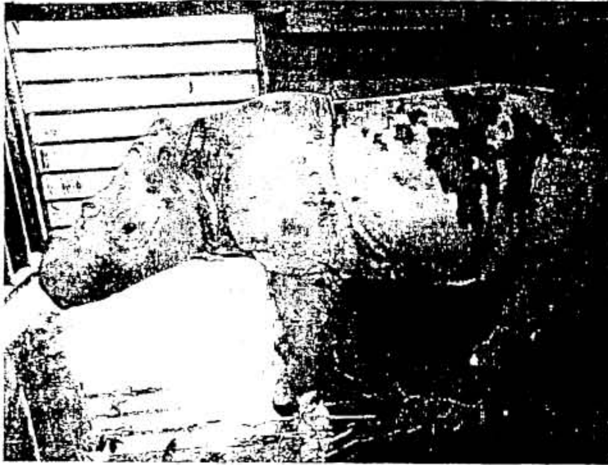
FACULTY OF VETERINARY MEDICINE
UNIVERSITI PUTRA MALAYSIA

REPORT ON THE PROJECT OF FECAL ANALYSIS OF REPRODUCTIVE HORMONE IN SUMATRAN RHINOCEROS



BY

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YAP KENG CHEE

19 January 2001

INTRODUCTION

The sole aim of the project is to be able in helping to determine the best time for mating of the captive Sumatran rhinoceros (*Dicterrhorinus sumatrensis*) at Sungai Dusun Sumatran Rhino Conservation Center (SRCC), Selangor Darul Ehsan through analysis of progesterone metabolite in rhino fecal using enzymeimmunoassay (EIA) technique.

It has been agreed that Universiti Putra Malaysia (UPM) will initially established the EIA techniques for analysis of fecal P4 metabolite using methods as adopted by Roser of Cincinnati Zoo, USA and Prof. Dr. Franz Schwarzenberger of Austria. UPM will also continue to analyze plasma P4 using radioimmunoassay (RIA) technique that will also be used as a control. To achieve this, UPM received:

1. Proper EIA protocols as followed by Roser and Franz methods. These include the extraction and analysis techniques.
2. From Prof. Dr. Franz Schwarzenberger - coating antibody, steroid antibody against 20-oxo-prgnanes, enzyme label (biotynilated steroid) and steroid standard progesterone.
3. 20 samples of lyophilized sieved Sumatran rhino (Emi) fecal samples that were collected during cycling/breeding interval (Oct-Dec 1999).
4. From Dr. Lynn Patton - P4 monoclonal antibody.
5. From International Rhino Foundation (IRF) - a sum of USD5,000.00 to do a pilot study on EIA.

MATERIALS AND METHODS

Collection of Plasma and Fecal Samples

Sungai Dusun SRCC was instructed to collect daily fecal samples and at least twice a week blood samples for a period of 3 months beginning on May 1st until July 31th 2000 from all animals namely, Mas Merah, Minah, Panjang, Rima, and Seputih. Blood samples were collected via coccigeal vein in a vacutainer tube containing sodium heparin as anti-coagulant, centrifuge at 3000 rpm for 10 minutes and plasma were separated and stored at –20°C before sending to UPM. Using a plastic bag, approximately 250g fresh feces were collected from all animals every morning and stored at –20°C.

Fecal Extraction

Extraction of fecal P4 metabolites was conducted base on the method as described in Appendix A.

Analysis of Samples

A. Plasma progesterone

Plasma samples were analyzed using RIA Coat-a-Count kit (DPC, USA). Briefly, the samples were analyzed according to the basic radioimmunoassay as indicated in the Coat-a-Count kit progesterone manual.

1. **Plain Tubes:** Label four plain (uncoated) 12x75 mm polypropylene tubes with T (total counts) and NSB (non-specific binding) in duplicate.

   Coated Tubes: Label fourteen Progesterone Ab-Coated Tubes A (maximum binding) and B through G in duplicate. Label additional antibody-coated tubes, also in duplicate, for controls and patient samples.

Calibrators	ng/mL	nmol/L
A (MB)	0	0
B	0.1	0.3
C	0.5	1.6
D	2	6.4
E	10	31.8
F	20	63.6
G	40	127.2

2. Pipet 100 µL of the zero calibrator A into the NSB and A tubes, and 100 µL of each of the calibrators B through G into correspondingly labelled tubes. Pipet 100 µL of each control and patient sample into the tubes prepared.
3. Add 1.0 mL of ^{125}I Progesterone to every tube and vortex.
4. Incubate for 3 hours at room temperature (15-28°C).
5. Decant thoroughly (except for total count tubes).
6. Count for 1 minute in a gamma counter.

Calculation of Results

To obtain results in terms of concentration from a logit-log representation of the calibration curve, first calculate for each pair of tubes the average NSB-corrected counts per minute:

Net Counts = Average CPM *minus* Average **NSB** CPM

Then determine the binding of each pair of tubes as a percent of maximum binding (MB), with the NSB-corrected counts of the A tubes taken as 100%:

Percent Bound = (Net Counts / Net **MB** Counts) x 100

With these formulas, final concentration of progesterone in ng/ml for samples and controls were calculated using a spreadsheet software.

B. Fecal progesterone metabolite

Fecal P4 metabolites were analyzed using method as described in Appendices B and C. The use of lyophilized fecal samples will be the most ideal than wet feces in term of standardizing variation. Due to inability to provide freeze drying machine for lyophilizing the sample, therefore an alternative way is to find the fecal moisture content. The method used is as follows:

a. Weigh 0.5g of wet feces and extract according to the extraction protocol (Appendix A).
b. Weigh approximately 1.4 – 2.0 g of excess wet feces and record the reading.
c. Dry the sample in an oven at 120°C for 2hr.
d. Cool down the dried feces.
e. Re-weigh dried feces and record the reading.
f. Calculate the moisture content:

$$\text{Moisture (\%)} = \frac{\text{Wet feces (g)} - \text{Dry feces (g)}}{\text{Wet feces (g)}} \times 100$$

g. Calculate weight of dry feces:

$$\text{Weight of dry feces (g)} = \text{Weight of wet feces} - (\text{Weight of wet feces} \times \text{\% moisture}) = 0.5 - (0.5 \times \text{\% moisture})$$

$$\text{Therefore, correction factor of fecal sample} = \frac{0.1}{\text{Weight of dry feces}}$$

FINANCIAL REPORT

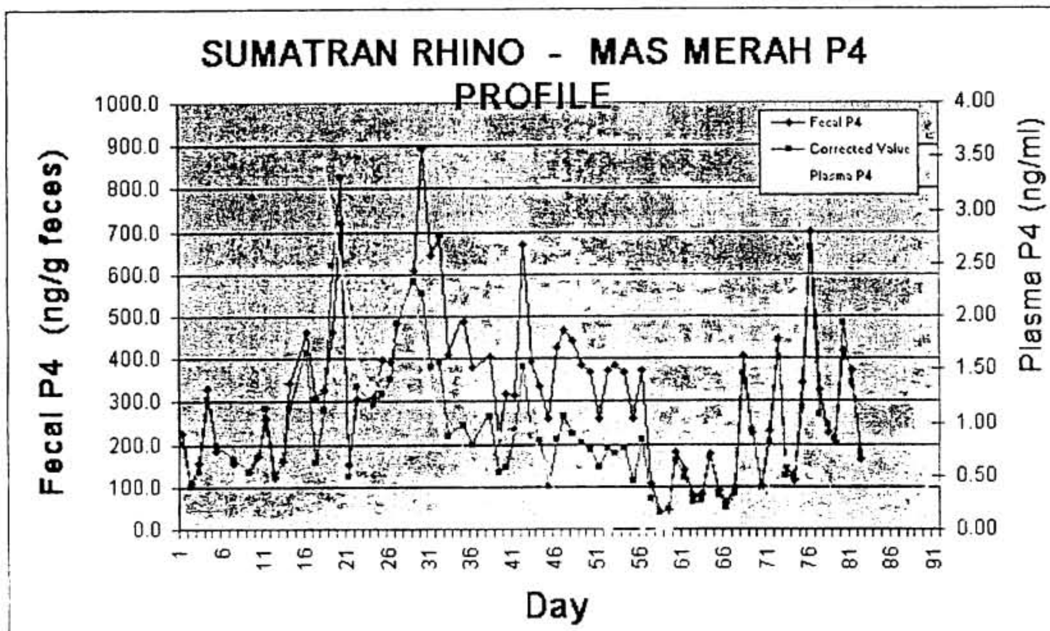
The financial report up to December 2000 for this project is as in Appendix D.

RESULTS AND DISCUSSION

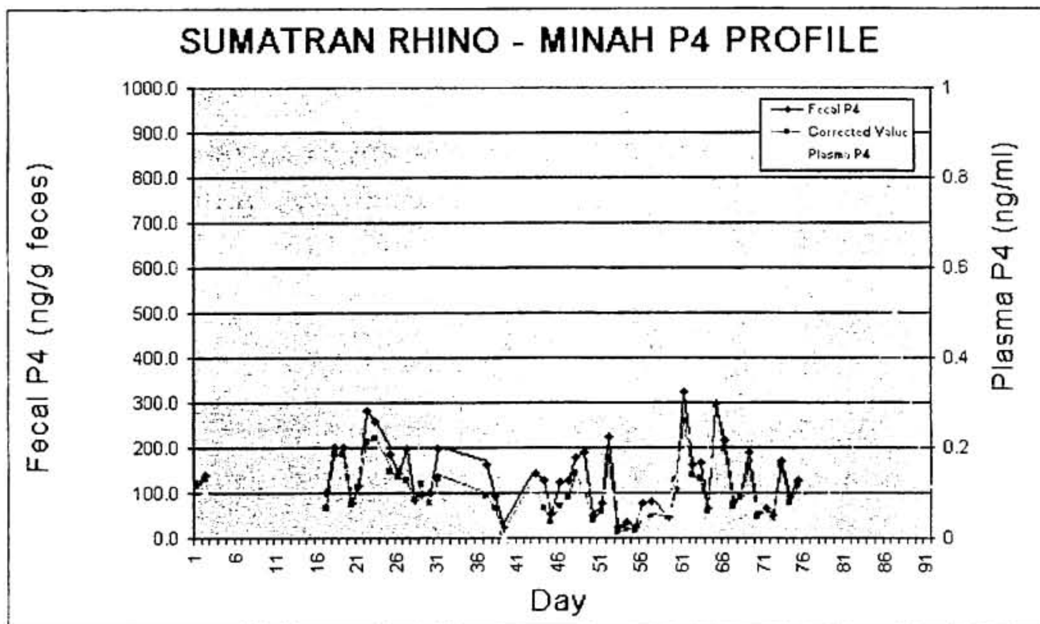
Plasma progesterone and fecal progesterone metabolites profiles are as presented in the graphs (A to E). Although initial problems regarding the protocol were encountered in this study, results from these data indicated that significantly correlated trends between plasma progesterone and corrected values of fecal progesterone metabolites were observed for all animals at $P<0.05$ (Seputih, $r=0.814$; Panjang, $r=0.722$; Rima, $r=0.626$; Mas Merah, $r=0.524$, and Minah, $r=0.508$).

Plasma progesterone levels for some rhinos revealed that the animals is not cycling as indicated by its low level. This can be seen in Minah, later part of Seputih and initial part of Panjang where the levels remain low (less than 0.4ng/ml) for more than 30 days. From the level of progesterone for Mas Merah, she is showing an irregular estrous cycle. Rima, however shows at least two estrous cycles as indicated by the high plasma progesterone level. The results also indicted that it is difficult to predict the precise duration of estrous cycle in this species as previously described. Therefore, it is inappropriate to predict the best time for mating in this species if it is solely determine by the duration of estrous cycle. In order to avoid injury during mating caused by improper introduction of animal, a combination of plasma progesterone determination and introduction of animals for mating should be implemented.

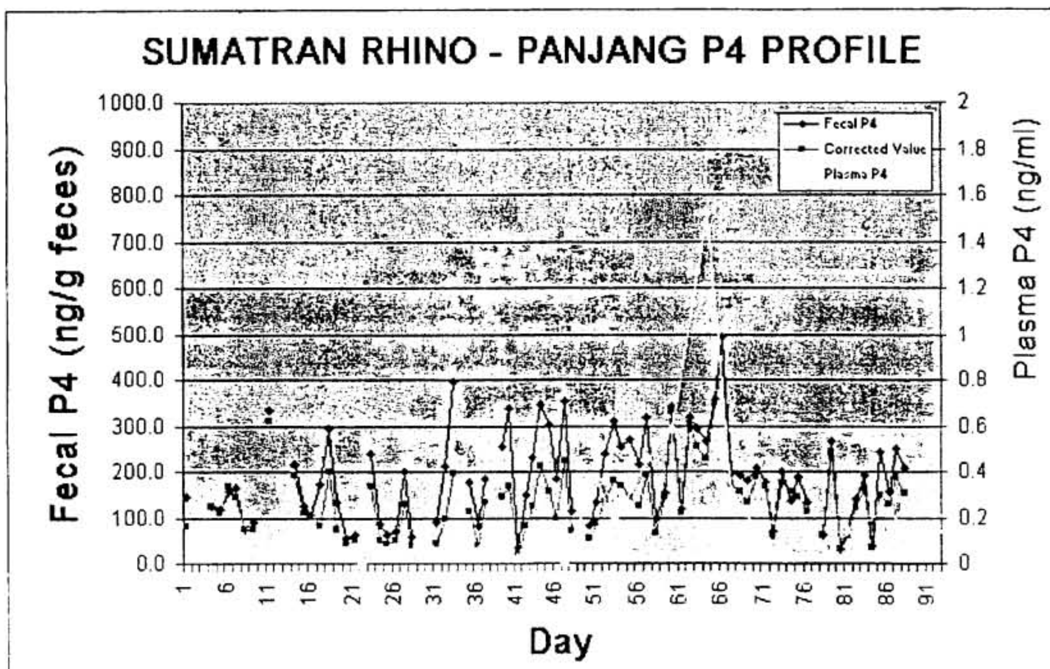
GRAPH A



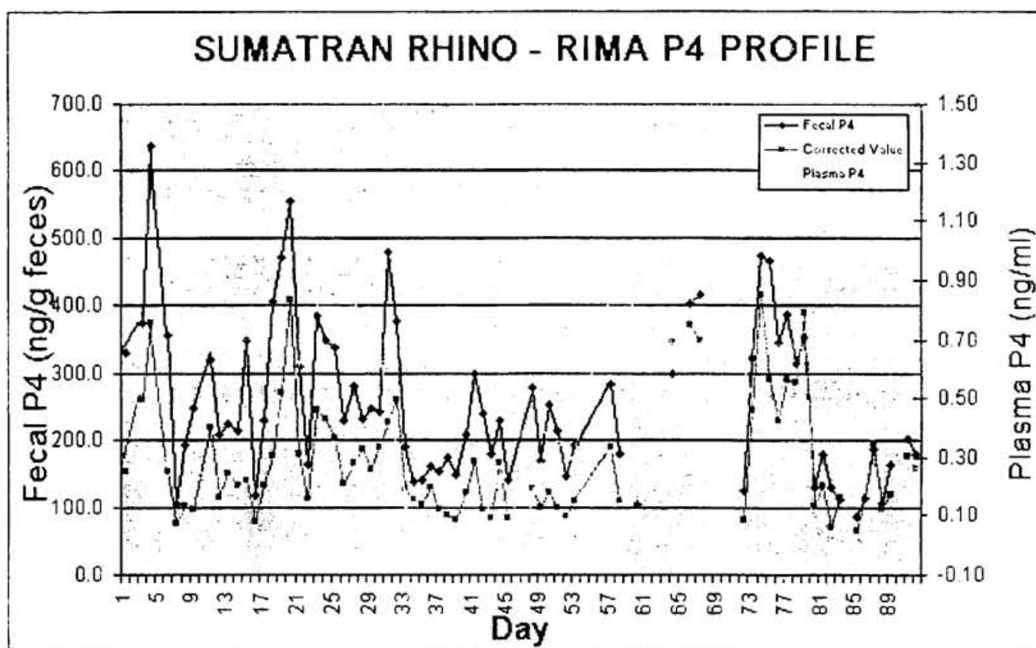
GRAPH B



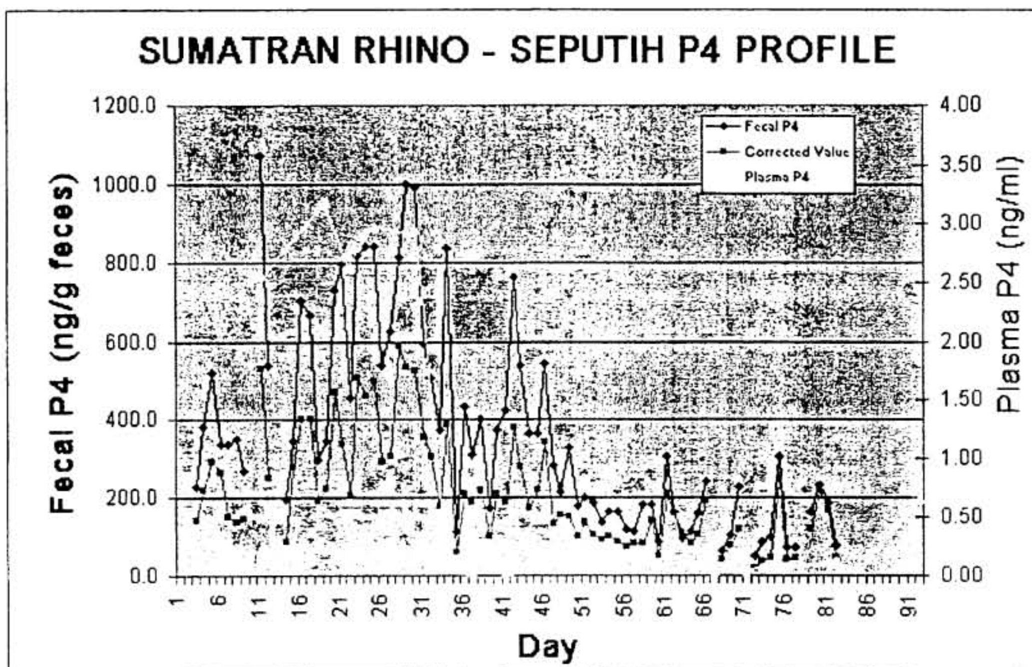
GRAPH C



GRAPH D



GRAPH E



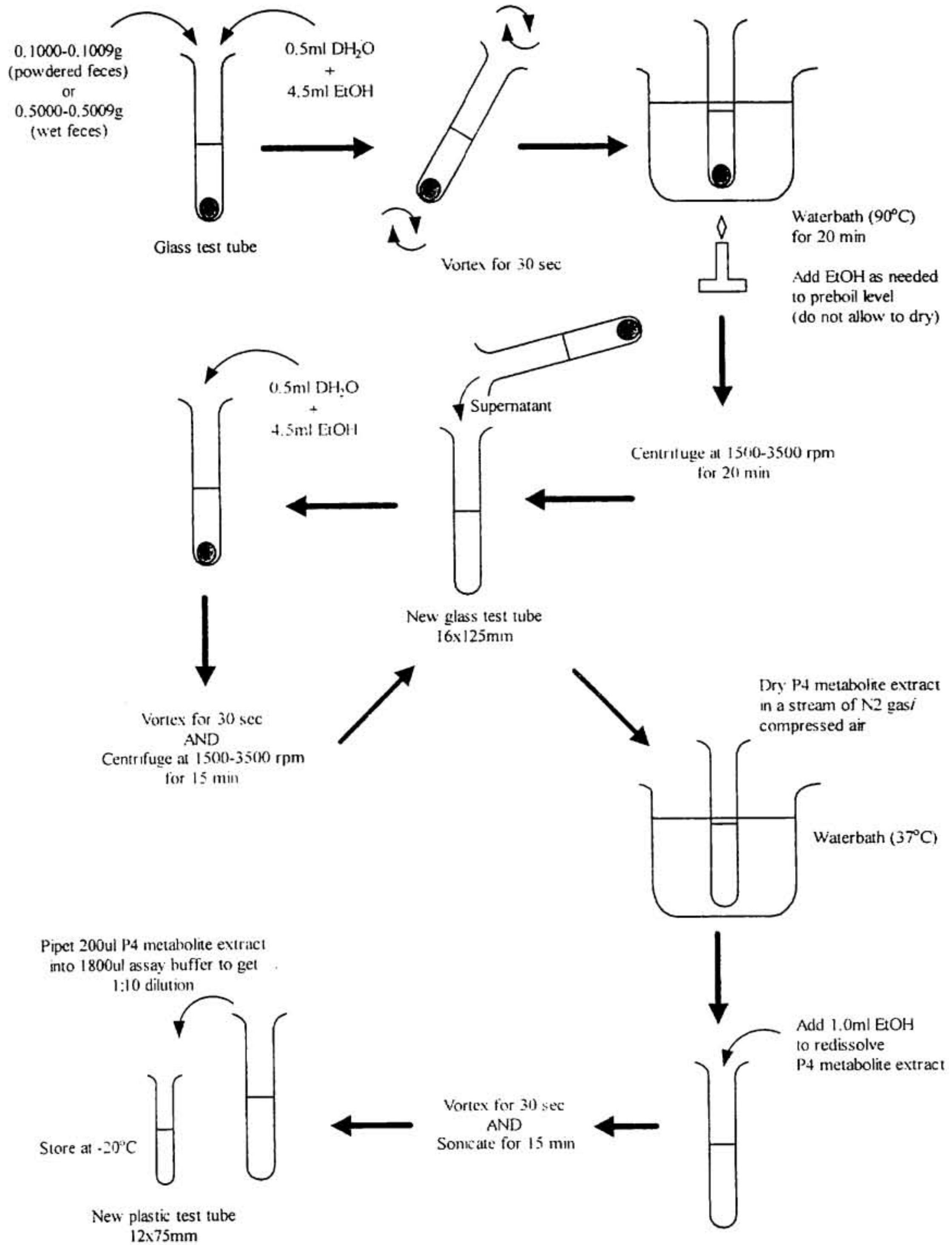
CONCLUSION

Based on the results as presented above, therefore, for determination of expected mating time, we would like to suggest that plasma progesterone level should be used. This will subsequently followed by introduction of animals for breeding. It is also appropriate to observe the other reproductive hormone such as estradiol, which is closely related to the time of estrus in these animals.

These comprehensive studies of the plasma progesterone and fecal progesterone metabolite levels give a promising indication for determining the best mating period of the Sumatran rhinos in captivity. Although fecal progesterone metabolite assay may not be as accurate as to predict the appropriate time for breeding, it is however the best method for monitoring the animal due to its easy collection of samples and non-invasive technique.

Appendix A

FECAL EXTRACTION



Appendix B

PROTOCOL FOR P4 EIA (Franz Technique) – per plate – RED
For Sumatran Rhino Fecal Extract
DAY 1

1. Plate Coating (Non-specific Ab)
   - use NUNC Maxisorb plates.
   - prepare a solution of 50 ug coating antibody (1 mg/ml affinity purified goat anti-mouse IgG) and dissolved in 25 ml coating buffer (anti-rabbit or anti-sheep Ab can be used, depending on the 2nd Ab).
   - **DO NOT COAT NSB wells (i.e. wells C1 & D1)** and dispense 250 ul/well of the antibody solution using the Eppendorf repeater pipet.
   - tap plates gently to ensure that coating solution covers well bottom.
   - cover MTP with plate sealer and incubate overnight at room temperature.

DAY 2

2. 2nd Plate Coating
   - Dump contents of plates.
   - **DO NOT COAT NSB wells** and dispense 300 ul/well of 2nd coating buffer.
   - cover MTP and store at room temperature. MTP can be used after 3 hrs and up to 4 weeks.

DAY 3

3. Standards (P4)
   - values used are: 500, 200, 80, 32, 12.8, 5.12 and 2.048 pg/well
   - use **assay buffer** for 0 standard.
   - mix 30 ul (1250 pg) standard stock in 45 ul assay buffer (1:2.5 dilution), mix well, make 7 serial dilutions of 1:2.5

4. Controls
   - High control and Low control can be used as it is.

5. Antibody
   - mix 80 ul stock AB2 (1:100) in 11.920 ml assay buffer a glass scintillation vial to give a working dilution of 1:15,000.

6. Biotin Label
   - mix 20 ul stock biotin labelled steroid (1:1000) in 11.980 ml assay buffer to give a working dilution of 1:600,000.

Note: *Standards, antibody and biotin labelled steroid need to equilibrate for* ***20 min*** *before use.*

7. Samples
   - dilute fecal extract in assay buffer to the appropriate dilution.

8. Plate Loading
   a) **Plate Washing** – decant and wash plate 3x with wash solution. Blot plate on paper towel and load immediately.
   b) **NSB** – pipette 150 ul assay buffer into wells C1 & D1.
   c) **Zero standard** – pipette 50 ul assay buffer.
   d) **Standards/Controls/Samples** - pipette **50 ul/well** of the appropriate diluted standards, controls and samples according to plate map.
   e) **Antibody** – dispense **100 ul** antibody solution into each well **except NSB**.

f) **Biotinylated Label** – dispense **100 ul** biotin labeled steroid into each well.

9. Incubation
- cover MTP with plate sealer and incubate **overnight at 4°C** with mild shaking.

DAY 4

10. Streptavidin (Enzyme Reaction)
   a) **Streptavidin Preparation – Immediately Before Use** – mix **WELL** 5 ul (100U/ml) Streptavidin-POD (2° Stock) conjugate into 30 ml assay buffer.
   b) **Plate Washing** – decant and wash plate 4x with **cold (4°C) wash solution** and between washing, shake for 10-30 seconds on MTP shaker. Blot plate on paper towel.
   c) **Streptavidin Loading** – dispense **250 ul** of enzyme solution in each well, cover and incubate MTP for 45 minutes at 4°C with mild shaking.

11. Substrate (Colour Reaction)
   a) **Substrate Preparation – Immediately Before Use** – mix **WELL** 500 ul 0.4% TMB and 100 ul 0.6% H_2O_2 into 30 ml substrate buffer.
   b) **Plate Washing** – as step 9b.
   c) **Substrate Loading** – dispense **250 ul** of substrate solution in each well, cover and incubate MTP at 4°C with mild shaking for 45 minutes (depending on colour intensity).

12. Stop Reaction
- dispense **50 ul** Stop Reagent (4M H_2SO_4). Blue colour turns into yellow.

13. Plate Reading
- plates can be read immediately or will keep for several hours.
- Reference Filter: 620 nm
- Interference Filter: 450 nm

Appendix C

PROTOCOL FOR P4 EIA (Metro Toronto Zoo Technique) – per plate – RED
For Sumatran Rhino Fecal Extract

DAY 1

1. Plate Coating (Non-specific Ab)
   - use NUNC Maxisorb plates.
   - prepare a solution of 50 ug coating antibody (1 mg/ml affinity purified goat anti- mouse IgG) and dissolved in 25 ml coating buffer (anti-rabbit or anti-sheep Ab can be used, depending on the 2nd Ab).
   - **Do not coat column 1 (except NSB's)** - start at A2 and go down each column (see plate map).
   - Dispense 250 ul/well of the antibody solution using the Eppendorf repeater pipette.
   - tap plates gently to ensure that coating solution covers well bottom.
   - cover MTP with plate sealer and incubate overnight at room temperature.

DAY 2

2. 2nd Plate Coating
   - Dump contents of plates.
   - **Do not coat column 1 (except NSB's)** and dispense 300 ul/well of 2nd coating buffer.
   - cover MTP and store at room temperature. MTP can be used after 3 hrs and up to 4 weeks.

DAY 3

3. Standards (P4)
   - values used are: 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.6 and 7.8 pg P4/well.
   - use **assay buffer** for 0 standard.
   - dilute standard stock (200 ng/ml **or** 10,000 pg/well, -20°C) 1:5 by using 100 ul of stock plus 400 ul assay buffer to prepare 2000 pg/well standard. Dilute this serially 2-fold using 200 ul stock plus 200 ul assay buffer.

4. Controls
   - High control and Low control can be used as it is.

5. Antibody
   - mix 100 ul stock Roser Ab (1:1,000) in 4.9 ml assay buffer in a glass scintillation vial to give a working dilution of 1:50,000.

6. Biotin Label
   - mix 50 ul stock biotin labelled steroid (1:5,000) in 4.95 ml assay buffer to give a working dilution of 1:500,000 **or** 20 ul stock (1:1,000) in 9.980 ml assay buffer.

Note: *Standards, antibody and biotin labelled steroid need to equilibrate for* ***20 min*** *before use.*

7. Samples
   - dilute fecal extract in assay buffer to the appropriate dilution.

8. Plate Loading
   a) **Plate Washing** – decant and wash plate 3x with wash solution. Blot plate on paper towel and load immediately.
   b) **NSB** – pipette **100 ul** assay buffer into wells A1 & B1.
   c) **Zero standard** – pipette **50 ul** assay buffer.

TIME DEPENDENT c) **Standards/Controls/Samples** – pipette **50 ul/well** of the appropriate diluted standards, controls and samples according to plate map.

TIME DEPENDENT d) **Antibody** – dispense **50 ul** antibody solution into each well **except NSB.**

TIME DEPENDENT e) **Biotinylated Label** – dispense **50 ul** biotin labeled steroid into each well.

9. Incubation
   - cover MTP with plate sealer and incubate **at RT for 3 hours** with mild shaking.

10. Streptavidin (Enzyme Reaction)
   a) **Streptavidin Preparation – Immediately Before Use** – mix **WELL** 5 ul (100U/ml) Streptavidin- POD (2° Stock) conjugate into 12 ml assay buffer.
   b) **Plate Washing** – decant and wash plate 4x with **cold (4°C) wash solution** and between washing, shake for 10-30 seconds on MTP shaker. Blot plate on paper towel.
   c) **Streptavidin Loading** – dispense **100 ul** of enzyme solution in each well, cover and incubate MTP for 45 minutes at 4°C with mild shaking.

11. Substrate (Colour Reaction)
   a) **Substrate Preparation – Immediately Before Use** – mix **WELL** 500 ul 0.4% TMB and 100 ul 0.6% H_2O_2 into 12 ml substrate buffer.
   b) **Plate Washing** – as step 9b.
   c) **Substrate Loading** – dispense **100 ul** of substrate solution in each well, cover and incubate MTP at 4°C with mild shaking for 45 minutes (depending on colour intensity).

12. Stop Reaction
   - dispense **50 ul** Stop Reagent (4M H_2SO_4). Blue colour turns into yellow.

13. Plate Reading
   - plates can be read immediately or will keep for several hours.
   - Reference Filter: 620 nm
   - Interference Filter: 450 nm

Appendix D

RESEARCH/PROJECT ALLOCATION FOR 2000
Researcher: Dr. Abd. Wahid Haron
Research Assistant: Mr. Yap Keng Chee
Project Title: Fecal Analysis for Reproductive Hormones in Sumatran Rhino
Project Code: 63552
Account No.: 63552

Financial Report (till 24 October 2000)

INCOME

DATE	ITEM	AMOUNT
14-May-00	Bank draft (no. 458183)	$18,500.00
14-May-00	Cash	$350.00
26-Sep-00	Bank draft (no. 562824)	$18,000.00
28-Sep-00	Bank draft (no. 562931)	$20,000.00
TOTAL		**$56,850.00**
MINUS FIXED EMOLUMENT		**$13,200.00**
BALANCE (to be distributed for expenditure)		**$43,650.00**

EXPENDITURE

Code	Item	Amount Allocated	Amount Spent	Balance
510000	Emolument (fixed)	$13,200.00	$1,500.00	$11,700.00
521000	Transportation (2.5%)	$1,091.25	$325.80	$765.45
527000	Supplies - chemical and consumables (70%)	$30,555.00	$14,963.50	$15,591.50
528000	Maintenance and minor modification (27.5%)	$12,003.75	$4,355.36	$7,648.39
	TOTAL	**$56,850.00**	**$21,144.66**	**$35,705.34**

Expenditure in detail

510000 – Emolument (amount allocated for emolument is fixed for 3 months (May-July 2000) as follows:

Name	Month	Amount/ month	Total
Dr. Abd. Wahid Haron	3	$2,000.00	$6,000.00
Mr. Yap Keng Chee	3	$1,500.00	$4,500.00
Additional research assistant - 1	3	$900.00	$2,700.00
TOTAL		$4,400.00	$13,200.00

DATE	NAME	DOCUMENT NUMBER	AMOUNT
11-Sep-00	Yap Keng Chee		$1,500.00
TOTAL			$1,500.00

521000 - Transportation

DATE	CLAIMER	DOCUMENT NUMBER	AMOUNT
19-May-00	Abd. Wahid Haron		$325.80
TOTAL			$325.80

527000 – Supplies (chemical and consumables)

DATE	SUPPLIER	PO NUMBER	AMOUNT
23-Mar-00	FC-Bios Sdn Bhd	63552050000002	$4,750.00
10-Apr-00	Asian Surgical Scientific Supplies	63552050000001	$984.50
09-May-00	Interscience Sdn Bhd	63552050000003	$1,021.00
10-May-00	Asian Surgical Scientific Supplies	63552050000004	$2,668.00
16-May-00	Antah Sri Radin Sdn Bhd	63552050000006	$720.00
29-Jun-00	Antah Sri Radin Sdn Bhd	63552050000007	$1,440.00
24-Oct-00	Antah Sri Radin Sdn Bhd	63552050000008	$3,380.00
TOTAL			$14,963.50

528000 – Maintenance and Minor Modifications

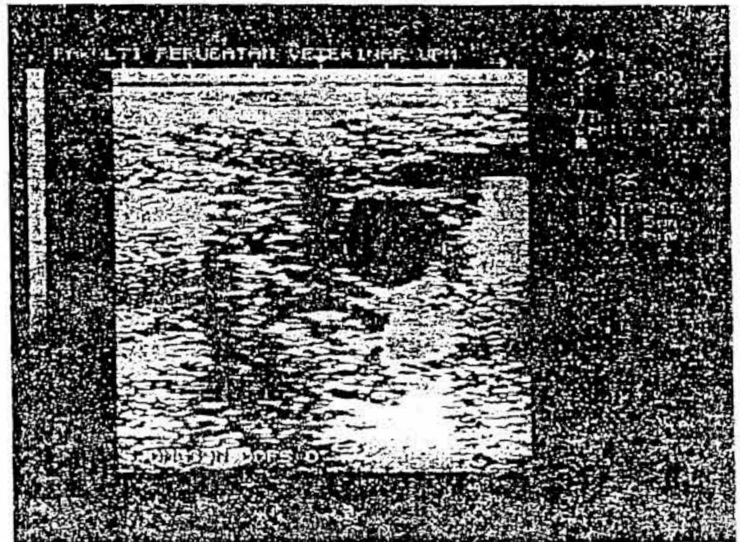
DATE	SUPPLIER	PO NUMBER	AMOUNT
11-May-00	FC-Bios Sdn Bhd	63552050000005	$4,355.36
TOTAL			$4,355.36

11/Dec/2000.

Panjang.

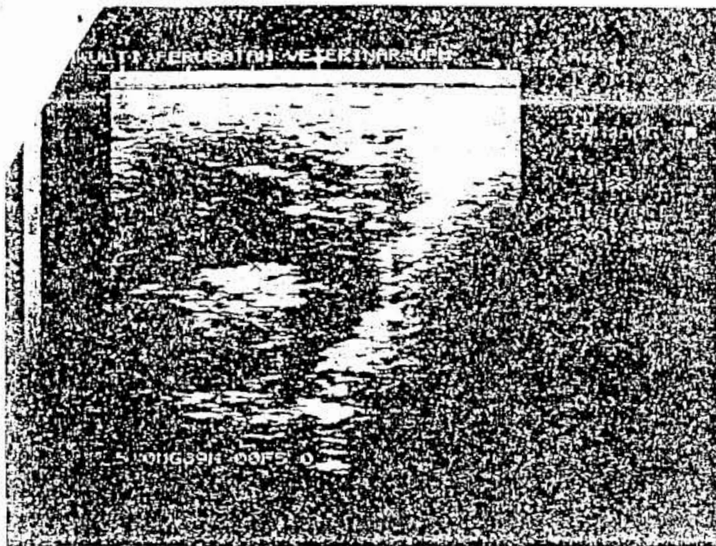
ultrasound scanning images.

PANJANG 7.

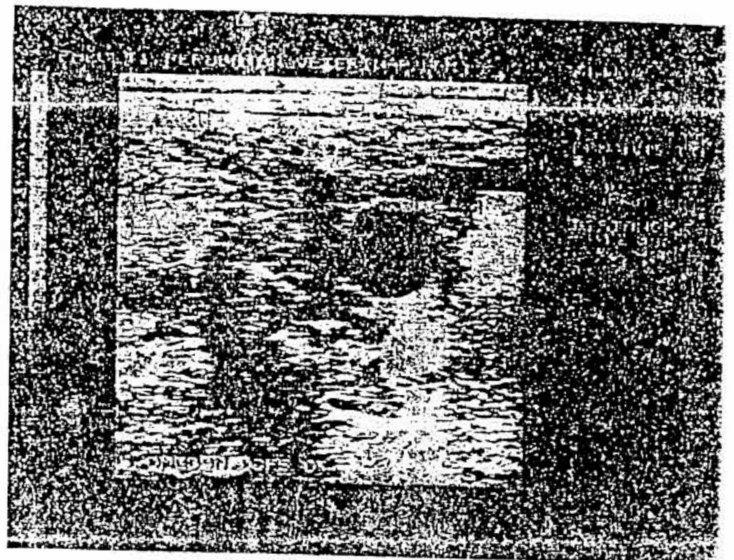


left ovary.

1.5 cm } Fol
1.6 cm }

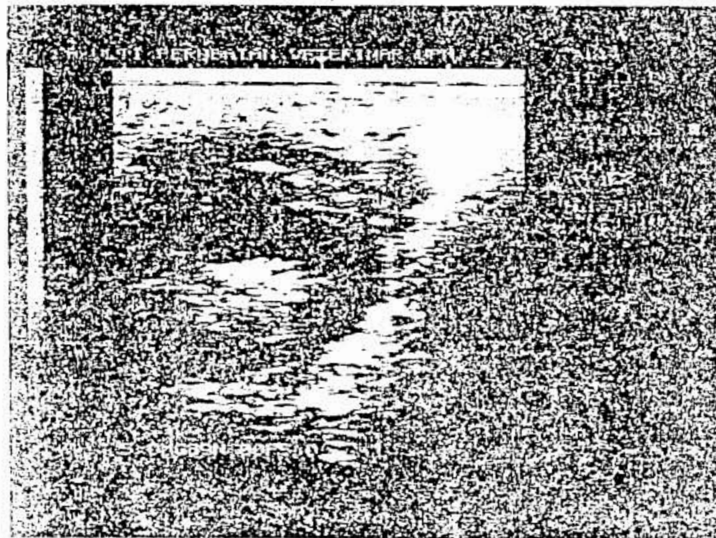


Right ovary 1.4 cm
1.6 cm



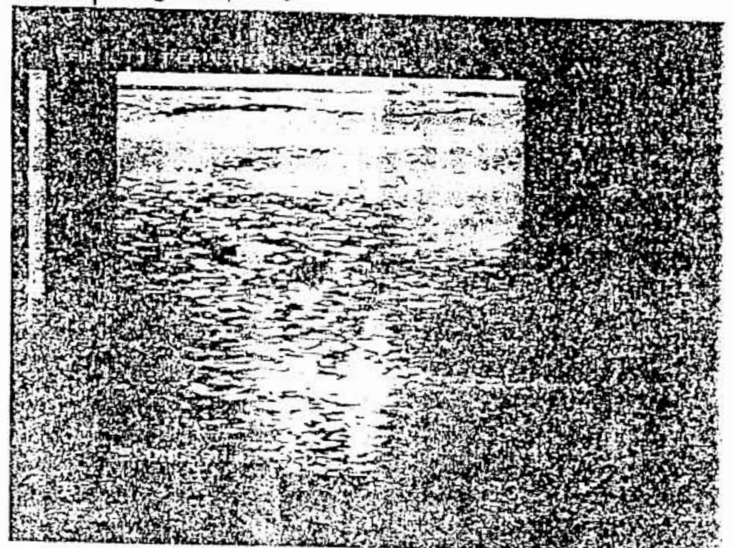
Left ovary

PANJANG 2



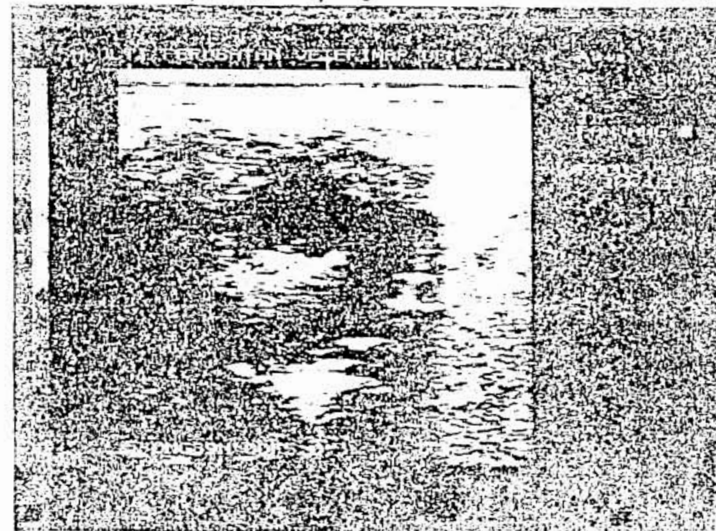
Right ovary 4.1 cm
4.8 cm

PANJANG 5

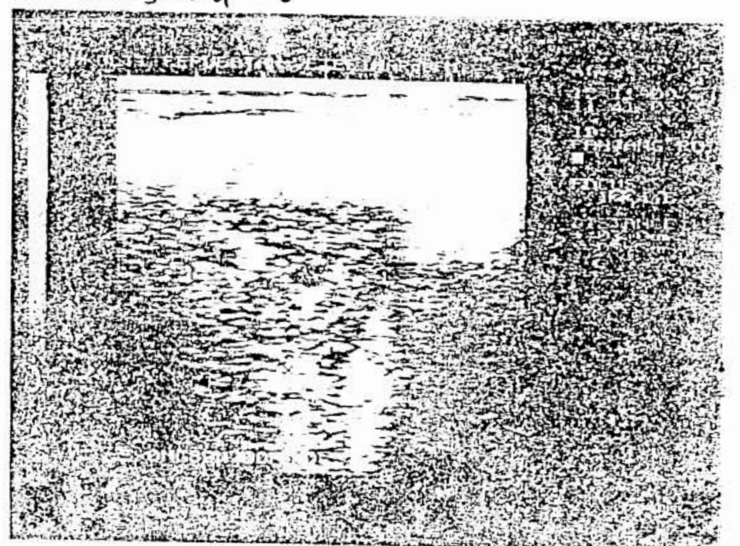


Right ovary 0.5 cm
0.4 cm } Fol

PANJANG 3



PANJANG 6



Right ovary