OVULATION INDUCTION FOR TIMED ARTIFICIAL INSEMINATION IN THE SUMATRAN RHINOCEROS (*Dicerorhinus sumatrensis*)

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Abstract

The development of artificial insemination (AI) in the Sumatran rhino has become a priority to overcome behavioral incompatibility between some rhino pairs and provide a means of genetic exchange among participating countries without the transfer of live animals. Because the Sumatran rhino is an induced ovulator, a proven ovulation induction protocol is essential for successful AI in this species. The goal of this study was to evaluate the ability of exogenous hormone administration to mimic the physiological responses previously documented in a naturally mating female Sumatran rhino that successfully reproduced. Thrice weekly rectal ultrasound exams were conducted to monitor a young, sexually mature rhino's ovarian activity. When a preovulatory follicle measuring 21 x 22 mm in diameter was observed (Day 0), the rhino was administered 500 µg gonadotropin releasing hormone (GnRH; Cystorelin; i.m.). After treatment, ultrasound exams were conducted every 12 h until ovulation was confirmed by follicular collapse, and then thrice weekly to monitor ovarian activity throughout the luteal phase and subsequent follicular phase. Urine samples were collected daily prior to GnRH administration, as often as possible during the 36 h period following GnRH, and then every other day through Day 12. Blood samples were collected 41 min and 10 days after GnRH treatment. The follicle was still present 36 h post-GnRH but had ovulated by 48 h post-GnRH (Day 2). Following ovulation, both ovaries remained relatively inactive until another pre-ovulatory follicle formed on Day 23. No GnRH was administered, and the follicle grew to >30 mm in diameter without ovulating. Compared to baseline serum luteinizing hormone (LH) concentrations in this female (1.37 ng/ml), LH had increased to 9.97 ng/ml 41 min following GnRH treatment, thereby confirming that the pituitary had responded. Urinary LH concentrations revealed more detail about the dynamics of the pituitary's response. From an initial baseline value of 962 pg/ng creatinine (Cr), urinary LH peaked at 2,288 pg/ng Cr 7 h post-GnRH and then dropped rapidly below baseline (161 pg/ng Cr) 11 h post-GnRH. On Day 1, LH had recovered to typical baseline concentrations. Serum progesterone concentrations on Day 0 and Day 10 were 0.32 and 1.25 ng/ml, respectively, confirming the formation of an active corpus luteum. In conclusion, changes in LH, progesterone and ovarian activity following specifically timed GnRH administration mimic those observed following natural mating in the Sumatran rhino. Therefore, this ovulation induction regimen should help pave the way for successful AI in this species.

Introduction

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is critically endangered with no more than 200 surviving in the wild and just 9 animals in captivity. The captive breeding program was initiated in the 1980s as part of a two-pronged approach consisting of ex situ protection and in situ propagation to ensure the species' survival (Khan et al., 1999). To-date, only three calves have been produced from one pair of rhinos at the Cincinnati Zoo & Botanical Garden. Therefore, four of the nine captive rhinos are closely related. Furthermore, behavioral incompatibility and physical challenges between male and female pairs has limited the success of natural mating among the remaining rhinos. Hence, the development of artificial insemination (AI) is deemed necessary to enhance current captive breeding efforts. AI in conjunction with sperm banking would provide an alternative to inbreeding without requiring the international transport of live rhinos. In addition, these technologies would provide a means of producing pregnancies in behaviorally incompatible pairs.

Sperm cryopreservation in the Sumatran rhino has already been achieved, and post-thaw sperm function appears adequate for producing embryos (O'Brien and Roth, 2000; Stoops et al., 2011). However, no offspring have been produced from these samples. Until now, AI simply was not necessary since natural mating among founder pairs was ongoing. Because the Sumatran rhino differs from other rhino species in being an induced ovulator (Roth et al., 2001; Roth, 2006), the next step in developing AI for this species is establishing an ovulation induction protocol. Although it is known that ovulation occurs following female-male interactions during a female's estrus, the exact stimulus has not been identified and is not dependent on successful copulation (Roth et al., 2001). Regardless, the sharp, short-lived endogenous release of luteinizing hormone (LH) that occurs following an introduction to a male is ultimately responsible for inducing follicular rupture. The timing and amplitude of this LH spike following mating has been documented in a fertile female (Roth et al., 2001). Additionally, gonadoropin releasing hormone (GnRH; Cystorelin) administration in an old, acyclic Sumatran rhino produced a spike in LH similar to that induced by natural mating (Patton et al., 1998). Therefore, the hypothesis of this study was that a GnRH injection delivered to a reproductively active female Sumatran rhino with a pre-ovulatory follicle would induce an LH surge and ovulation in a manner that mimics that of a female allowed to mate naturally.

Methods

A sexually mature, 5 ½ yr old female Sumatran rhino at the Cincinnati Zoo & Botanical Garden previously conditioned to voluntarily allow rectal ultrasound examinations was used for this study. Thrice weekly ultrasound exams were conducted to monitor ovarian activity prior to treatment. When a fresh preovulatory follicle was observed (Day 0), the rhino was administered 500 µg GnRH (Cystorelin; i.m.) in the neck. After treatment, ultrasound exams were conducted

every 12 h until ovulation was confirmed by follicular collapse, and then thrice weekly to monitor ovarian activity throughout the luteal phase and subsequent follicular phase. Urine samples were collected non-invasively from the stall floor after the female squirted urine or by catching a sample into a cup on a pole during periods of free-flow urination to monitor LH concentrations. Samples were collected daily prior to GnRH administration, as often as possible during the 36 h period following GnRH, and then every other day through Day 12. Blood samples were collected from a vein on the outer pinna (back of the ear) using a tourniquet, 23 ga x 1 inch butterfly catheter and 6 cc syringe. Samples were obtained 41 min after GnRH treatment to validate an LH surge had occurred, and again on Day 10 to confirm a luteal phase had resulted.

Urine samples were divided into two equal aliquots, with half frozen immediately and later thawed and analyzed for creatinine concentrations (Taussky 1954). The remaining urine was concentrated via ultrafiltration, resuspended 1:1 in EIA buffer and stored at -80°C until analyzed for LH concentrations by the EIA methodology reported by Stoops et al., 2004. Serum progesterone was extracted and concentrations determined by EIA following the protocol described by Roth et al., 2001 and using monoclonal antibody CL425 (provided by C. Munro, University of California, Davis, CA).

Results

A pre-ovulatory follicle measuring 21 x 22 mm in diameter and 6.74 mm in circumference was observed on March 9th (Day 0), just 10 days after initiating the study. This follicle was still present 36 h post-GnRH measuring 22 x 24 mm in diameter and 7.11 mm in circumference but had ovulated by 48 h post-GnRH (Day 2). Following ovulation, both ovaries remained relatively inactive through Day 20. On Day 22, a 17 x 19 mm follicle was present and grew to pre-ovulatory size (22 x 21 mm) by Day 23. No GnRH was administered, and the follicle continued to grow achieving a size of 32 x 29 mm on Day 32 without ovulating.

Baseline serum LH concentration in this female on Day 10 of the study was 1.37 ng/ml. Serum LH increased from baseline concentrations to 9.97 ng/ml 41 min following GnRH treatment on Day 0. Serum progesterone concentration on Day 0 was 0.32 ng/ml, but it had increased to 1.25 ng/ml by Day 10 following treatment (eight days post-ovulation).

Urinary LH concentrations were useful in tracking pituitary activity over time following GnRH administration. From an initial baseline value of 962 pg/ng Cr the morning of GnRH treatment (Day 0), urinary LH increased each hour peaking at 2,288 pg/ng Cr 7 h post-GnRH. Urine LH concentrations then declined rapidly reaching concentrations below baseline (161 pg/ng Cr) by 11 h post-GnRH. By the next day (Day 1), LH concentrations had recovered to typical baseline concentrations (range, 681 – 1,155 pg/ng Cr).

Discussion

This project demonstrates the value of studying animals in captivity to learn more about a species' physiological mechanisms. Because this rhino was conditioned to allow serial ultrasound exams and blood collection and could be managed under conditions that allowed serial urine sample collections, the effects of this ovulation induction trial could be characterized in significant detail proving that it does elicit similar follicular and endocrine responses to those observed following natural mating.

Sumatran rhinos do not reliably exhibit behavioral signs of estrus, but previous research on one female proved revealed a 21-day estrus cycle, pre-ovulatory follicles ranging in size from 19 to 25 mm in diameter on the day of estrus and ovulation occuring > 24 but < 48 h after mating. (Roth et al., 2001; 2004; Roth 2006). This previously published information served as a valuable resource in developing the methodologies for this study. As a result, the female rhino's physiological responses to treatment were very similar to those previously described in the naturally mating rhino. Because this female was young and had never been paired with a male, this ovulatory cycle was the first she had experienced, but it was followed by an apparently normal luteal phase and the natural development of another pre-ovulatory follicle 23 days later. Although this cycle length is slightly longer than the 21 days described previously, some variation among individuals and even between cycles within individuals would be expected, and a 23 day cycle certainly could be considered within normal range limits for this species.

Based on a previous report, the Sumatran rhino's serum LH concentrations remain at baseline (.5 to .7 ng/ml) throughout most of her reproductive cycle except following the introduction to a male, after which her LH spikes to levels as high as 20 ng/ml (Roth et al., 2001). Baseline serum LH concentrations were slightly higher in this study and could reflect individual animal variation but more likely resulted from a change in methodologies. The previous study utilized a radioimmunoassay developed for elephants (Brown et al., 1999), whereas in this study an EIA protocol was used (Stoops et al., 2004). Regardless, compared to baseline concentrations, serum LH was significantly elevated just 41 minutes following GnRH treatment, thereby validating that the pituitary responded to the GnRH treatment. Similarly, baseline serum progesterone concentrations were slightly higher in this study compared to previous reports (0.3 vs. < 0.1 ng/ml), likely due to slight variation in antibody sensitivity in the EIA system employed. However, the sample collected 10 days after GnRH treatment contained progesterone concentrations equal to those reported for peak luteal concentrations in a naturally mating, ovulating female (Roth et al., 2001; 2004).

Although the female rhino was conditioned for blood collection prior to the study, collecting serial samples throughout the day would have been difficult and unacceptable from an animal welfare perspective. Therefore, urine was collected as a back up to serum in hopes that the dynamics of systemic LH concentrations could be monitored non-invasively throughout the trial. The results indicate that this technique is an accurate, valuable method of assessing LH in

Sumatran rhinos. Previous assessments of serum LH concentrations at different intervals after mating suggest that the ovulatory surge occurs very quickly, peaking 1 h after mating (>20 ng/ml) but declines very rapidly with concentrations reduced to 3.5 ng/ml by 4 h (Roth et al., 2001). The urinary LH profile established herein is in agreement with the previous report. Given an expected lag time between circulating serum concentrations and excretion into the urine, it is not surprising peak LH concentrations occurred at 7 h instead of 1 h post-GnRH, but similar to the pattern reported in serum, concentrations decreased rapidly after achieving peak concentrations and resumed baseline concentrations by the next day.

Overall, results from this trial are very encouraging. Follicular and hormonal dynamics mimicked those previously reported for a regularly cycling, naturally mated Sumatran rhino that proceeded to produce three viable calves. Therefore, this ovulation induction protocol appears promising as a tool for performing AI in Sumatran rhinos that fail to breed naturally.

ACKNOWLEDGMENTS

This study was supported by grants from the Institute of Museum and Library Services (LG-26-08-0155-08) and the Mohamed bin Zayed Species Conservation Fund. The authors are also grateful for the additional help and support provided throughout the study by rhino care staff members Libby Krouskop. Lissa Browning and Mark Bachmann.

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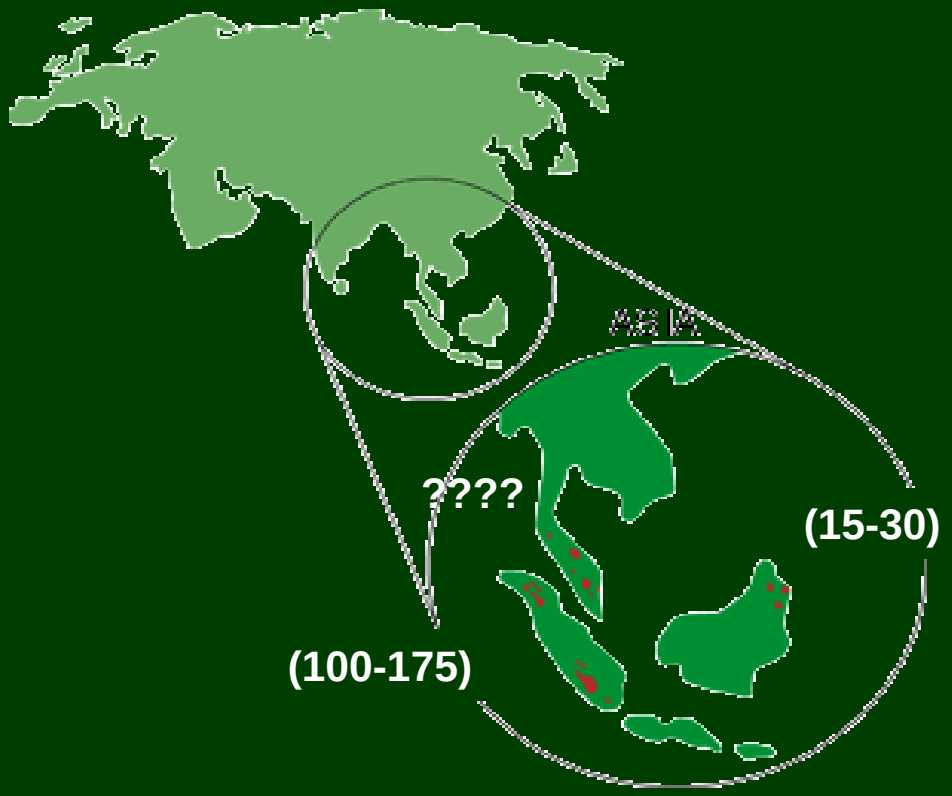
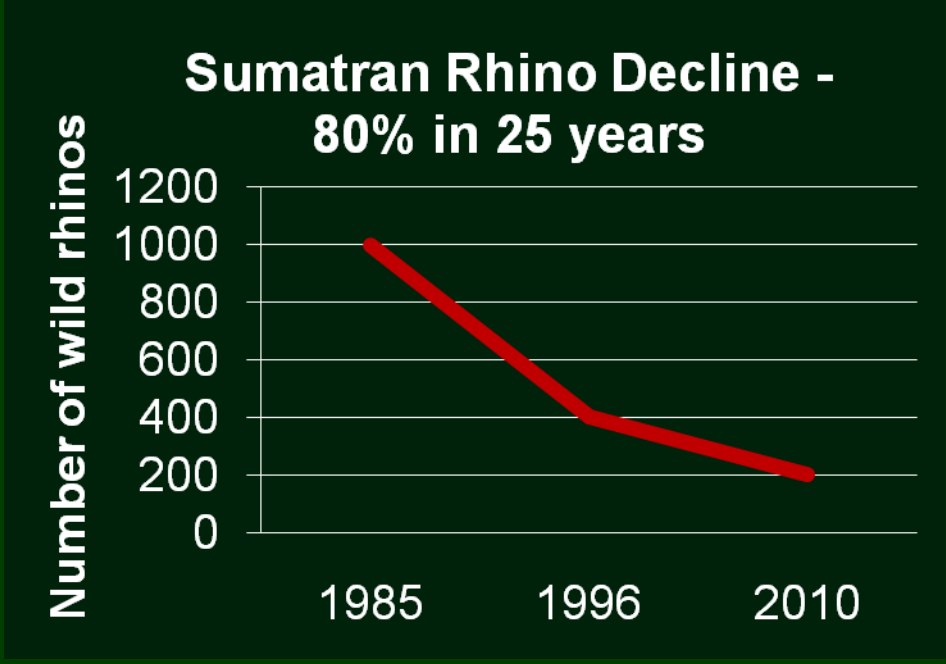
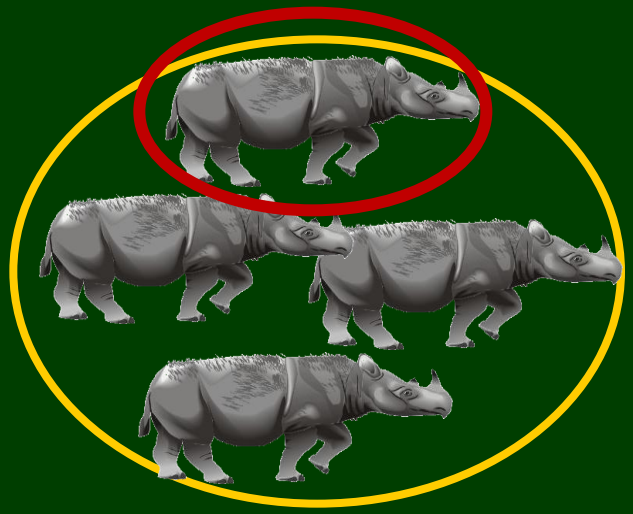
The Status of the Sumatran Rhino – Wild Population



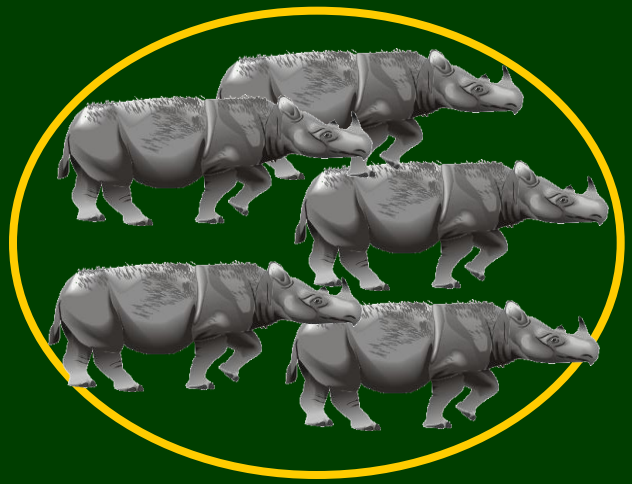


Peninsular Malaysia – 0??
Sumatra – 100-175
Sabah Malaysia – 15-30

Sumatran Rhino Captive Population Past 15 years

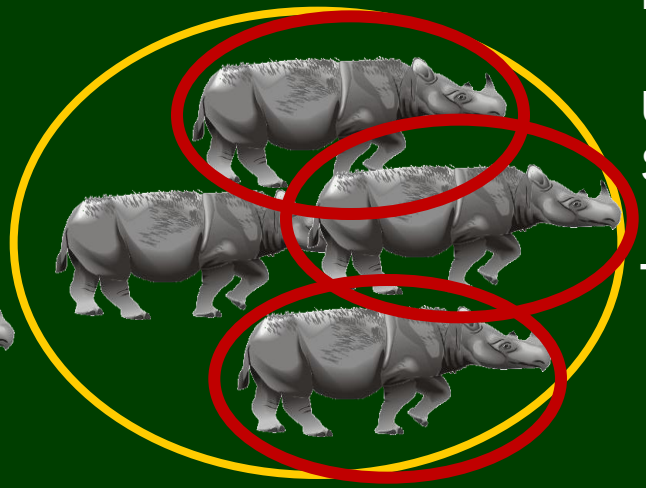


Sumatra, Indonesia



Sabah Malaysia

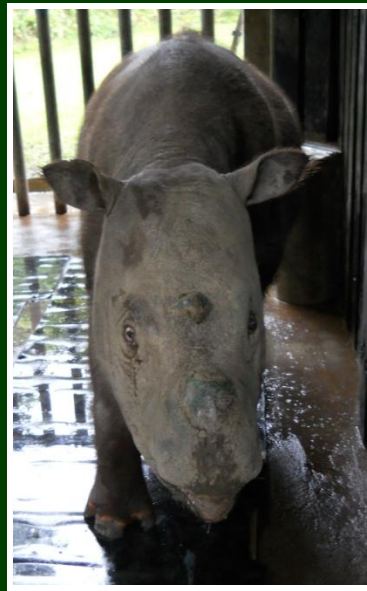
Penninsular Malaysia



United States

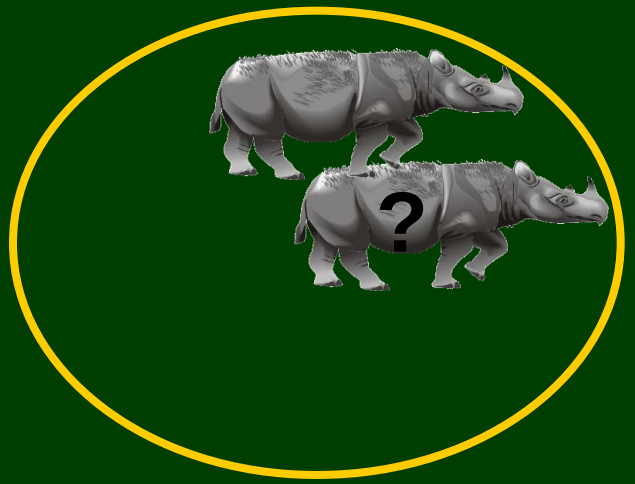
Country	1997	2003	2011
Sumatra, Indonesia	3	2	4
Sabah, Malaysia	5	2	2
Penninsular Malaysia	8	0	0
United States	3	4	3
TOTAL	**19**	**8**	**9**

Today's Captive Sumatran Rhinos

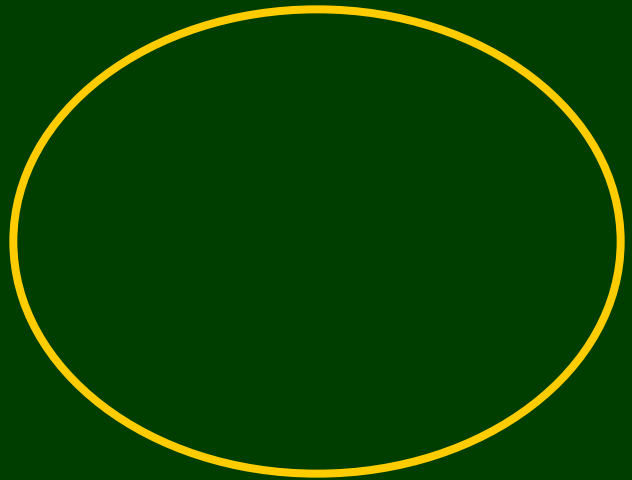


Current Challenges of the Captive Program

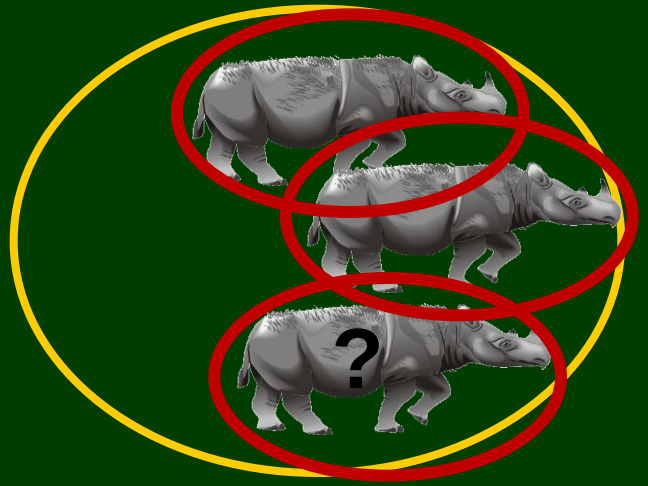
Sumatra, Indonesia



Sabah Malaysia



Penninsular Malaysia



United States

- Low animal numbers
- Most related
- Demographic
- Physical disabilities
- Behavioral incompatability
- Governments unwilling to send rhinos out of country

Could AI provide part of the solution?

What we already know:

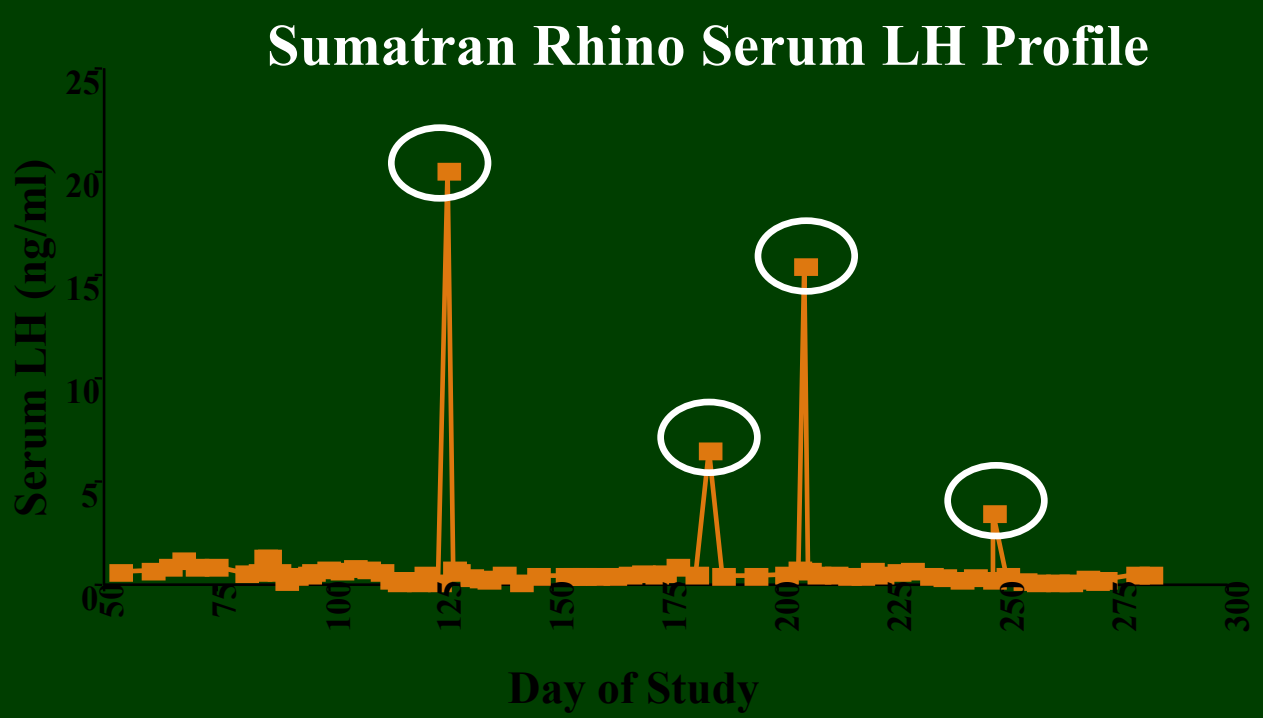
- Cryopreserved sperm appears viable (O'Brien & Roth, 2000; Stoops et al., 2011)
- Basic reproductive physiology has been mapped out (Roth et al., 2001; 2004)

- ✓ Estrous behavior unreliable
- ✓ Induced ovulator
- ✓ Cycle is irregular if not ovulating
- ✓ Estrous cycle approximately 21 days
- ✓ Pre-ovulatory follicle is 19-24 mm
- ✓ Anovulatory follicles often grow larger

Ovulation Induction Protocol Essential

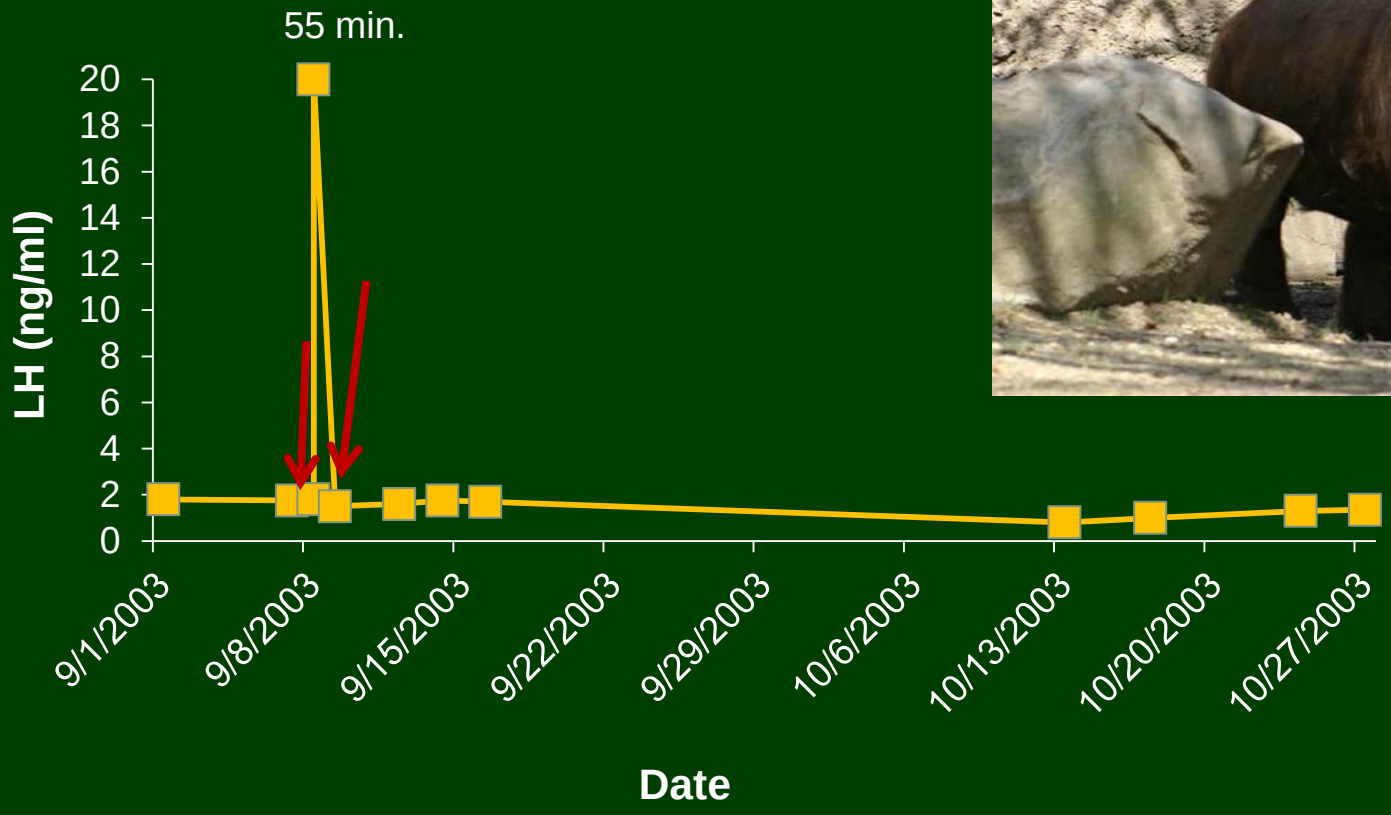
Preliminary data from naturally mating female

- Sharp increase in LH following mating
- LH rise appears short-lived
- LH returns to baseline levels by next day
- Female ovulates between 24 and 48 h after LH surge



Preliminary data from old, acyclic female

- GnRH (Cystorelin) treatment induced LH spike
- Pituitary response similar to that after natural mating
- Inactive ovaries did not respond



Study design

Prior to GnRH administration

- Ultrasound 3X/wk
- Collect urine daily

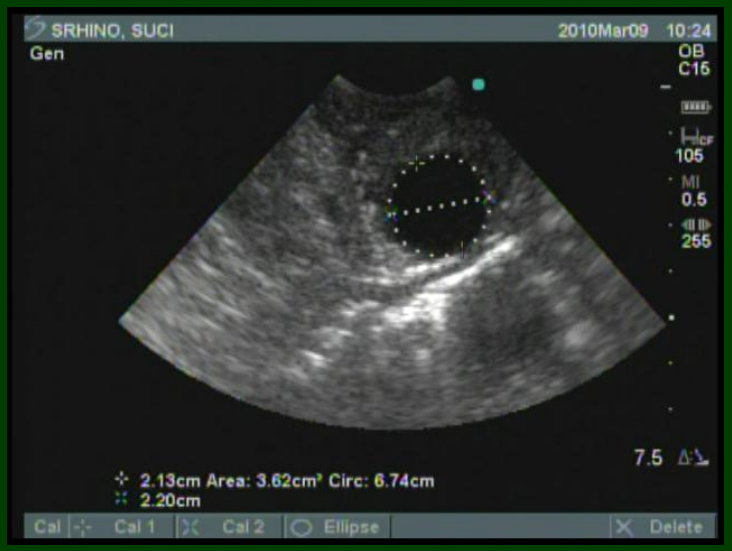
Day of GnRH (Cystorelin; 500 µg; im)

- Ultrasound every 12 h until ovulation confirmed
- Collect all urine samples for 36 h
- Collect blood ~30 min after GnRH to confirm LH spike (EIA)

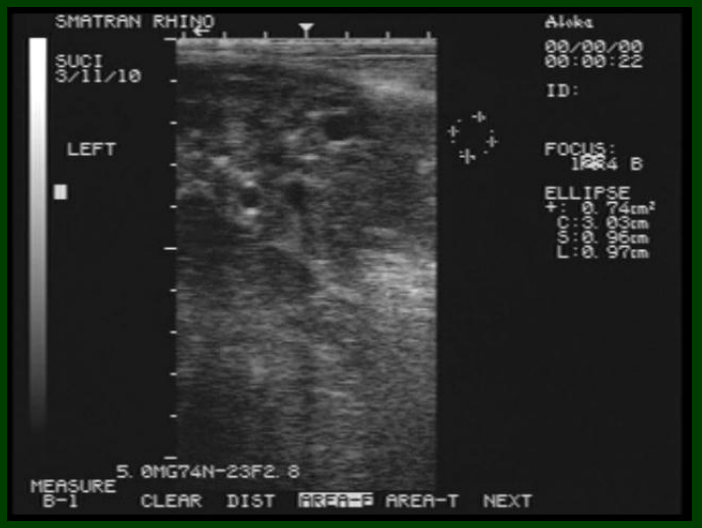
After GnRH treatment period

- Ultrasound 3X/week until next pre-ovulatory follicle is observed
- Collect urine every other day
- Collect blood at Day 10 to confirm luteal phase (progesterone EIA)

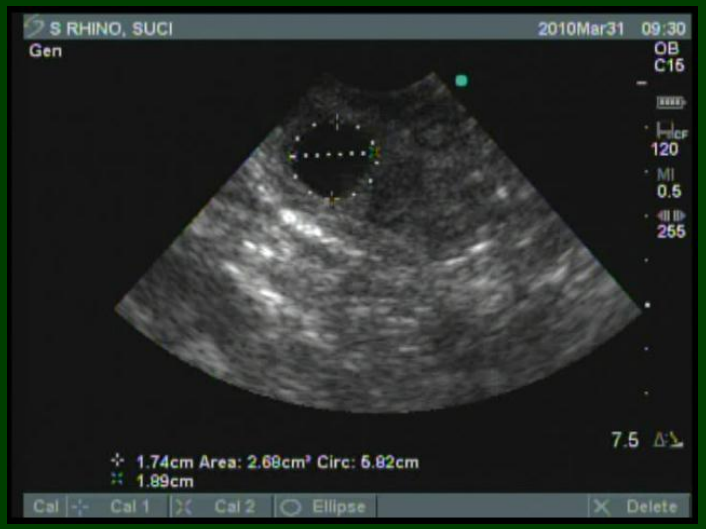
Results - Ultrasound



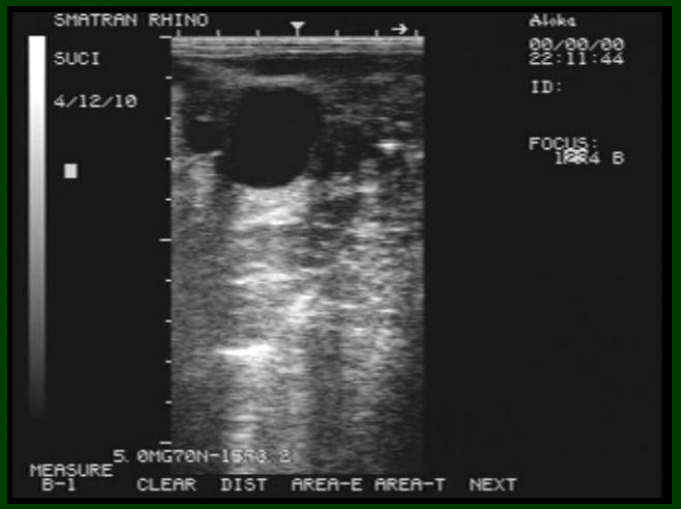
Pre-ovulatory follicle (21 x 22 mm) on left ovary the morning of GnRH treatment



***T**he left ovary following ovulation 48 h after GnRH treatment*

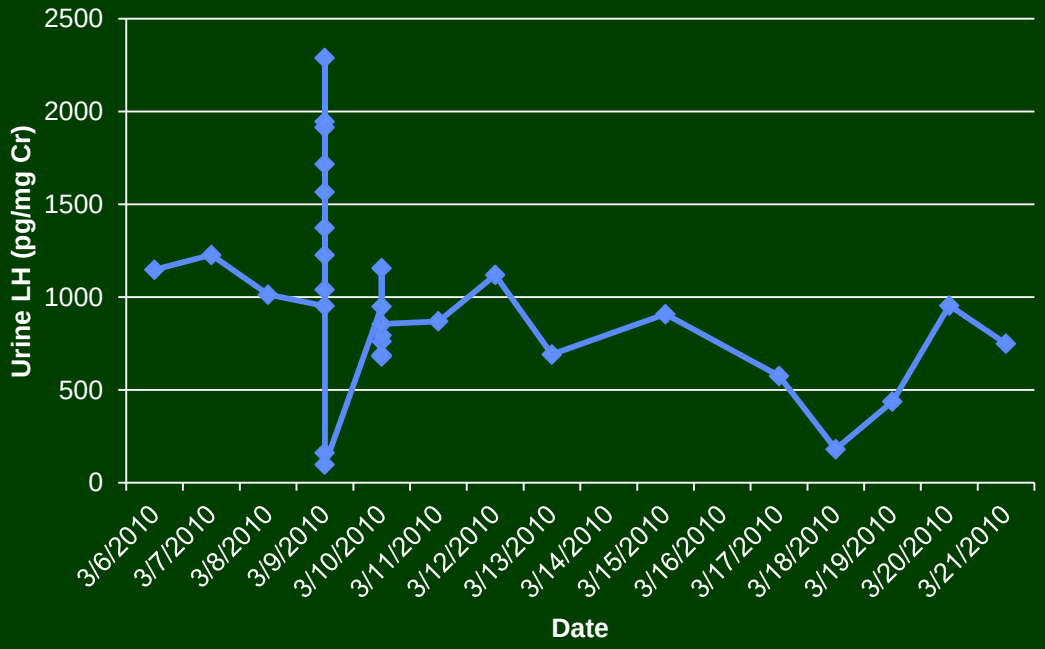


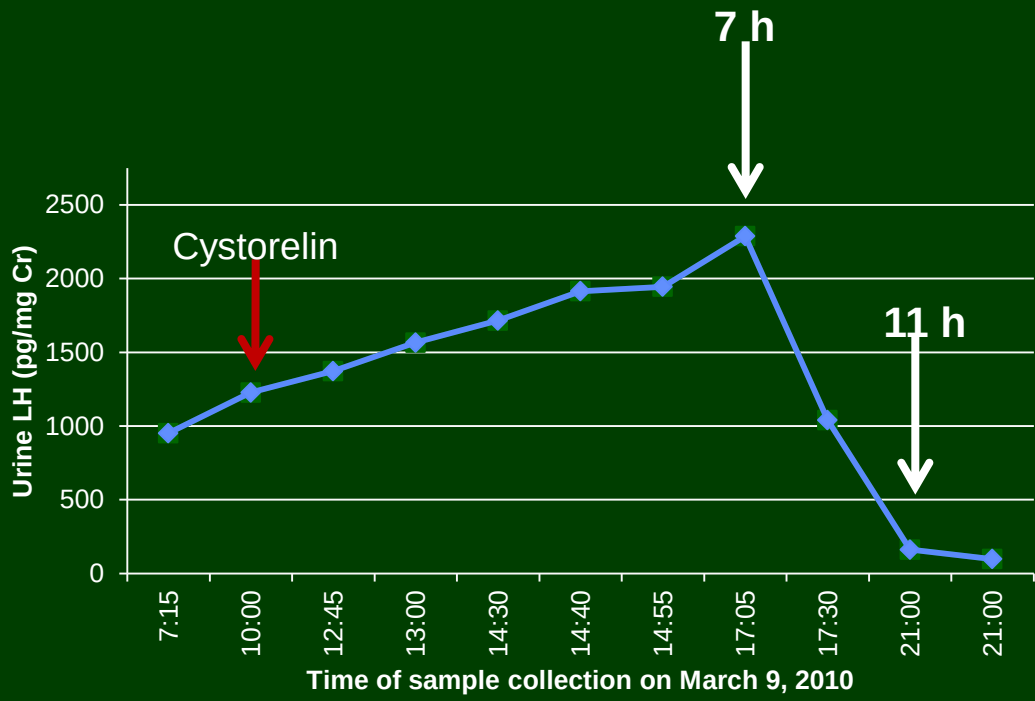
On Day 22, a 17 x 19 mm follicle was present and grew to pre-ovulatory size (22 x 21 mm) by Day 23.



The follicle continued to grow achieving a size of 32 x 29 mm on Day 32 without ovulating.

Results - Urinary LH





Urinary LH concentrations in samples collected during the ovulation induction trial.

Detailed data from serial urine samples collected throughout the day of Cystorelin injection.

Results – Serum Hormones

	Stage of Cycle	
	Estrual (41 min post-GnRH)	Luteal
Date of Sample	3/9/10	3/19/10
LH (ng/ml)	9.97	1.37
Progesterone (ng/ml)	0.320	1.25

Conclusions:

- Cystorelin treatment stimulates LH release by the pituitary
- The pattern of the LH response to Cystorelin appears similar to that for a naturally mating female
- Timing of ovulation after Cystorelin treatment is similar to that observed in a naturally mated female post-copulation
- Urine samples can be used to non-invasively monitor changes in LH concentrations in Sumatran rhinos

Event timeline for naturally mated female Sumatran rhino (top) compared to that for a Cystorelin treated female (bottom)

Day -1 New follicle developing	Day 0 POF Mated LH spike	Day 1 POF Baseline LH	Day 2 Ovulation Baseline LH	Day 10 Baseline LH Progesterone elevated	Day 19 New follicle developing	Day 21 POF	
Day -1 New follicle developing	Day 0 POF GnRH LH spike	Day 1 POF Baseline LH	Day 2 Ovulation Baseline LH	Day 10 Baseline LH Progesterone elevated	Day 22 New follicle developing	Day 23 POF	Day 33 Very large anovulatory follicle

SUMMARY

Follicular and hormonal dynamics mimicked those previously reported for a regularly cycling, naturally mated Sumatran rhino. This ovulation induction protocol appears promising as a tool for performing AI in Sumatran rhinos that fail to breed naturally.

ACKNOWLEDGMENTS

Funding provided by:

- Institute of Museum and Library Services National Leadership Grant (LG-26-08-0155-08)
- The Mohamed bin Zayed Species Conservation Fund

Outstanding assistance provided by:
Cincinnati Zoo rhino care staff members Libby Krouskop, Lissa Browning and Mark Bachmann.


Thank You
Questions?