efforts, there are instances when ovarian down regulation is the desired outcome. Down regulation of ovarian function has been used as a strategy to treat reproductive tract tumors in seven African white and Indian rhinoceroses greater than 23 years of age [24]. A gonadrotropin releasing factor (GnRF) vaccine (Improvac, Zoetis; 450 μg) was administered at 0, 4 and 16 weeks, with boosters given every 6–8 months. The authors reported that the vaccine suppressed ovarian and luteal activity while effectively reducing tumor size and associated bloody vaginal discharge. Given the extremely high risks associated with surgeries required to remove these sometimes life-threatening tumors, and the difficulties in removing them laparoscopically [51], hormonal down-regulation is a much more palatable approach.

5. Ovulation induction

Effective ovulation induction protocols for all managed rhinoceros species are desired for enhancing AI success and reducing luteinized follicle formation that results from anovulatory cycles and can contribute to irregular cyclicity. Furthermore, Sumatran rhinoceroses are induced ovulators requiring an exogenous stimulus to ovulate if interaction with a male rhinoceros is not allowed. Finally, in the acyclic white rhinoceros that produces only immature follicles, exogenous hormone treatment is necessary to induce both follicular growth and ovulation.

Early efforts to induce ovulation in rhinoceroses followed treatments to stimulate ovarian activity in isolated cases and were largely ineffective or inconclusive (Table 1) [46]. Now, several horse and cattle products have been tested more rigorously for inducing ovulation in rhinoceroses, namely hCG and GnRH analogues (gonadorelin, histrelin, deslorelin), the latter of which have been administered in saline, oil and implants via hand injection (im or sc) pole syringe or darting. Timing of administration has been based on prior hormone treatment for stimulating follicular growth, size of a dominant follicle, rhinoceros behavioral estrus, hormone concentrations or a combination of these indicators. In most cases, ovulation has been confirmed by ultrasonography or pregnancy, but in some cases, it was inferred based on elevated progesterone following treatment.

5.1. African black rhinoceros

Whereas, ovarian superstimulation attempts have been reported for black rhinoceroses [37], little to no information has been published on ovulation induction. Recent trials involving the use of gonadorelin or histrelin have been attempted in parous, cycling black rhinoceroses (Table 2; Schook, unpublished results). Histrelin (1.5 mg im) induced ovulation within 48 h in 5 of 6 attempts. Gonadorelin (200 μg im) administration was only attempted once and was not successful at inducing ovulation though follicle diameter at the time of treatment was larger (4.6 cm) than in most attempts with histrelin.

5.2. African white rhinoceros

Most ovulation induction attempts in the African white rhinoceros have been conducted in conjunction with estrus synchronization or ovarian stimulation trials followed by AI or attempted breeding. A thorough summary of this work was reported by Hermes et al. [30]. In short, a GnRH implant appeared most successful at inducing ovulation (93.3%). However, both hCG and GnRH biorelease were moderately successful (66.7% and 62.5%, respectively) and were somewhat disadvantaged since they were only tested on females that were acyclic prior to estrus induction treatment, whereas the implant was used to treat six cycling females in addition to eight acyclic females. It is impossible to discern when ovulation was directly attributed to treatment and when it would have occurred spontaneously without treatment. Several rhinoceroses were confirmed ovulating within 24 h of treatment, and therefore were likely responding to their own internal trigger and not the exogenous hormones administered to induce ovulation. Ovulation in response to these hormones is expected >24 and < 48 h after injection in most species, including rhinoceroses. Interestingly, hCG was more likely to result in a ~30 d luteal phase when ovulation was successful in contrast to the GnRH biorelease and implant which more frequently resulted in a ~70 d luteal phase. These extended luteal phases are unusual when compared to well established natural estrous cycles of the African white rhinoceros which, in their entirety, last 30 to 35 or 65–70 d, with the interluteal phase occupying ~10 d of the cycle [3,5,8]. Regardless, females treated with GnRH implants have conceived and carried term pregnancies following AI [28,29]. More recent efforts at inducing ovulation in both acyclic and cyclic females using the GnRH analogue gonadorelin have produced promising results despite its shortened stimulus compared to implants and biorelease forms [49]. Subsequent estrous cycles following ovulation induction using gonadorelin were shown to occur with equal frequency of either 30 (55%) or 70 (45%) days, similar to natural cycle lengths.

5.3. Indian rhinoceros

Indian rhinoceroses that are not breeding naturally often develop anovulatory follicles which impede AI success [10,22]. Early attempts to induce ovulation in the Indian rhinoceros during natural cycles with high dosages of gonadorelin (500 μg im) were not very encouraging. Although two of five attempts resulted in ovulation, the timing (8–12 h post-administration) suggested that ovulation was triggered naturally prior to treatment (Table 2) [10,22]. Timing of treatment is critical, but follicle size cannot be used to properly time injections in this species, therefore behavioral estrus serves as the primary cue. Unfortunately, behavioral estrus can be unreliable because some Indian rhinoceroses experience silent estrus [11] and others exhibit estrous-like behavior on more than one occasion during a cycle [10]. Mistimed injections were likely to blame for anovulatory results in early trials, and later work with gonadorelin and both short and long-acting deslorelin have demonstrated much higher ovulatory success (>90%) by 40–48 h post-treatment (Table 2) [22].

5.4. Sumatran rhinoceros

The most detailed studies of natural ovulation have been conducted in the Sumatran rhinoceros, the only known obligate induced ovulator within the rhinoceros taxon. Strategic serum sample collections at times prior to and after copulation clearly demonstrated the sharp, but short-lived increase in LH following mating, with ovulation occurring within 48 h [7]. Given the brief, natural LH surge in this species, the short-acting GnRH analogue, gonadorelin, was the hormone of choice for extensive studies on ovulation induction for timed AI [31]. Gonadorelin proved to be highly effective at inducing ovulation in Sumatran rhinoceroses at doses ranging from 50 to 500 μg (0.073–0.733 μg/kg) administered

Table 2
Ovulation success following induction trials employing various hormones, dosages, methods of delivery and timing of treatment in each rhinoceros species.

Species	Treatment	Dose/delivery	Follicle size (cm)	Attempts	Ovulated (%)	Reference
African black rhinoceros	GnRH analogue (gonadorelin)	200 μg im	4.4 × 4.6[a]	1	0	
(*Diceros bicornis*)	GnRH analogue (histrelin)	1.5 mg im	4.4 × 4.5[a]	1	100	
	GnRH analogue (histrelin)	1.5 mg im	3.9 to 4.2[a]	5	80	
African white rhinoceros	GnRH analogue biorelease	3 mg im in oil	3.2–3.4[b]	8	62.5	[30]
(*Ceratotherium simum*)	GnRH implant	4.2 mg sc	3.4–3.8[b]	15	93.3	[30]
	hCG	10,000 IU im	3.2–3.8[b]	12	66.7	[30]
	GnRH analogue (gonadorelin)	250 μg im	3.0 to 3.1	11	100	[49]
Indian rhinoceros	GnRH analogue (gonadorelin)	500 μg im	>10.0	5	40	[10,22]
(*Rhinoceros unicornis*)	GnRH analogue (deslorelin)	3.75 μg im	14.2 to 14.6	2	100	[22]
Sumatran rhinoceros	GnRH analogue (gonadorelin)	500 μg im	2.0 × 2.1	1	100	[31]
(*Dicerorhinus sumatrensis*)		100 μg im	1.6 × 2.0 to 2.2 × 2.3[c]	11	100	
		50 μg im	1.7 × 2.3; 1.8 × 2.2[c]	2	100	
		25 μg im	2.0 × 2.2[c]	1	0	
		10 μg im	2.0 × 2.1[c]	1	0	
		100 μg sc	1.9 × 2.0 to 2.0 × 2.1[c]	3	100	
		250 μg vaginal	2.1 × 2.0[c]	1	0	
		250 μg rectal	1.9 × 2.2[c]	1	0	
		500 μg vaginal	1.9 × 2.1[c]	1	0	
		500 μg rectal	1.9 × 2.1[c]	1	0	

[a] Schook, unpublished results.
[b] 24 h post-treatment.
[c] Roth, unpublished results.

during a natural cycle when a follicle reached the pre-ovulatory size of ~19–22 mm diameter (Table 2) [31]. Both shoulder im and foot sc injections were effective, but two attempts each at rectal and vaginal delivery of 250 or 500 μg failed to induce ovulation (Table 2). On a few occasions, serial urine samples were collected following treatment to measure the LH response (Fig. 4) [10] which appeared similar to that described for serum LH during natural mating [7] and that reported for urinary LH in the Indian rhinoceros following induced [10] or natural, spontaneous ovulation (Fig. 2); concentrations increased within a couple hours of injection and then dropped back down to baseline by 6–8 h post-injection. Following successful treatments, ovulation always occurred within 48 h of injection, and the subsequent luteal phase was always normal resulting in a 20–21 d cycle. The shortest time from treatment to ovulation was 33 h, and when two pre-ovulatory size follicles formed, both would ovulate in response to treatment.

6. Overcoming pregnancy loss

Early embryo loss, miscarriages and abortions have been reported in all four managed rhinoceros species [2,7,11,18,19,22,23]. There is no definitive proof that luteal insufficiency leads to early embryo loss in rhinoceroses, but it has been suggested as a contributing factor, and there is mounting evidence that supplemental altrenogest can help afflicted rhinoceroses carry a pregnancy to term. The first report of altrenogest use in rhinoceroses was in 1995 [18] providing anecdotal evidence that the supplement supported a term pregnancy in an aged black rhinoceros after abortions at three different stages of gestation. Since then, altrenogest has been used on numerous African black, Indian and Sumatran rhinoceroses with a history of confirmed pregnancy loss, and most females have carried a term pregnancy while supplemented, including two black rhinoceroses (2 pregnancies) [2,18; Dr. Chris Miller, personal communication], three Indian rhinoceroses (4 pregnancies) [11,22; Dr. Gabriela Mastramonaco, personal communication] and two Sumatran rhinoceroses (3 pregnancies) [6; Dr. Dedi Candra and Dr. Zulfi Arsan, personal communication]. Not surprisingly, some supplemented rhinoceroses still fail to carry a pregnancy to term [11]. In all reported cases, the equine dosage of altrenogest (0.044 mg/kg) has been used. This dosage of altrenogest does not inhibit ovarian follicular development in Asian rhinoceroses as both Indian and Sumatran rhinoceroses that did not conceive or lost embryos while on the supplement proceeded to develop pre-ovulatory follicles [11; Dr. Dedi Candra, personal communication]. Furthermore, endocrine monitoring does not reveal any evidence of progesterone deficiency in rhinoceroses that experience embryo loss/early abortion compared to those that carry natural pregnancies to term without supplement [2,23]. Therefore, it has been proposed that altrenogest functions qualitatively (i.e., via different modes of action on the maternal reproductive tract or developing embryo) versus quantitatively to help maintain rhinoceros pregnancies [23], but the actual mechanistic pathway has yet to be elucidated.

7. Conclusions

The application of reproductive science and its associated technologies to the study of rhinoceros ovarian function has benefitted the managed breeding programs substantially while increasing our understanding of the basic reproductive physiology of this taxon. Our ability to directly or indirectly monitor natural ovarian function has been key to the success of breeding Sumatran rhinoceroses [6,7] and has facilitated the same in Indian rhinoceroses [11]. Furthermore, it has proved essential for timing both AI in Indian rhinoceroses [22] and treatment to overcome anovulation in all four managed rhinoceros species (Sumatran rhinoceroses, [31]; Indian rhinoceroses, [10,22]; African white rhinoceroses, [49]; African black rhinoceroses, Table 2). Controlling ovarian function is instrumental for planning fixed time ART procedures [28–30,37] and necessary for overcoming ovarian dysfunction that results in irregular cycles and hinders natural breeding in African white rhinoceroses [30,49]. Finally, some of these same technologies developed and validated on rhinoceroses maintained in zoos have since been applied to free-ranging rhinoceroses, thereby enhancing our comprehension of these in situ populations, their reproductive activities and the challenges they face [12–15,52].

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