Characterization of the reproductive cycle in the Sumatran rhinoceros (*Dicerorhinus sumatrensis)*

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Introduction: The Sumatran rhinoceros is one of the most critically endangered species in the world, with less than 750 animals estimated to survive in 35 or more localities throughout Southeast Asia. The Department of Wildlife and National Parks, Malaysia has made a long term commitment to the conservation of the Sumatran rhino with active field conservation programs and the establishment of a captive breeding center. There currently is a total of 7 animals in captivity in West Malaysia. The captive breeding center at Sungai Dusun holds 1.4 animals and the Melaka Zoo is holding 0.2 animals. Although, great effort is being made to breed these animals, this goal faces major restrictions given the lack of basic information on the reproductive biology and behavior of the Sumatran rhinoceros.

This project was designed to help the Malaysian Department of Wildlife and National Parks develop the technology required to effectively monitor the reproductive cycle in their population of captive Sumatran rhinos. To fulfill this goal, the project supported the technical training of Mhd. Suri (Sumatran Rhino Captive Breeding Unit, Sungai Dusun) in endocrine analyses.

Training: Mhd. Suri spent 7 weeks in the United States and Canada, training in Drs. Brown and Goodrowe's laboratories. During this period he became proficient in both radioimmunoassay and enzyme immunoassay techniques. He was able to apply these skills to the analysis of urine and fecal samples collected from the female rhinos at Sungai Dusun.

Results of analyses: Summary reports from both laboratories are provided in Appendix I. Both radioimmunoassay and enzyme immunoassay techniques were able to measure estrogen and progesterone metabolites in the feces, however, urine steroid concentrations were extremely low and unable to be accurately measured. Hormonal profiles from the four females revealed similar trends. The only female that appeared to be exhibiting ovarian activity was Panjang. Panjang demonstrated estrogen elevations at approximately days 23, 46, and 73. The profiles for the remaining three females do not provide an overall clear picture. Both Putih and Rima demonstrated elevated estrogen levels around day 50, with Rima experiencing a prolonged elevation to day 75. Estrogen and progesterone levels for Mas Merah appear to be at baseline throughout the collection period. For all four females, progesterone levels were very low and there does not appear to be a progesterone peak following an elevation of estrogen which would suggest ovulation. Several differences in the profiles between RIA and EIA analysis can be attributed to the fact that the RIA analysis measured only one estrogen metabolite, whereas EIA analysis measured total estrogens and that fecal extraction techniques were different. HPLC analysis on fecal and urine extracts is being done through Dr. Goodrowe's laboratory to determine the specific hormonal metabolites present.

These results should be considered preliminary information and provide the basis for more in-depth analyses. Based on recent studies, it is probable that the storage of fecal material in ethanol contributed to steroid leakage resulting in low steroid concentrations. Based on creatinine levels and low steroid concentration in the urine, it is possible that the samples were subjected to freeze-thaw damage. Both summary reports recommend: 1) the storage of feces without ethanol and 2) ensure adequacy of freezing system so that urine and feces do not undergo a series of freeze/thaw cycles.

Proposed continuation of project: We would like to propose that Dr. Goodrowe and her laboratory assistant, Astrid Bellem, continue to work with the Department of Wildlife and National Parks towards characterizing the reproductive cycle of the Sumatran rhino. This would encompass the following: 1) provide additional training in endocrinology to learn to interpret hormonal profiles, 2) provide assistance in establishing an EIA laboratory and 3) provide assistance in establishing a behavioral observation protocol to correlate with endocrine profiles.

APPENDIX I

Summary of work conducted for Sumatran rhino endocrine analyses
Conservation and Research Center - National Zoo
November - December 1994

M.S.M. Suri, L. Graham and J. Brown

Fecal and urine samples from 4 female Sumatran rhinos were processed and analyzed for concentrations of estrogen and progestogen metabolites.

Methods

Fecal analysis:

Fecal samples were collected and stored frozen in tubes containing 100% ethanol. Samples were air dried to remove the ethanol and then lyophilized. Dried fecal samples were pulverized and ~0.2 g of well-mixed powder boiled in 5 ml of 90% ethanol:distilled water for 20 min (in 16 x 125 mm glass tubes). Samples were centrifuged at 500 x g for 10 min, the supernatant recovered and pellet resuspended in another 5 ml of 90% ethanol. Samples were then vortexed for 1 min and re-centrifuged. Both ethanol supernatants were combined (16 x 125 mm tubes), dried completely, redissolved in 1 ml methanol, sonicated in an ultrasonic glass cleaner for 30 sec and re-vortexed (15 sec). Samples were diluted (~1:40 for estradiol; 1:200 for progestogens) in phosphate-buffered saline (0.01 M PO_4, 0.14 M N_aC1, 0.5% BSA, 0.01% N_aN_3) before analysis.

Fecal estradiol and progestogen metabolites were quantified using validated radioimmunoassays similar to those previously described [Brown et al., 1994; Biol. Reprod. 51:776-786]. Assay sensitivities, based on 90% of maximum binding, were 2 pg/ml and 5 pg/ml for the estradiol and progesterone assays, respectively. Intra- and interassay coefficients of variation were <10% for both assays. All data were expressed on a per g dry fecal weight basis.

Urine analysis:

Urine samples were diluted 1:2 to 1:6 in phosphate buffer and analyzed for estrone-sulfate and pregnanediol-glucuronide concentrations using previously described methods [Monfort et al., 1989; J. Reprod. Fert. 85:203-212: Monfort et al., 1991; J. Reprod. Fert. 91:155-164]. All urine values were indexed by creatinine concentrations to account for fluctuations in fluid intake.

Results

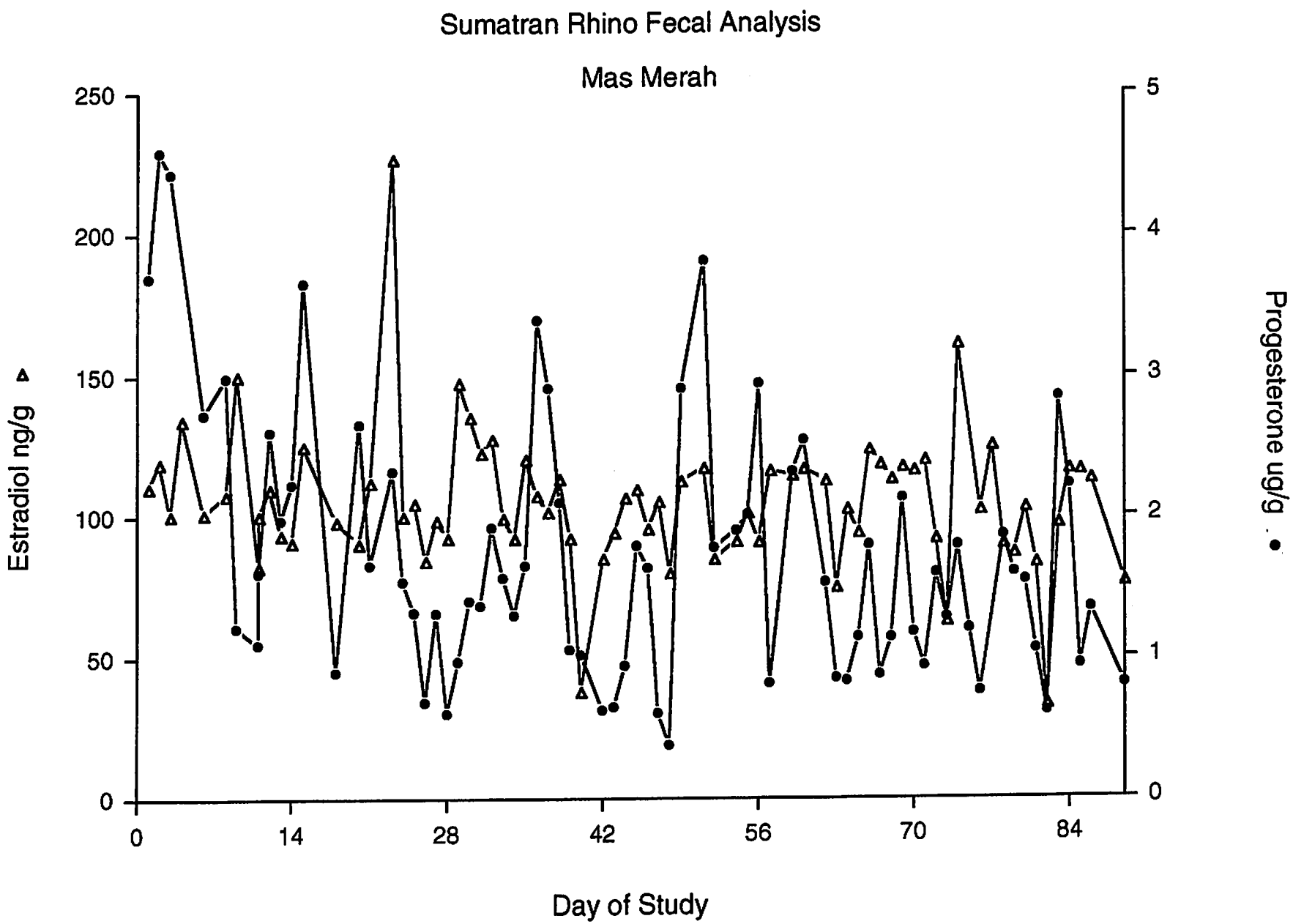
Fecal analysis:

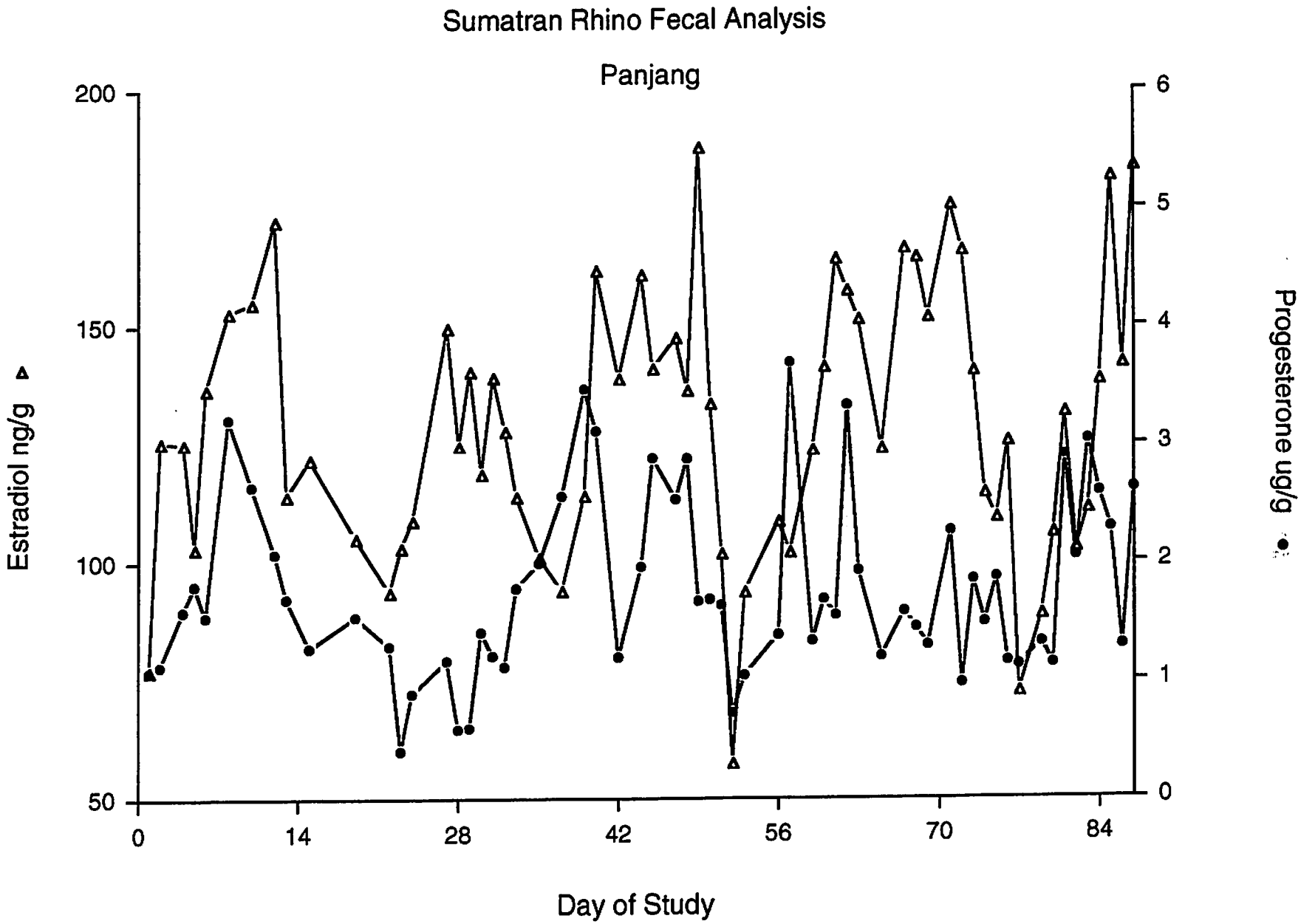
Although the data were quite variable, Panjang appeared to demonstrate 3 estrous cycles of about 25-30 days in length based on changes in progestogen concentrations. In contrast, no regular cycles of estradiol excretion were observed. Estradiol concentrations fluctuated and were elevated during both the follicular and luteal phases. In the other 3 females (Mas Merah, Rima, Putih), there does not appear to be discrete cycles. In general, overall concentrations of progestogen metabolites are low for the luteal phase compared to other species, but this just may be species variability. These estradiol metabolite profiles definitely were not consistent and no conclusions can be drawn from these data.

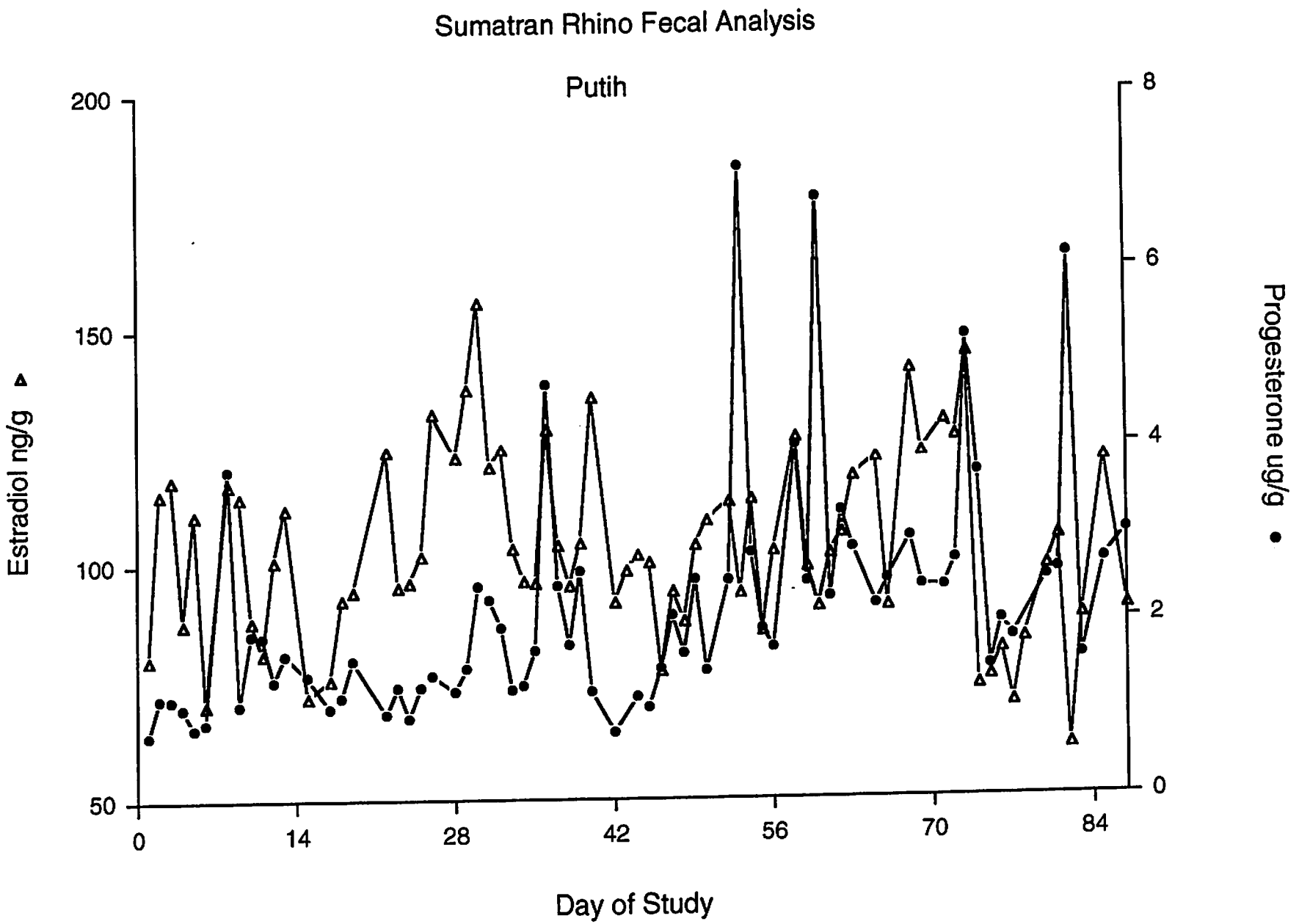
Because the fecal samples were stored in ethanol for an extended period of time before processing, it is possible that steroid leakage occurred. This would account for the overall low concentrations of steroid and inconsistent results. Future studies should emphasize collecting feces soon after defecation and storing them in tightly-capped tubes or ziplock bags at freezer temperatures (~-20°C).

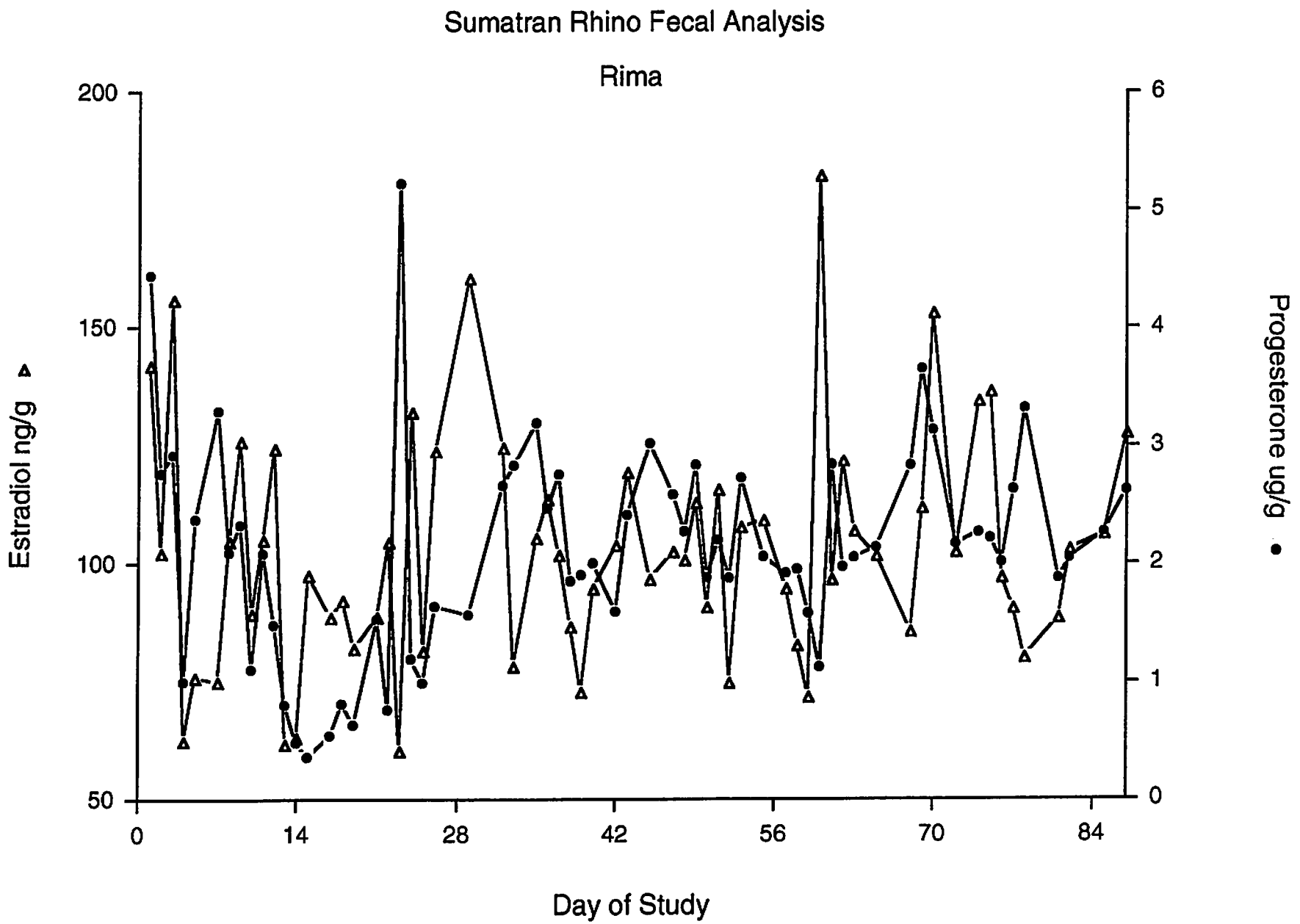
Urine analysis:

Urine was not collected on a routine basis, therefore no conclusions can be drawn from these analyses. There were no discernible profiles of either estrone-sulfate or pregnanediol-glucuronide concentrations in these females. In addition, overall steroid concentrations were very low and in most cases creatinine values approached the limit of detection. This suggests that something may have happened to the urine (i.e., frequent thawing and/or warming) to cause steroid and creatinine damage. This data has not been presented. Future studies should involve collecting urine free-catch or off of enclosure floors (even dirt flooring is okay as long as samples are centrifuged to remove debris) and storing it frozen.


Sumatran Rhino Fecal Analysis
Mas Merah
Estradiol ng/g ▲
Progesterone ug/g ●
Day of Study
0
50
100
150
200
250
0
1
2
3
4
5
0
14
28
42
56
70
84


Sumatran Rhino Fecal Analysis
Panjang
Estradiol ng/g ▲
Progesterone ug/g ●
Day of Study
200
150
100
50
6
5
4
3
2
1
0
0
14
28
42
56
70
84


Sumatran Rhino Fecal Analysis
Putih
Estradiol ng/g ▲
Progesterone ug/g ●
Day of Study
200
150
100
50
8
6
4
2
0
0
14
28
42
56
70
84


Sumatran Rhino Fecal Analysis
Rima
Estradiol ng/g ▲
Progesterone ug/g ●
Day of Study
200
150
100
50
6
5
4
3
2
1
0
0
14
28
42
56
70
84

Summary of work conducted for Sumatran rhino endocrine analyses
Metro Toronto Zoo
December 8-January 6, 1994/95

M.S.M. Suri, A. Bellem, K. Auckland and K.L. Goodrowe

All hormonal analyses at the Metro Toronto Zoo were conducted using enzyme-immunoassay (EIA) techniques and M.S. M. Suri recieved training in the technical aspects of urine and fecal EIA hormonal analyses.

For the urine assays, 100 µl from each of 8 urine samples from 4 females were pooled together, serially diluted from neat to 1:4,000 and analyzed to determine if parallel displacement curves against the standard curves could be generated for each of the following assays: pregnanediol-3-glucuronide, estrone conjugates (estrone glucuronide and estrone sulfate) and 20α-hydroxy-progesterone. Only very poor displacement curves were obtained, indicating that measurable immunoreactive hormones were present only in very low concentrations and likely would not be accurately measured. Therefore, EIA was not conducted on urine samples.

However, because only a small number of samples was available from each female, it is possible that only baseline values were obtained. While clean-catch samples are preferred, urine aspirated from the ground and centrifuged to remove particulate matter also can be used in the assay systems. Frequent collection might provide more accurate results.

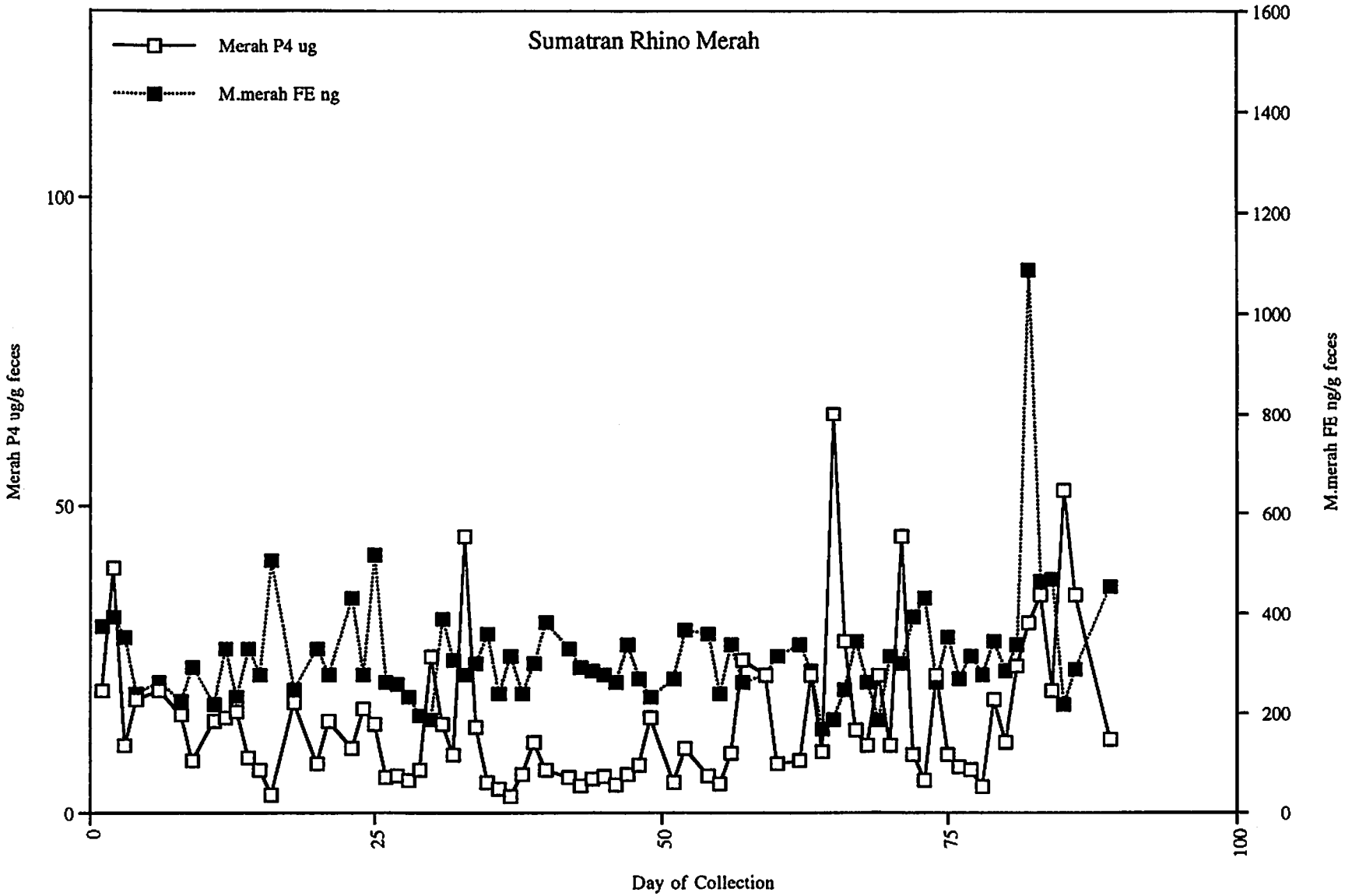
For fecal assays, all dried, crushed fecal samples (prepared at the endocrine laboratory of the National Zoological Park in Front Royal, VA) were extracted using a method involving buffer, alcohol and overnight shaking. Extracts were stored frozen until use. Extracts were pooled, as described above for urine and serially diluted from neat to 1:4,000 to determine if parallel displacement curves against the standard curves could be generated for each of the following assays: fecal progesterone, pregnanediol and total estrogens. Displacement curves were obtained for fecal progesterone and estrogens, but only at low dilutions. Nevertheless, all extracts were analyzed and the data plotted as a function of time.

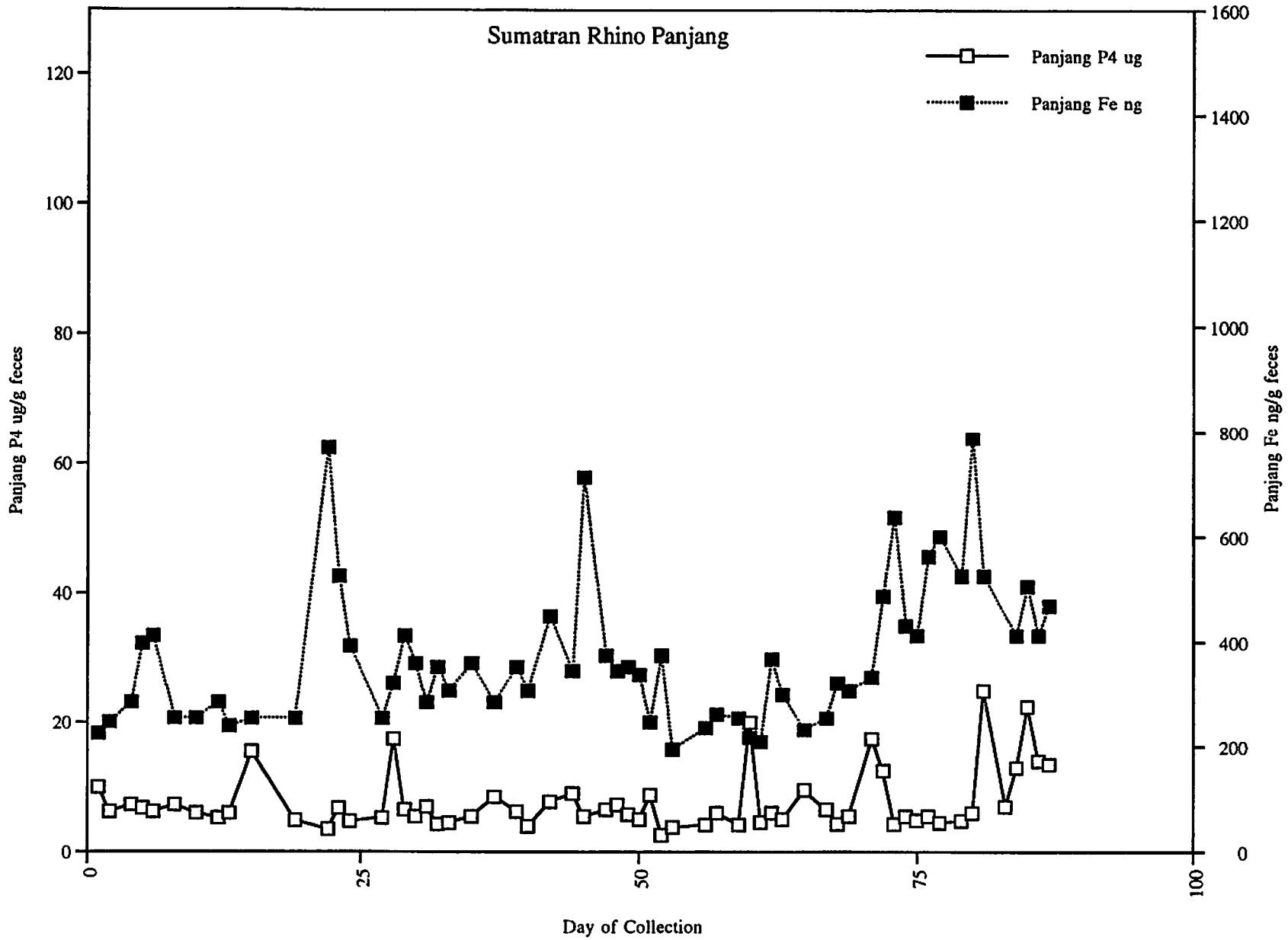
There was extreme variation in hormone concentrations between individuals, particularly with reference to fecal estrogens. Results from fecal estrogen analysis with Panjang are suggestive of ovarian cyclicity, with elevations at ~ days 23, 46 and 73, but there are no concurrent elevations in progesterone signalling ovulation, as would be predicted based on other rhino species. Although no apparent cycles were present Putih, some pronounced elevations in estrogens were observed, particularly around day 50, suggesting ovarian activity (i.e. follicular development). Again, progesterone levels were low, with the exception of a 3 day elevation around days 5-7, but the significance of this phenomenon is unclear. Interestingly, Putih demonstrated ovarian activity at ultrasound examination, as described by Schaeffer et al., 1994. In general, estrogen concentrations from Rima were not suggestive of cyclicity, with the exception of a prolonged elevation between days 50 and 75. The significance of this event is unclear. Again, there were no increases in progesterone signalling ovulation. Samples from Mas Merah all appear to be at baseline levels.

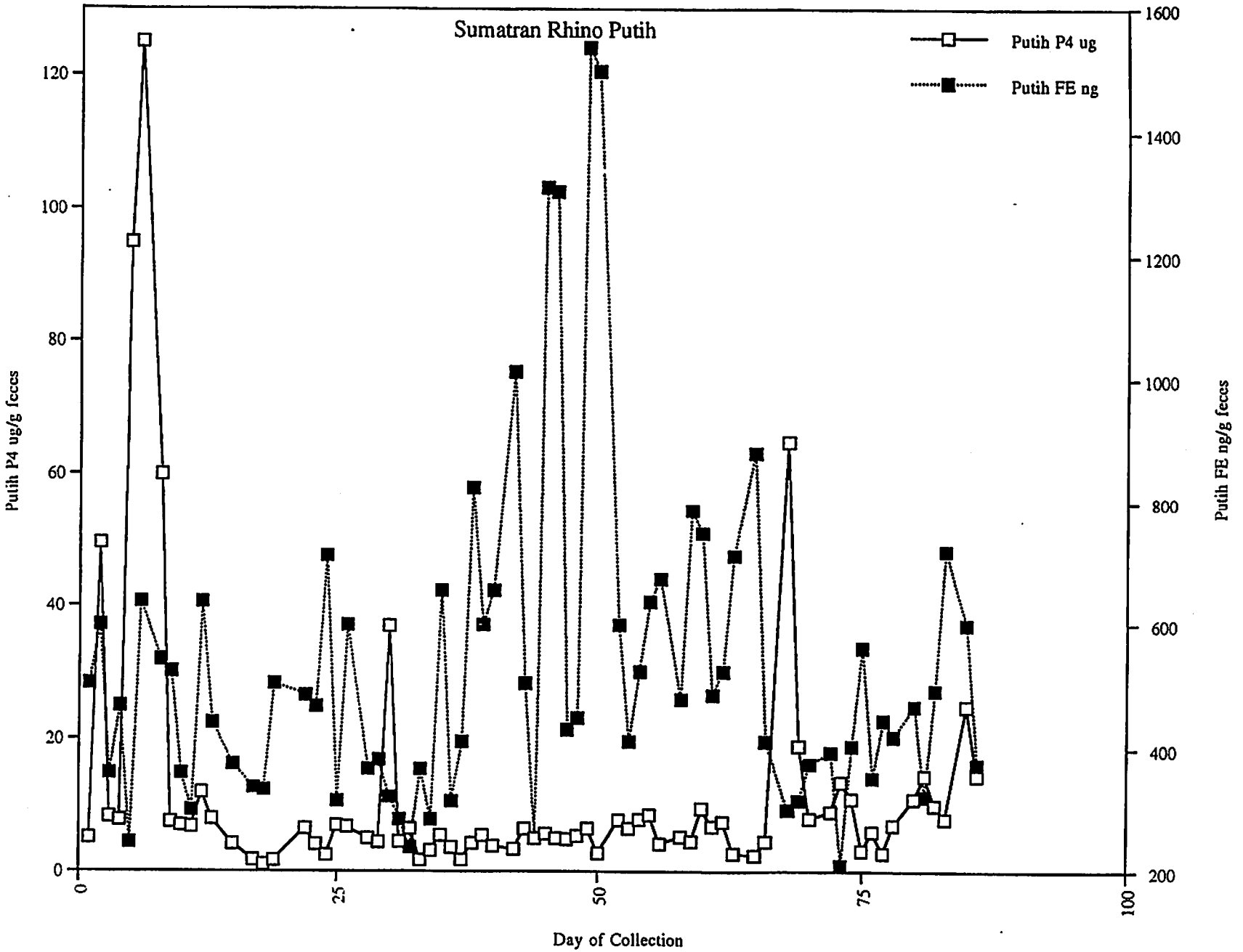
Recommendations for continued study:

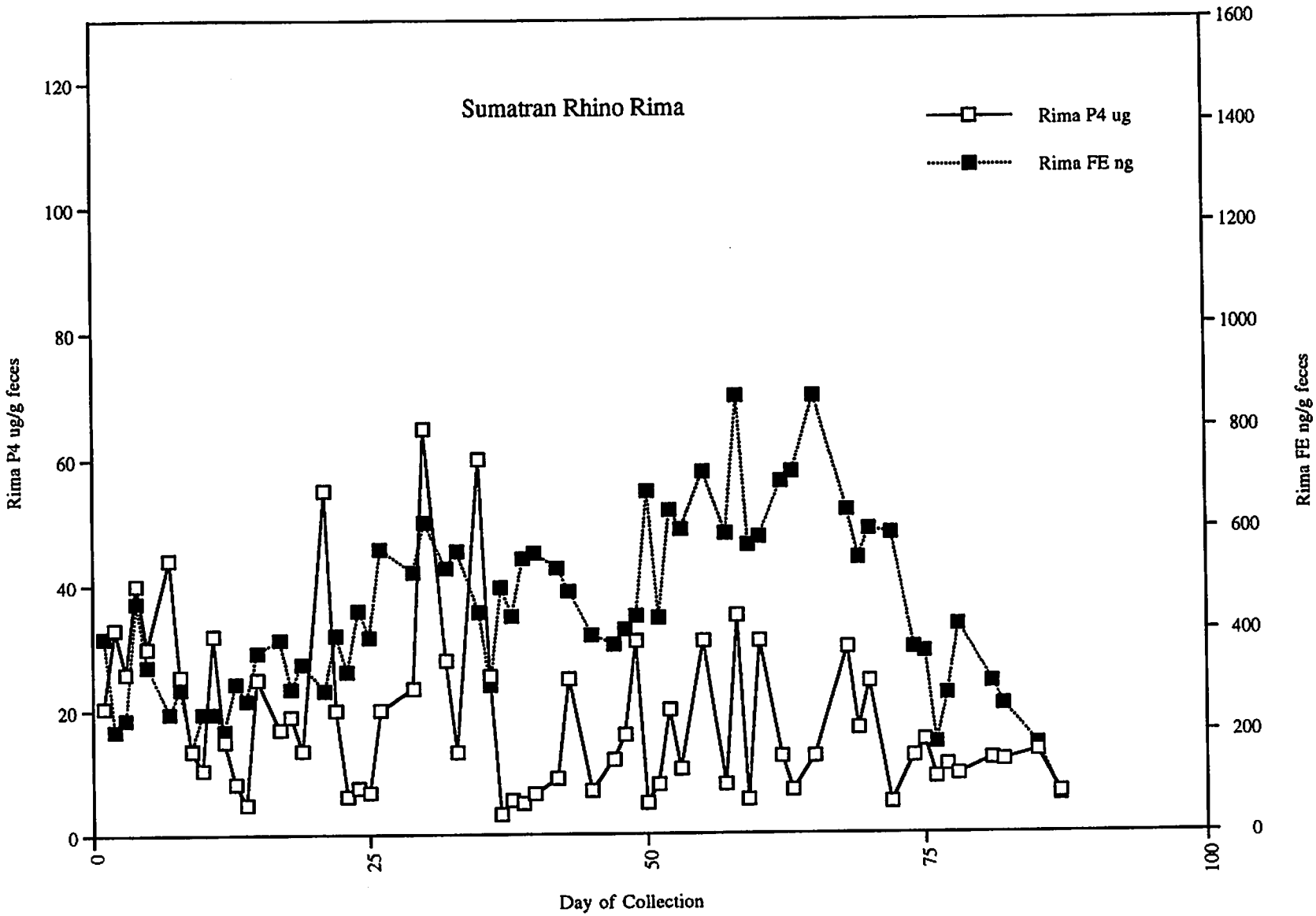
1. Perform HPLC analysis on fecal extracts and urine as a first step to provide more specific information regarding the properties of the hormonal metabolites present. Arrangements have been made for this through the University of Guelph, Ontario.
2. Collect daily fecal samples from all 4 females for a period of at least 6 months. If there are seasonal changes associated with rainfall patterns, or if the cycles are longer than expected, this prolonged collection period would allow for these factors to be taken into account.
3. Store the fecal samples frozen, but without alcohol. It is unknown what effects alcohol has on long term storage and stability of steroid hormones.

4. Compare results of hormonal analysis after extraction of dried, crushed fecal samples to wet fecal samples to determine if the more rapid method of extraction (from wet feces) is suitable.
5. Collect daily urine samples (either clean-catch or aspirated off the ground) for a period of 6 months. Analysis of more numerous samples will provide clearer information on the feasibility of urinary hormonal analysis.
6. If power outages occur, keep the freezer doors shut and the samples likely will stay frozen unless it is unseasonally hot or if the outage lasts for more than 24 hours.
7. Examine the diet of the animals for types and amounts of browse, fruits, hay, pellets, vitamin supplements, etc. and compare this information to the diets of other captive rhinos which have successfully reproduced.
8. Provide staff at Sungai Dusun with educational training in endocrinology, to enable them to interpret hormonal analyses.
9. Attempt to ultrasound all females at least once during the collection period, to gain information on ovarian activity which possibly could be correlated with endocrine results.
10. Establish a practical behavioral observation protocol which staff at Sungai Dusun can incorporate into their daily duties. This information likely would be extremely valuable for enhancing interpretation of endocrine results at this point in time.
11. Establish an EIA lab at Sungai Dusun with assistance from Metro Toronto Zoo staff.


Sumatran Rhino Merah
Merah P4 ug
M.merah FE ng
Merah P4 ug/g feces
M.merah FE ng/g feces
Day of Collection
0
50
100
0
25
50
75
100
0
200
400
600
800
1000
1200
1400
1600


Sumatran Rhino Panjang
Panjang P4 ug
Panjang Fe ng
Panjang P4 ug/g feces
Panjang Fe ng/g feces
Day of Collection
0
20
40
60
80
100
120
0
200
400
600
800
1000
1200
1400
1600
0
25
50
75
100


Sumatran Rhino Putih
Putih P4 ug
Putih FE ng
Putih P4 ug/g feces
Putih FE ng/g feces
Day of Collection
0
20
40
60
80
100
120
200
400
600
800
1000
1200
1400
1600
0
25
50
75
100


Sumatran Rhino Rima
Rima P4 ug
Rima FE ng
Rima P4 ug/g feces
Rima FE ng/g feces
Day of Collection
0
20
40
60
80
100
120
0
200
400
600
800
1000
1200
1400
1600
0
25
50
75
100