REPRODUCTIVE BIOLOGY OF THE SUMATRAN RHINOCEROS *Dicerorhinus sumatrensis* (FISCHER 1814)

MUHAMMAD AGIL

POSTGRADUATE SCHOOL
BOGOR AGRICULTURAL UNIVERSITY
2007

STATEMENT OF THE ORIGINALITY OF THE DISSERTATION AND INFORMATION RESOURCES

Accordingly, I declare that all statement in the dissertation with the title of **REPRODUCTIVE BIOLOGY OF THE SUMATRAN RHINOCEROS *Dicerorhinus sumatrensis* (FISCHER 1814)** are original and obtained from my own research results under supervision of the advisory committee. All data and information resources cited in the dissertation are stated clearly and written in the list of references and these can be objectively assessed. This dissertation is never proposed to take a degree at any other universities.

REPRODUCTIVE BIOLOGY OF THE SUMATRAN RHINOCEROS *Dicerorhinus sumatrensis* (FISCHER 1814)

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PREFACE

The research is proposed due to the situation in which the Sumatran rhinoceros is in extremely serious threatened and very limited basic reproductive biology of the species is available. Moreover, although the species has been kept since the early 19th century but the breeding program of the species was not successful, no animal bred successfully until one offspring was born in 2001 in Cincinnati Zoo, USA. There are a number of reasons for this failure in captive breeding, such as a lack of understanding of the reproductive biology which is probably the most important factor of failure, in addition to limited number of male rhinos and a lack of knowledge on breeding management.

Knowledge on reproductive biology and the information on reproductive status of the species are urgently needed in order to support the success of breeding programs of the species. On the other hand, propagation programs of the species are really important as a part of *ex-situ* conservation and to support *in-situ* conservation programs, and its population is decreasing rapidly due to illegal hunting (poaching) and habitat destruction. To date, this dissertation will provide information on the reproductive biology of the Sumatran rhinoceros and to discuss the development of some possible methods for assessing reproductive status in the female and male rhinos.

Last but not least, I do hope this dissertation could produce valuable information that could support the success of conservation programs of the Sumatran rhinoceros and possibly for the other rhinos. Finally, this dissertation is dedicated to my beloved wife 'Dasa Pratiwi' and my daughter 'Annisa Vigilanty Pratiwi' in appreciation of their tireless support of my work, and to the community who are interested and committed to the conservation of specifically the Sumatran rhinoceros and generally the other rhinos.

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Above all, I wish to express my sincere thanks to my supervisor Prof. Dr. J. Keith Hodges for his generous support, invaluable advice, interest and encouragement throughout my study period.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

RINGKASAN

MUHAMMAD AGIL. Reproductive Biology of the Sumatran Rhinoceros (*Dicerorhinus sumatrensis*, FISCHER 1814). Dibimbing oleh BAMBANG PURWANTARA sebagai ketua komisi pembimbing, HADI S. ALIKODRA, J. KEITH HODGES dan MOZES R. TOELIHERE sebagai anggota komisi pembimbing.

Badak sumatera adalah badak yang sangat terancam punah dengan polulasi hanya tinggal 300 ekor dan tingkat penurunan populasi sangat tinggi, berkurang 50% dalam satu dekade terakhir. Jumlah badak sumatera dipenangkaran juga sangat sedikit hanya sembilan ekor tersisa sampai tahun 2007 dan progam pengembangbiakan badak sumatera juga dianggap belum berhasil walaupun sudah ditangkarkan sejak satu abad yang lalu karena baru menghasilkan tiga anak dari satu induk di penangkaran. Kegagalan progam pengembangbiakan badak sumatera terutama disebabkan karena sangat terbatasnya pengetahuan tentang biologi badak sumatera dan tidak adanya metoda yang tepat untuk memonitor status reproduksi badak sumatera. Dalam studi ini dipelajari proses metabolisme dan eksresi hormon reproduksi steroid untuk pengembangan metode monitoring status reproduksi badak sumatera, dipelajari siklus reproduksi badak betina dan status kesuburan pada badak jantan, dan dipelajari gambaran hubungan antara parameter hormon reproduksi dengan perubahan perilaku seksual dan tanda-tanda siklus estrus.

Badak sumatera betina mengekskresikan hormon oestrogen lebih banyak melalui urin (67,8%) dibandingkan melalui feces (32,2%), sedangkan hormon progesterone hampir semuanya diekskresikan melalui feces (99,4%). Metabolit oestrogen dalam urin adalah oetradiol-17β glucuronide, dan oestrone dalam feces, sedangkan metabolit progesterone dalam feces terdiri atas tiga metabolit utama, yaitu dua metabolit dalam bentuk pregnanediol dan satu metabolit sebagai 5-reduce 20-oxo pregnane. Pemeriksaan metabolit hormon secara non-invasiv dari feces dan urin dapat digunakan untuk memonitor status reproduksi pada badak sumatera. Ekskresi metabolit E2 dan progesteron ke dalam urin ditemukan dengan jeda waktu (*time lag*) 24 jam, sedangkan jeda waktu ekskresi metabolit ke dalam feces terjadi 48-72 jam.

Pemeriksaan 5-P-3OH immunoreaktif dalam feces dan E2 dalam urin dapat digunakan untuk memonitor siklus estrus dan kebuntingan pada badak sumatera. Panjang siklus estrus badak sumatera adalah 23-24 hari dengan rata-rata fase folikular 6 hari dan fase luteal 17 hari. Lama kebuntingan pada badak sumatera adalah 475 hari dan kebuntingan awal dapat didiagnosa dengan hanya menggunakan sampel tunggal dari feces dengan mengukur metabolit progesteron dalam feces setelah hari ke 60 masa kebuntingan. Konsentrasi 5-P-3OH immunoreaktif dalam feces

pada fase folikel adalah 7,8 ± 3,8 μg/g feces kering, sedangkan konsentrasi hormon pada fase luteal adalah dua sampai tiga kali lipat lebih tingggi 17.3 ± 6.4 μg/g feces kering dibandingkan fase folikuler. Konsentrasi E2 immunoreaktif meningkat 3-6 kali pada fase folikel 28,2-335,8 ng/mg Cr (nilai median 96,7 ng/mg Cr) dibandingkan pada fase luteal 8,9-191,9 ng/mg Cr (nilai median 33,4 ng/mg Cr). Konsentrasi 5-P-3OH immunoreaktif meningkat signifikan (46,3 μg/g feces kering) di atas konsentrasi tertinggi pada fase luteal setelah hari ke-60 periode kebuntingan, dan akan terus meningkat selama periode kebuntingan mencapai konsentrasi maksimum antara 10-20 kali (344,2 – 787,1 μg/g faeces kering) dari konsentrasi fase luteal.

Pengamatan aktivitas ovarium dengan ultrasonogafi dan pengamatan perubahan penampakan vulva memberikan informasi yang berguna untuk monitoring siklus etrus apabila pemeriksaan metabolit hormon tidak memungkinkan. Monitoring harian perubahan penampakan vulva dan pemunculan perilaku seksual berguna untuk menentukan waktu yang tepat sebagai prediksi awal waktu penggabungan badak untuk kawin. Perilaku kawin (*mating behaviour*) selalu tampak teramati apabila folikel mencapai ukuran folikel dominan berdiameter 19-25 mm. Konsentrasi 5-P-3OH immunoreaktif signifikan lebih rendah pada saat dimana perilaku seksual tampak teramati dibandingkan pada saat dimana perilaku seksual tidak muncul.

Studi ini menghasilkan data pertama kali tentang karakterisasi ejakulat segar dari badak jantan yang diperoleh dari koleksi semen secara berbantuan. Kombinasi metode koleksi semen AGM, PM dan AV menghasilkan stimulasi ejakulat tertinggi, walaupun kualitas semen tampak lebih baik apabila menggunakan metode koleksi semen dengan kombinasi PM dan AV tanpa menggunakan AGM. Dibandingkan dengan badak lain, volume ejakulat dan konsentrasi sperma hasil koleksi dengan metode yang sama pada badak Sumatera lebih rendah dibandingkan dengan badak lainnya. Hasil analisa semen pada badak jantan ”Torgamba” di SRS menunjukkan badak jantan tersebut memiliki potensi reproduksi yang rendah ditandai dengan kejadian *oligozoospermia* karena konsentrasi spermanya rendah dan *oligospermia* karena volume ejakulatnya rendah

Metode monitoring status reproduksi secara non-invasiv yang dikembangkan dapat digunakan untuk mengevaluasi karakterisasi fungsi reproduksi badak sumatera seperti karakterisasi siklus estrus, deteksi kebuntingan dini, memonitor masa kebuntingan, dan mengetahui kelainan fungsi reproduksi. Metode tersebut juga dapat mendukung progam konservasi badak sumatera dengan menentukan waktu penggabungan yang tepat untuk meningkatkan keberhasilan pengembangbiakannya dan juga dapat digunakan untuk menentukan tingkat kesuburan populasi badak sumatera di habitatnya dengan menentukan kebuntingan pada individu badak sumatera hanya menggunakan sampel feces tunggal dengan menganalisa hormon progestinnya.

ABSTRACT

MUHAMMAD AGIL. Reproductive Biology of the Sumatran Rhinoceros (*Dicerorhinus sumatrensis*, FISCHER 1814). Under supervision of BAMBANG PURWANTARA as a chairman, HADI S ALIKODRA, J. KEITH HODGES and MOZES R. TOELIHERE as members of the Advisory Committee.

The Sumatran rhinoceros is the most critically endangered rhinoceros species, with fewer than 300 animals left worldwide and an extremely high rate of decline (50% of wild population lost over the last decade). Very few Sumatran rhinos are to date kept in captivity only about 9 animals distributed worldwide. Moreover, although the species has been kept since the early 19th century, captive breeding program is not really successful because only three offspring have been born in captivity. The first offspring was born in 2001 in Cincinnati Zoo, USA. There are a number of reasons for this failure in captive breeding, such as a lack of understanding of the reproductive biology and there were no reliable methods for monitoring reproductive status in the Sumatran rhinoceros. Therefore, the study was focused on the development of appropriate methods for monitoring reproductive status non-invasively and to study various reproductive aspects of the species.

The female Sumatran rhinoceros excreted oestrogen predominantly into urine (67.8%), whereas 14C-progesterone metabolites were almost exclusively (99.4%) eliminated via the faeces. Oestradiol-17β glucuronide is the only abundant oestrogen in urine, whereas oestrone is the major oestrogen in faeces, with oestradiol-17β being less abundant. Progesterone metabolites are more complex and resulted in the excretion of three major metabolites, two pregnanediols, one of which was 5β-pregnane-3α, 20α-diol, and a 5-reduced 20-oxo pregnane, being identified as 5β-pregnane-3α-ol-20-one. E2 and progesterone metabolites were excreted into urine within the first 24 h (time lag). In contrast, progesterone metabolites were excreted into faeces occurred within 48 hours, while oestrogen metabolites were eliminated into faeces within 72 hours.

Measurement of 5-P-3OH in faeces and E2 in urine can be used for monitoring oestrous cycles, determining oestrous cycle disorders and pregnancy in the Sumatran rhinoceros. 5-P-3OH immunoreactive levels during the presumed luteal phase (17.3 $\pm$ 6.4 µg/g dry faeces) were on average two to three fold higher compared to the presumed follicular phase (7.8 $\pm$ 3.8 µg/g dry faeces). E2 immunoreactive concentration during follicular phase is higher 3-6 fold (28.2-335.8 ng/mg Cr, median value 96.7 ng/mg Cr) compared to E2 level during luteal phase (8.9-191.9 ng/mg Cr, median value 33.4 ng/mg Cr). Oestrous cycle length in the Sumatran rhinoceros is about 23-24 days, with the length of follicular and luteal phase about 6 days and 17 days, respectively. Pregnancy length

was recorded for 475 days, and early pregnancy could be detected after day-60 according to 5-P-3OH level using single faecal sample. 5-P-3OH arose significantly (46.3 μg/gr dry faeces) after day-60 above the highest 5-P-3OH values during luteal phase. Since then, 5-P-3OH level steady increased during pregnancy up to 10-20 fold (344.2-787.1 μg/gr dry faeces) higher than luteal level.

Ultrasound examinations of ovaries and observation on changes in vulval appearance provide useful information on female reproductive status which can be used as a tool for monitoring the oestrous cycle when endocrine measurement is not possible. Monitoring daily changes in vulval appearance and occurrence of initial stages of sexual behaviour are useful to determine a proper timing for pairing since intensive vulval coloration and interest between the sexes usually occurs a few days prior to ovulation. Mating behaviour always occurred when follicle develop to reach dominant size (∅ 19-25 mm). 5-P-3OH levels were on average two fold lower during the period when sexual behaviour occurred compared to when it not occurred and the difference in levels was highly statistically significant.

The study has provided the first data on the characterization of the fresh ejaculates obtained from artificial semen collection methods. A combination of AGM, PM and AV yielded a higher success rate in stimulating ejaculate compared to the other collection methods, however semen quality appeared to be better when PM and AV without AGM was applied. In comparison to other rhino species using the same semen collection methods, the volume of ejaculates and sperm concentration was very low. The results indicate that the male Torgamba presumably has a low fertilization capacity as a result of low sperm concentrations, so called *oligozoospermia* and low ejaculate volume, so called *oligospermia*.

Non-invasive monitoring reproductive status can be used to evaluate reproduction in the Sumatran rhinoceros e.g., characterization of oestrous cycle, early pregnancy detection, pregnancy monitoring, and to determine reproductive disorder. This study could contribute and support breeding success of the Sumatran rhinoceros as part of the conservation program of the species through providing reliable information on proper time for pairing. The established method could facilitate and support field work on investigating fertility status of wild rhinos using single faecal sample to determine pregnancy in the rhino population through progestins analysis.

GENERAL INTRODUCTION

Background

The Sumatran rhinoceros is the most critically endangered rhinoceros species. With fewer than 300 animals left worldwide and an extremely high rate of decline (50% of wild population lost over the last 15 years) (Anonymous 2005a), the species is listed on Appendix I of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and in the red list of the International Union for the Conservation of Nature and Natural Resources (IUCN) (Anonymous 2004). Very few Sumatran rhinos are to date kept in captivity (about 9 animals distributed worldwide). Moreover, although the species has been kept since the early 19th century (year 1800 until now, total 96 animals), no animal bred successfully until one offspring was born in 2001 in Cincinnati Zoo, USA (Anonymous 2005b). There are a number of reasons for this failure in captive breeding, such as a lack of understanding of the reproductive biology, limited number of male rhinos and a lack of knowledge on breeding management. Of these reasons, the lack of understanding of the animal's reproductive biology is probably the most important. In fact, there is virtually no knowledge on the reproductive biology of the Sumatran rhinoceros. Information in this area would, however, be important to regulate the pairing of animals in the female oestrous period as a basis for appropriate and successful breeding because generally the animals show a very violent courtship. Problems in pairing have also resulted in a number of serious injuries and on one occasion death. Furthermore, we do not know whether the animals held in captivity are fertile. Thus, the situation for the Sumatran rhinoceros is extremely serious and there is an urgent need to acquire information on the reproductive biology of the species and on factors limiting its reproductive success in the captive environment.

In this respect, basic knowledge on the characteristics of the ovarian or oestrous cycle and methods for its monitoring are essential since the breeding success depends on reliable information on female fertility. Unfortunately, up to now there are no available methods for assessing reproductive status in the female Sumatran rhinoceros. The availability of such methods, however, is important for the establishment of a successful breeding program as it allows the identification of appropriate breeding animals and recognition of reproductive disorders (Hodges 1992). Particularly, if assisted reproductive technologies (i.e., AI, ET, and IVF, etc.) are to be considered an option for improving breeding, then accurate information on the endocrine status of the female is needed, e.g., the exact time of ovulation which is necessary for successful timing of AI or the recovery of oocytes for IVF, and the time when the uterus is synchronous with the stage of embryo development in case that ET is carried out (see Hodges & Hearn 1983; Holt *et al*. 1988; Wildt 1989; Monfort *et al*. 1993; Brown *et al*. 1994 for references).

Since many exotic species are easily stressed, difficult to handle and also potentially dangerous, invasive methods for monitoring reproductive status are usually impracticable and undesirable. Non-invasive methods are more appropriate and the only feasible approach for long-term studies on reproductive assessment in most exotic animals (Hodges & Heistermann 2002). Since all aspects of reproduction are mediated through hormones, an analysis of endocrine status is the most effective approach and, undoubtedly, the most precise of the indirect methods of monitoring female reproductive function (Hodges 1985). The broad application of these methods to a wide range of species is related to the development and application of methods for measuring reproductive hormones and their metabolites in animal excreta (Lasley & Kirkpatrick 1991). The application of non-invasive methods based on urinary and faecal hormone measurements can improve natural and artificial breeding of those species endangered in the wild and which breed poorly in

captivity as has been demonstrated for a variety of species (Brown *et al*. 1994; Schwarzenberger *et al*. 1996a; Möstl and Brunner 1997; Garrott *et al*. 1998; Morrow & Monfort 1998; Velloso *et al*. 1998; Fieβ *et al*. 1999; Stoops *et al*. 1999; Ziegler *et al*. 2000; Hamasaki *et al*. 2001; Palme *et al*. 2001; Ishikawa *et al*. 2002; Crofoot *et al*. 2003). With respect to natural breeding, non-invasive assessment of reproductive status helps to determine the precise time when mating should take place in cases where the male and female animals have to be brought together for only a short mating period, as in the case of the Sumatran rhinoceros (see above).

In contrast to the situation for other species of rhinoceros, endocrine methods for assessing reproductive status in the Sumatran rhinoceros have not been developed yet. In the Indian rhinoceros, Kassam & Lasley (1981) identified oestrone sulphate as the major urinary oestrogen and were able to use this hormone to monitor the ovarian cycle. The most abundant urinary progesterone metabolite was identified by Hindle *et al.* (1988) as being pregnanediol-3-glucuronide (PdG) and the simultaneous measurement of these two steroids provided valuable information on ovarian function and pregnancy in this species (Kasman *et al*. 1986; Hodges & Green 1989).

The level of PdG immunoreactivity in both African black and white rhinoceros is, however, either extremely low or undetectable in urine during the oestrous cycle. Furthermore, although oestrone conjugates could be measured, the pattern of excretion showed no correlation with reproductive events (Ramsay *et al*. 1987; Hodges & Hindle 1989). Failure to adapt assay methods from the Indian to the African species thus indicated the need for more basic studies of hormone metabolism and excretion.

In 1990, Hindle and Hodges carried out a radiometabolism study that identified the major urinary oestrogen and progesterone metabolites as being conjugated oestradiol-17β and conjugated 20α-hydroxyprogesterone, respectively, in the white rhinoceros. The follow up

development of specific assays for their measurement led to the first description of the ovarian cycle in both the Black and White African rhinoceros and led to improved captive breeding of these species (Hindle *et al*. 1992). Later Schwarzenberger *et al.* (1996b) established group-specific enzyme immunoassays for the measurement of 20-oxo-pregnanes in faeces and showed that these can be reliably used for monitoring ovarian activity in the Black rhinoceros. In contrast to the other species of rhinos mentioned above, there is no information on the metabolism and excretion of reproductive hormones in the Sumatran rhinoceros and therefore no method was available to non-invasively monitor female reproductive function in this species.

Apart from the inability to monitor reproductive status, the breeding failure of the Sumatran rhinoceros in captivity is also probably based on the fact that only a limited number of males are maintained in captivity and that there are no data on fertility status available. Semen analysis is one of the methods that can be used for assessing fertility in the male. Regarding semen analysis, there is a need for obtaining proper ejaculate through semen collection techniques. Although, Stover *et al.* (1981); Schaffer *et al.* (1990); Roth *et al.* (1999); and O'Brien & Roth (2000) have reported on the establishment of semen collection methods and semen analysis in rhinoceros species and other wild animals, unfortunately, there have been no methods established for assessing fertility in the male Sumatran rhinoceros.

Knowledge of the reproductive anatomy is also important in terms of the development of assisted reproductive technology in the species. For successful application of assisted reproductive technologies, it is important to understand the anatomy and function of the reproductive organs. For instance, in the application of *in-vitro* fertilization (IVF), understanding the anatomy and physiology of the ovary is required for a successful ova-pick up technique. On the other hand, as stated by Holt *et al*. (1988); Hodges (1992); Monfort *et al.* (1993); Heistermann *et al.*

(1995) the development of techniques for artificial insemination (AI), oocyte maturation, IVF and embryo transfer (ET) depends on an understanding of the basic knowledge on the reproductive physiology of the species in question and the availability of practical and reliable methods for monitoring reproductive function. This is exemplified by the success story of AI in Eld's deer (*Cervus eldi thamin*) and Blackbuck (*Antilope cervicapra*) which was supported by the availability of basic information on female reproductive physiology as required for proper timing of AI (black buck: Holt *et al*. 1988; Eld's deer: Monfort *et al*. 1993).

Research on the mentioned aspects of the reproductive biology of the Sumatran rhinoceros is urgently needed given the critical status of the species both in the wild and in captivity. By providing such information, it is hoped that this study will contribute both directly and indirectly to the long-term conservation of this highly endangered species.

Identification of Problems

From a conservation point of view, a number of problems have been identified contributing to the failure of the propagation of the Sumatran rhinoceros. These are:

1. Information on gross anatomy of the reproductive organs in the Sumatran rhinoceros is limited, which is important in understanding general reproductive function, and to support assisted reproductive technologies.
2. A reliable and appropriate method for assessing reproductive status in the Sumatran rhinoceros does not exist yet which is important for longitudinal monitoring of the reproductive status of the species and identification of appropriate females for captive breeding.
3. Information on ovarian cycle characteristics is lacking, which is important to provide a basis for establishing successful breeding programs of natural and eventually artificial breeding.

4. An evaluation on the relationship between hormonal profiles during the ovarian cycle and sexual behaviour and morphological changes has been overlooked in the Sumatran rhinoceros, although this may provide a more practical method for monitoring female reproductive status.
5. Information on fertility in the male is not available due to the almost complete lack of studies in males resulting from the unavailability of reliable methods for assessing male fertility parameters.

General Objective

The main aim of the study is to carry out research leading to the development of appropriate methodologies to obtain accurate and reliable information on various aspects of the reproductive biology of the Sumatran rhinoceros through (1) to study hormone metabolism and excretion in the female Sumatran rhinoceros, (2) to study endocrine characterisation of reproductive status in the female Sumatran rhinoceros, and (3) to study male's breeding potency.

Specific Objectives

The study on hormone metabolism and excretion in the female Sumatran rhinoceros will involve a radiometabolism study with specific objectives are (1) to determine the time course and distribution (route of excretion) of ^{3}H-labelled oestradiol-17β and ^{14}C-labelled progesterone metabolites in urine and faeces of the Sumatran rhinoceros and (2) to provide information on the identity of the major urinary and faecal metabolites of both hormones. The objectives of the study on endocrine characterisation of reproductive status in the female Sumatran rhinoceros are (1) to establish and biologically validate reliable methods of urinary and faecal hormone analysis for monitoring female ovarian function, (2) to

apply these methods to assess the fertility status and thus reproductive potential of individual females of the captive population, (3) to provide information on ovarian cycle length, (4) to describe changes in oestrogen and progestin excretion during an individual pregnancy, and (5) to examine the relationship between changes in genital morphology and sexual behaviour and endocrine patterns in a single female. Lastly, the objectives of the study on male's breeding potency are (1) to establish an appropriate semen collection method and (2) to characterize ejaculate and semen quality in a single male.

Outcome of the Study

The study will be expected to provide an important contribution directly or indirectly towards the breeding program and conservation of the Sumatran rhinoceros by providing (a) appropriate and reliable method for assessing reproductive status in the female, (b) reliable information on the fertility status of a substantial number of captive females currently held, (c) information on the fertility status of a single male rhino through the assessment of semen quality. With respect to conservation of the Sumatran rhino, the research will presumably support the propagation program from the existing captive population specifically in the Sumatran Rhino Sanctuary (SRS), Way Kambas National Park, Lampung-Indonesia which keeps the largest number of captive Sumatran rhino in the world.

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REVIEW OF LITERATURE

The Sumatran Rhinoceros and its Taxonomy

In former times, perhaps million years ago, rhinos existed in the form of many species, were widely distributed throughout the world and lived in high abundance. Nowadays, only five species of rhinoceros exist worldwide. Two species (black and white) survive in Africa, while three species (Indian, Javan and Sumatran) are distributed on the Asian continent. Based on the history, the surviving rhinoceroses are called "the precious representatives of the glorious heritage and history of the rhino family on our planet" (Anonymous 2007). The Sumatran rhinoceros is one of the three species of rhinoceros in Asia being unique and critically endangered (Anonymous 2004; 2007).

In English, this species is commonly referred to as the Sumatran rhinoceros. The scientific name is *Dicerorhinus sumatrensis* (G. Fischer 1814). This means that it belongs to the genus Dicerorhinus, which also includes many fossil species from Europe and Asia (Anonymous 2005). Dicerorhinus is derived from the Greek; *di*, meaning "two"; cero, meaning "horn" and rhinos, meaning "nose", while sumatrensis refers to the Indonesian island of Sumatra (Anonymous 2005; 2007).

The first person who described an Asiatic two horn rhinoceros (from Sumatra) was Bell in 1793. It was, however, 20 years later that Fisher (1814) provided the scientific name for the Sumatran rhinoceros that is now commonly used (Strien 1974). Nevertheless, in former times there were more than four names of the species, the most widely used being: *Rhinoceros crossi*-GRAY 1854; *Rhinoceros lasiotis*-BUCKLAND 1872; *Ceratorhinus niger*-GRAY 1873 and *Ceratorhinus blythii*-GRAY 1873 (Strien 1974)

Groves (1967) reported that around the 1960's there were only three Sumatran rhinoceros subspecies i.e., *Dicerorhinus sumatrensis*

sumatrensis FISHER, 1814, distributed in Sumatra and Peninsular Malaya; *Dicerorhinus sumatrensis harrisoni* GROVES, 1965, present in Borneo (Kalimantan) and *Dicerorhinus sumatrensis lasiotis* BUCKLAND, 1872, occurring in the Northern part of Burma (Myanmar), Assam and the Eastern part of Pakistan. However, according to the International Rhino Foundation (IRF), to date only two subspecies exist, namely: *Dicerorhinus sumatrensis sumatrensis*, the so called Western Sumatran rhinoceros; and *Dicerorhinus sumatrensis harrissoni* the Eastern Sumatran rhinoceros (Anonymous 2007).

Biology and ecology of the Sumatran Rhinoceros.

The Sumatran rhinoceros is well known as the smallest and the most primitive form of the existing species of rhinoceros. Body length is about 200-300 cm, the shortest among the rhinos as described by Nowak (1991) and Anonymous (2007) (c.f., Javan rhino 200-400 cm; black rhino 300-380 cm; white rhino 380-500 cm; and Indian rhino 300-380 cm). The Sumatran rhino has also the lowest body weight which is only about 800 – 1000 kg (Strien 1986; Anonymous 2005), compared to 1800-2700 kg for the Indian rhinoceros; 1800kg for the African white rhinoceros; 900-2300 kg for the Javan rhinoceros; and 800-1350 kg for the African black rhinoceros (Anonymous 2007).

D. sumatrensis has two horns that are located above the thick nasal bones, the anterior horn is much longer than the posterior one. The horns are developed from skin with no connection to the skull, although the bone on the skull can be its foundation. The horny material is composed of ceratinized cells growing from epidermis covering a cluster of long dermal papillae. The horns grow during the life and redevelop if lost (Fowler 1986).

Figure 1. Sumatran rhinoceros (*Dicerorhinus sumatrensis*), the smallest rhinoceros species. The whole body is covered by hair (the hairy rhino). Documented at Sumatran Rhino Sanctuary, Way Kambas National Park, Lampung Indonesia (Doc. SRS 2007).

The general appearance of the Sumatran rhino's body is mostly different compared to that of the other rhino species. The skin is relatively soft, and rather thin (maximum only 16 mm), and only has two major skin folds; one just behind the fore-legs surrounding the trunk and the other one over the belly and flank, not on the back, just before the hind-legs (Evans in Strien 1974). The hair-cover is one of the major characteristics of the Sumatran rhino which also discriminates the species from the other rhinoceros. Therefore, the Sumatran rhino is very commonly called the "hairy rhinoceros" (Nowak 1991; Anonymous 2007).

There is no reliable information concerning the age of sexual maturity of the Sumatran rhinoceros, due to the lack of studies on hormonal function and sperm production in the young rhinos. Nevertheless, information on the growth of the young animal was reported from the calf born in Calcutta zoo in 1889 and also from looking

at photographs of one of the animals in Vienna zoo. From these data, Strien (1974) suggested that one might come to the conclusion that *D. sumatrensis* reaches full grown size at about 2.5 to 3 years. However, for one female born in Malaysia, the sexual maturity was recorded to appear at the age of 6-7 years (Anonymous 2007). Nowak (1991) suggests that *D. sumatrensis* probably does not begin to breed before until at least an age of 7-8 years. In contrast to the Sumatran rhinoceros, there is reliable knowledge on the age of sexual maturity of the other rhinoceros species (Indian rhino: ♀ 4-5 years, ♂ 7-9 years, Wirz-Hlavacek *et al*. 1997; Tomasova 1998; African black rhino: ♀ 6-7 years, ♂ 7-8 years, Mentis 1972; Bertschinger 1994; African white rhino: ♀ 4-5 years, ♂ 6-7 years, Mentis 1972).

The longevity record of the Sumatran rhinoceros has been reported by Strien (1974) to be 32 years and 8 months for an animal in captivity. According to reports from IRF, the lifespan of the species is assumed to be 35-40 years (Anonymous 2007).

The Sumatran rhino is a typical browser with a very varied diet, that of a great diversity of the plant species in tropical forest. The rhinos do not feed grass, except bamboo. The largest part of the diet consists of leaves, twigs, sapling and small branches, and also includes fruit (wild mangoes), figs, bamboo and all plants found in the secondary growth (Nowak 1991). The Sumatran rhino lives in the dense tropical rain forest, where it is found from lowland up to highland (Anonymous 2005; 2007). Concerning social structure, the Sumatran rhinoceros is the only absolutely solitary living rhino among the rhinoceros species (Anonymous 2007). Male and female have each own territory.

Distribution and population in the wild

The historical distribution of the Sumatran rhinoceros was found from Indonesia up to the North-Eastern states of India. SOS Rhino reported that the species has been distributed in Indonesia (the island of

Borneo and Sumatra), Malaysia, Western Thailand, Burma (Myanmar), Laos, Cambodia and Vietnam, with few older records in the North-Eastern states of India (Anonymous 2005).

The current distribution of the Sumatran rhino population is limited mainly to the Malay Peninsula, on the Indonesian island of Sumatra, and on Borneo (Sabah and Sarawak, Malaysia). There are some reports of unconfirmed occurrence of the Sumatran rhinoceros in several other countries and regions such as India (Manipur and Nagaland area), in the south and north of Myanmar, Laos (forest near Da Lat), Thailand (Krachan isthmus and Phu Khieo Wildlife Sanctuary), and Indonesia (Kalimantan, status not determined) (Anonymous 2005; 2007).

At the AsRSG (Asian Rhino Specialist Group) meeting in 1993, the population size of the Sumatran rhinoceros in the wild was estimated to be about 356-495 individuals. The population was decreased by 1995 to approximately about only 300 rhinos left in the wild (Foose *et al*. 1997). Perhaps the largest number of the subspecies *sumatrensis* living on the island of Sumatra is only about 100-200 animals. According to IRF reports, the size of the population has decreased rapidly by about 50% in the last 15 years due to poaching and destruction of available habitat (Foose *et al*. 1997; Anonymous 2007).

Conservation status of the species

Compared to other rhino species (except the Javan rhinoceros, *Rhinoceros unicornis*), the population size of the Sumatran rhinoceros is very small, with less than 300 individuals left (see above). Unfortunately, captive propagation of the species has been of very limited success since it started a century ago. In fact, apart from the failure of captive breeding, the biology of the species is little known.

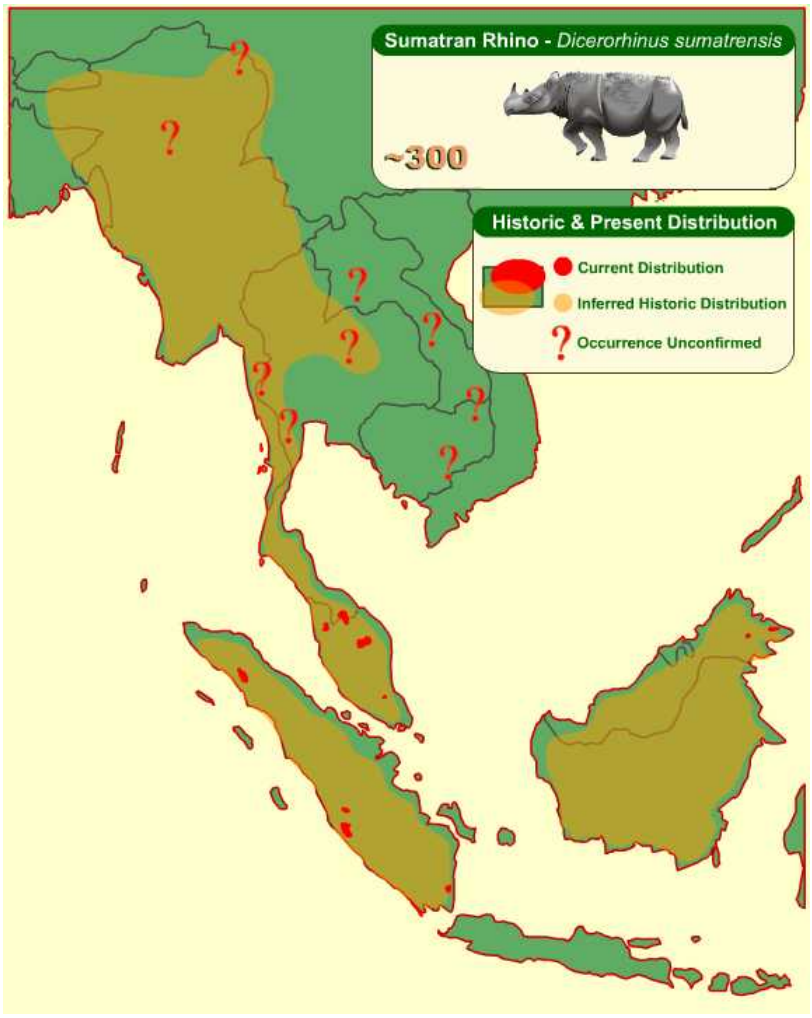


Figure 2. Distribution map of the Sumatran rhinoceros, current, inferred historic and unconfirmed occurrence (see the legend, Anonymous 2007).

According to CITES (Convention on International Trade in Endangered Species of Flora and Fauna), the Sumatran rhinoceros is listed in APPENDIX I, indicating the animal is in the category of an endangered species (Anonymous 1994). IUCN (the world conservation union) lists the Sumatran rhinoceros in "the 2003 IUCN red list of threatened species" with the status of species being "critically endangered" (Anonymous 2004). In contrast, for the white rhinoceros, a proposal from South Africa was even been considered at the 9th CITES conference 1994 to downlist the status of the African white rhinoceros to Appendix II of CITES (Anonymous 1995).

Captive propagation programs

The first record on the captive Sumatran rhino program was given by Reynold (1961) who stated that most of the animals were brought into captivity around the turn of the century. Only one rhino bred successfully and gave birth to one offspring in captivity in Calcutta in 1889, and two other captive females which conceived in the jungle gave birth in Calcutta zoo in 1872 and 1895 (Strien 1974). Since captive breeding was not successful in former times, a large program to propagate the Sumatran rhino in captivity has been developed since 1984. A total of 40 rhinos have been captured in Indonesia (Sumatra island), Peninsula Malay, and Sabah (Borneo, Malaysia) for the captive program during the period of 1984-1995, and one rhino died during the capture (Foose 2006).

Since 1800, a total of 96 Sumatran rhinos have been exhibited in 49 collections spread over 20 countries (until the end of 1994). The first animal arrived in Hamburg, Germany in January 1872. The Sumatran rhinos were exported out of Indonesia during the 19th century until 1909, but all of these had died in 1916 (Anonymous 2005). However, since 1984 until 1995, a total of 39 Sumatran rhinos have been distributed to captive facilities in Indonesia, Malaysia (Peninsula Malaya, and Sabah), Europe (England) and USA (two different places). Unfortunately, there were only five rhinos left in captivity until 2005. Recently, two offspring have been born in Cincinnati Zoo in 2001 and 2004, and two young females have been captured from the wild at the end of 2005 to save them from poaching (Anonymous 2007).

Due to the almost failure in captive breeding of the Sumatran rhinoceros, a decision was made in 1994 to establish a Sumatran rhino sanctuary (SRS) in Way Kambas National Park, Lampung Indonesia in the origin of the Sumatran rhino habitat (Anonymous 1994). In 1998, one male Sumatran rhino (Torgamba) was sent back from Port Lympne Zoo in England to SRS. At the same time, two female Sumatran rhinoceroses were also sent back from the Zoo in Indonesia to SRS.

Reproductive biology of the rhinoceros species

Detailed knowledge of the reproductive biology is important in order to support the breeding success of the species. In this respect, knowledge on reproductive anatomy, behaviour, and reproductive status of the animals is particularly important for maximizing natural breeding or establish artificial breeding programs including application of assisted reproductive technology (Heistermann *et al.* 1995b).

However, information on the reproductive biology of the Sumatran rhinoceros is very limited, and for many aspects, it does not even exist. For instance, there is absolutely no information on the gross anatomy of the reproductive organs and on semen quality and characteristics, and very limited information exists on ovarian cycle characteristics. In contrast, there is much information on the reproductive biology of the other rhino species (African black rhino: Ramsay *et al*. 1987; Hindle *et al*. 1992; Brown *et al*. 1994; Berkeley *et al*. 1997; Garnier *et al*. 1998; 2002; Radcliffe *et al*. 2001; African white rhino: Hindle & Hodges 1990; Hindle *et al*. 1992; Radcliffe *et al*. 1997; Patton *et al*. 1999; Indian rhino: Kassam & Lasley 1981; Kasman *et al*. 1986; Schaffer *et al*. 1990; Roth & O'Brien 2000; Schwarzenberger *et al*. 2000). In the case of the Indian rhinoceros, this information has helped to establish and apply assisted reproductive technologies such as artificial insemination (Roth & Stoops, 2006 personal communication).

Information on reproductive behaviour in the rhinoceros species is generally very limited. Only a few researchers have reported about reproductive behaviour in the Indian rhinoceros (Stoops *et al*. 2004), African black rhino (Patton *et al*. 1999), and African white rhinoceros (Hindle *et al*. 1993). Oestrous behaviour in the Indian rhino was identified by specific changes in behaviour, including a marked decrease in appetite, an increase in overall activity and vocalization and frequent urine spraying associated with vulva winking. The Indian rhinoceros shows a behavioural oestrous that lasts for 2 days. In contrast, description on reproductive

behaviour in the male rhinoceros does almost not exist, although, there is one report from Stoops *et al*. (2004) in the male Indian rhinoceros. Interestingly, here the male shows behavioural changes that preceded behavioural oestrous of the female by 2-3 days. These changes included reduced appetite overnight, an increase in vocalization (whistling and roaring), and extreme resistance to leaving the barn where the female was held.

Information on the oestrous cycle in the Sumatran rhinoceros is very limited, which is contrasting to the situation in the other rhino species, for which abundant data on the ovarian cycle and pregnancy are available for African white rhinoceros (Hindle *et al*. 1992; Schwarzenberger *et al*. 1994; 1998; Schwarzenberger & Walzer 1995; Radcliffe *et al*. 1997; Patton *et al*. 1999; Brown *et al*. 2001), African black rhinoceros (Hindle *et al*. 1992; Schwarzenberger *et al*. 1996b; Garnier *et al*. 1998; 2002; Radcliffe *et al*. 2001), and Indian rhinoceros (Kassam & Lasley 1981; Kasman *et al*. 1986; Roth & O'Brien 2000; Schwarzenberger *et al*. 2000; Stoops & Roth 2004). To date, there are only three publications providing information on the reproductive cycle of the Sumatran rhinoceros. Heistermann *et al*. (1998) and Roth *et al.* (2001; 2004) have described the ovarian cycle length to be about 21-25 days, although each report was based on only a single female. No information exists about the length of the follicular or luteal phase, the relationship between sexual behaviour and hormones profiles during the cycle and no longitudinal data on ovarian activity are available.

In the African white rhinoceros, two types of oestrous cycles have been described; type I cycles described by a duration between 4 and 10 weeks and type II cycles with a duration of 10 weeks or more (see Schwarzenberger *et al*. 1998 and Patton *et al.* 1999). Schwarzenberger *et al.* (1998) described the follicular phase for both cycle types being 16.7$\pm$1.7 days, whereas the luteal phase is reported to be 16.8$\pm$2.0 days and 58.7$\pm$4.8 days for type I and type II cycles, respectively. The African

black rhinoceros has an oestrus cycle length of 21-26 days as reported by Hindle *et al.* (1992), Schwarzenberger *et al*. (1993), Radcliffe *et al.* (2001) and Garnier *et al*. (2002), consisting of a follicular and luteal phase of 9 and 18 days, respectively (Garnier *et al*. 2002). On the other hand, the Indian rhinoceros is the only rhinoceros species for which a prolonged follicular phase of 14-16 days has been reported (Schwarzenberger *et al*. 2000; Stoops *et al*. 2004). Schwarzenberger *et al*. (2000) also described diestrus length that is indicated by basal steroid levels between declining 20-oxo-pregnanes and subsequently increasing estrogens to be 11.4±1.2 days. This parameter was never reported in the other rhinoceros species.

Due to the limited numbers of female Sumatran rhinoceros bred and giving birth in captivity, the only information on gestation length in the Sumatran rhino comes from a single female (Emi) in Cincinnati Zoo which produced two offspring in 2001 and 2004. Gestation length was recorded to be 475 days for the first offspring (Roth T.L., personal communication, Roth *et al.* 2004), which is almost similar to the 14-16 months gestation length reported for the African black (Schwarzenberger *et al.* 1993; Berkeley *et al*. 1997; Garnier *et al.* 1998; 2002) and Indian rhinoceroses (Kasman *et al.* 1986; Schwarzenberger *et al.* 2000). Gestation length in the African white rhinoceros is slightly longer (15 to 17 months, Schwarzenberger *et al.* 1998; Patton *et al.* 1999).

The reproductive biology of the male rhinoceros is largely overlooked, and only a few studies have been performed in this respect (see Platz *et al*. 1979; Schaffer *et al*. 1990; 1998; Hermes *et al*. 2005; Roth *et al*. 2005). Although assessment of semen quality has historically been used to predict potential fertilizing capability in mammals, studies on semen parameters in the rhinoceros species are limited. That is possibly because there were no methods available to collect semen from rhinoceros until Platz *et al*. (1979) succeeded in semen collection in the African black rhinoceros yielding 18-48 ml ejaculates using penile stimulation. Later, Schaffer *et al*. (1990) reported the first information on

semen quality in the Indian rhinoceros using different semen collection methods. Although, the highest volume of ejaculate (30-60 ml) was obtained using an artificial vagina, sperm concentration was poor. In contrast, rectal stimulation followed by penile massage yielded ejaculates of improved semen quality with respect to total sperm number and motility (Schaffer *et al*. 1990). Roth *et al*. (2005) showed that use of electroejaculation was successful to collect semen from the Indian rhinoceros and the African black and white rhinoceros. Electroejaculation in these rhinoceros species yielded high ejaculate volumes (36-200 ml), high total sperm numbers (1.8-66.4x10^6/ml ejaculate), and increased proportion of motile (60-90%), and structurally normal sperm (28-74%). Recently, Hermes *et al*. (2005) reported that sperm motility and morphology in the African white rhinoceros correlates positively with size of the accessory glands. Interestingly, semen quality in the African white rhinoceros was also associated with group size, suggesting that the social environment influences functional reproductive parameters and that change of territorial status in the white rhinoceros improves semen quality. In contrast, only one study on semen collection and sperm characteristics and only from a single male was reported for the Sumatran rhinoceros by O'Brien & Roth (2000). Semen was collected using post-coital technique, which yielded high ejaculate volumes (104 ml), with high total sperm numbers (2.5x10^9/ejaculate), which were moderately motile (60 %), but showed a high degree of morphological abnormalities (~60 %). To date, other techniques of semen collection have not been tested in the Sumatran rhinoceros. More knowledge in this area would not only be of comparative value, but may also be of practical importance if assisted breeding using artificial insemination is considered an option in the future breeding management of the species.

Methods of Assessing Reproductive Status

The importance of the methods

An assessment of female reproductive status is important in order to support breeding programs for many species in captivity. In this respect, the information gained is particularly important for maximizing natural breeding by providing the basis for an improved breeding management. Furthermore, it is the important key in the recognition and treatment of reproductive disorders. The ability to predict and detect the time of ovulation, to diagnose early pregnancy, to monitor foetal well being and to predict the time of parturition may help to improve fertility, reduce pregnancy loss and increase neonatal survival (Heistermann *et al*. 1995b). From a conservation point of view, knowledge of reproductive status of animals is also beneficial as it provides information on reproductive potency of the species and thus helps towards assessing the reproductive viability of the captive population.

Hodges (1986) described that the choice of methods for assessing reproductive status in exotic species depends on a variety of factors:

a. Accuracy and reliability are essential, since in many cases important management decisions arise from the outcome of the assessment
b. Methods need to be versatile, since comparative studies on exotic animals require multispecies application
c. Methods should produce valid and informative results from a small number of observations
d. The most important factor is the need for practicality, which means that the method has to be simple and economical and should not require the use of sophisticated laboratory equipment

There is a need to develop a specific method of assessing reproductive status for each species, because species differ with respect to what kind of information is most reliable and useful in reflecting

reproductive function. Therefore, Hodges (1996) states that the choice of methods depends largely on the type of information required, the species in observation and circumstances under which the study will be carried out. Several possible methods for assessing reproductive status in exotic animals are shown in Table 1.

Invasive methods

Invasive methods involve intensive and excessive contact with the animals. In many cases, excessive restraint and chemical immobilization is required (Hodges & Heistermann 2002). Therefore, the methods are not suitable and preferable for longitudinal studies or for monitoring reproductive status in free ranging animals which are likely to be stressed and difficult to handle.

The disadvantage of laparoscopy (endoscopy) and laparotomy is that both are surgical procedures requiring immobilization and anaesthesia, and in most cases frequent and repeated application is impractical. However, the main advantage of laparoscopy (endoscopy) is that it enables direct visualization of the reproductive organs and reduces any potential problems of interpreting information on reproductive status obtained by more indirect methods (Hodges 1996). Laparoscopy (endoscopy) allows the detection of ovulation within a short interval of time (probably less than 6 hours), and thus, has been particularly useful for predicting the time of ovulation (Wildt *et al*. 1977a). Moreover, laparoscopy has been useful to monitor ovarian function in carnivores in which there has been limited success in the development of more non-invasive procedures (see Wildt *et al*. 1977b; Wildt & Seager 1979; Wildt 1980, for references). Nowadays this method is no longer the only method of monitoring the oestrous cycle and detection of time of ovulation in carnivores, since several researchers have been successful in

Table 1. Possible methods for assessing reproductive status in exotic animals*

Non-invasive methods	Invasive methods
A. No animal contact required	
1. Behavioural observation	1. Laparoscopy
2. Urine sample**	2. laparotomy
3. faecal sample**	3. Blood sample**
B. Capture and/or restraint required	
1. Saliva sample**	
2. Milk sample**	
3. Vaginal cytology	
4. Ultrasonography	

*) Adapted from Hodges (1996)

**) Methods involving hormone analysis

establishing assays for faecal steroid hormones in several carnivores (cats: Shille *et al*. 1984, Brown *et al*. 1994b; clouded leopard: Brown *et al*. 1995; cheetah: Brown *et al*. 1996). However, to some extent laparoscopy or endoscopy is still useful to support successful application of artificial/assisted reproductive technology in some species (swine: Ratky *et al*. 1998; rhesus monkey: Hewitson *et al*. 1998; 2002; red deer: Asher *et al*. 2000).

Measurement of hormones in blood is still probably the most informative and widely used method of the indirect techniques for assessing reproductive function in laboratory and domestic animals (Hodges 1996). There can be situations in which non-invasive method do not exists, due to the lack of suitable methodologies, or where husbandry practices and/or degree of animal training are of sufficient level that blood

collection represents little additional risk and stress (Hodges *et al*. in press). For example, monitoring reproductive status in Asian and African elephants in North America is done by blood progestin analysis (Brown 2000). Longitudinal monitoring of steroid and protein hormones during the oestrous cycle and pregnancy using blood samples has also been used in a number of wildlife species, including rhinos (Berkeley *et al.* 1997; Roth *et al*. 2001; 2004), tapirs (Brown *et al*. 1994a), beluga whales (Robeck *et al.* 2005), mithuns (Mondal *et al*. 2005), yaks (Sarkar & Prakash 2006), buffalo (Mondal & Prakash 2004), and felids (Brown 2006). The advantages include fewer problems associated with samples preparation (less need for complicated extraction, hydrolysis, etc.), no need for indexing concentration, real-time reflection of hormonal status (little or no time lag) and the possibility to monitor short-term endocrine changes (Hodges *et al*. in press). However, with the development of simplified procedures for the measurement of hormones in urine and faeces, blood sampling is no longer necessary and unacceptable for the majority of exotic species. Furthermore, since many wildlife species are easily stressed, intractable and also potentially dangerous, the repeated capture and restraint necessary for regular blood sampling is both undesirable and impractical (Hodges 1985). Moreover, the stress involved in such procedures can have negative effects on reproductive function of the animals and thus can be contradictory to what is wanted.

Non-invasive methods

Invasive methods for monitoring reproductive status are impractical for most exotic species, including the rhinos. Non-invasive methods, based on the measurement of hormone metabolites in urine and faeces are therefore preferable, particularly as they have been shown to be as accurate and reliable as blood hormone analysis for monitoring the reproductive cycle in many animal species (for review see Heistermann *et al*. 1995b; Hodges *et al*. in press). Therefore, to date urinary and/or

faecal hormone analysis is widely used for monitoring reproductive status in many species of both domestic and wildlife (felids: Brown *et al*. 1994; farm animals: Schwarzenberger *et al*. 1996a; African black rhino: Schwarzenberger *et al*. 1996b; bitch: Möstl & Brunner 1997; elk: Garrott *et al*. 1998, Stoops *et al*. 1999; oryx: Morrow & Monfort 1998; maned wolves: Velloso *et al*. 1998; Asian and African elephant: Hodges 1998, Fieβ *et al.* 1999, Czekala *et al*. 2003; Hanuman langur: Heistermann *et al*. 1995a; Ziegler *et al*. 2000; sika deer: Hamasaki *et al*. 2001; mare: Palme *et al*. 2001; brown bear: Ishikawa *et al*. 2002; great hornbill: Crofoot *et al*. 2003). Analyzing steroid metabolites non-invasively can also provide other information, such as on gender of the conceptus in elephants (Meyer *et al*. 2004) and in the redfronted lemur (Ostner *et al*. 2003). In addition, some researchers have reported that the hormone profile generated from analysis of faecal and urinary steroid metabolites had a good correlation to female genital morphology and sexual behavioural changes (bonobo: Heistermann *et al*. 1996; Barbary macaques: Möhle *et al*. 2005; long-tailed macaques: Engelhardt *et al*. 2007; white-handed gibbon: Barelli *et al*. 2007).

Apart to urinary excretion, large amounts of steroids are excreted into faeces. In fact, in several mammals (e.g., many of the Felidae: Shille *et al*. 1990; Brown *et al*. 1994), faecal excretion predominates. For instance, while in the Sumatran rhinoceros (see chapter III) and in the African elephant (Wasser *et al*. 1996) estrogens are primarily excreted into urine, progestagens are predominantly excreted into faeces. While in the initial years of the development of non-invasive endocrine methodologies, urinary analysis was the focus of interest, there has been considerable recent interest in application of faecal hormone analysis for monitoring reproductive status in exotic species. The main advantage of this strategy is that faecal samples can be collected more easily under certain captive conditions, e.g., in group living animals, but particularly in the wild situation. Sometimes, faecal hormone analysis is the only way to obtain

reliable information on reproductive status (see Perez *et al*. 1988; Ziegler *et al*. 1989; Heistermann *et al*. 1995b for references). In the majority of situations, faecal sampling also provides the only feasible option for longitudinal studies in the wild and it is predominantly for this reason that interest in faecal assay methodologies has increased so markedly over the last 5-10 years (Hodges *et al*. in press).

Hormones are also found in saliva. However, salivary steroid concentrations usually are significantly lower than circulating levels, because only the unbound fraction is present. Czekala *et al*. (1996) and Gomez *et al*. (2004) demonstrated salivary steroid analysis to be useful to monitor reproductive status in the African black rhinoceros and the Indian rhinoceros, respectively, although, Gomez *et al.* (2004) also reported that not all the hormone assay systems tested were effective. In contrast, Fenske (1996) and Atkinson *et al.* (1999) reported the limited usefulness of salivary analysis for assessing reproductive function, with poor correlation observed between circulating and secreted concentrations. The inability to detect biologically relevant immunoactivity in saliva may be due to assay matrix effects (Hodges *et al*. in press).

Since non-invasive monitoring is preferably conducted by analyzing urine and faeces as mentioned above, knowledge on hormone metabolism and excretion in each species of interest is certainly important. Since, species can largely vary in this respect (Hodges 1992; Palme *et al*. 1993; Heistermann *et al*. 1998; Brown 2006), a careful validation of hormone measurements for each species is required if the results are to be accurate and reliable with respect to reproductive assessment (Heistermann *et al*. 1995a).

Metabolism and Excretion of Steroid Hormones (Reproductive Hormones)

If the levels of hormone are to be regulated in response to various needs, there must be mechanisms for hormones to be cleared from the circulation once they are released. The steroid hormones are metabolically altered inside tissues and the products are excreted by the kidneys and/or the gastrointestinal tract (Baxter *et al*. 1987). Steroids are metabolized by many tissues including liver, kidney, muscle and blood (Schulster *et al*. 1976). They are also metabolized in the intestine where the caecum is probably the major site for steroid transformation, and most of the steroidal transformations by intestinal bacteria in the gut are reductive (Honour 1984). The major site of steroid metabolism however is the liver.

Progesterone metabolism was found to be more complex with a higher degree of species variation than that of oestradiol. For example, pregnanediol-3-glucuronide (PdG) and pregnanetriol-3glucuronide are the major urinary metabolites in hominoid and several platyrrhine monkey species (Axelson *et al*. 1984; Lasley & Kirkpatrick 1991). In contrast, cercopithecines do not excrete large amounts of either of these metabolites but instead excrete androsterone and related conjugates (Liskowski & Wolf 1972), whereas, catarrhine monkeys would appear to produce both C-21 and C-19 metabolites of progesterone (Shideler *et al.* 1993b). Moreover, Hindle & Hodges (1990) and Hindle *et al*. (1992) reported that the African white rhinoceros excretes mainly 20-dihydroprogesterone (20-DHP) sulphate as progesterone metabolite in the urine, while the African black rhinoceros excretes 20-DHP as a glucuronide into urine. The Indian rhinoceros, on the other hand, excretes predominantly pregnanediol glucuronide (Kasman *et al.* 1986). The Sumatran rhinoceros metabolizes progesterone mainly into pregnanolones, which are excreted almost completely into faeces (Heistermann *et al.* 1998,

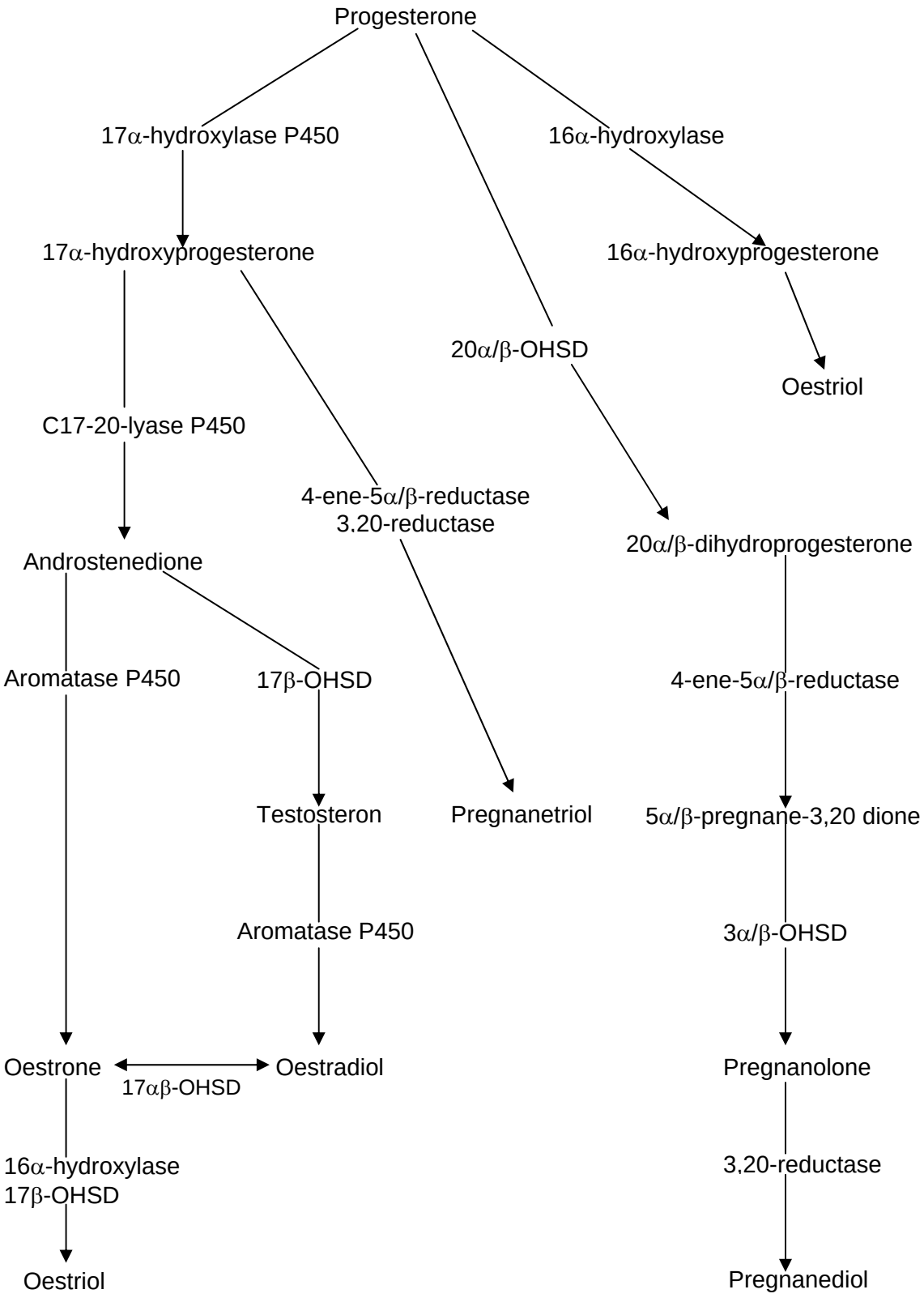
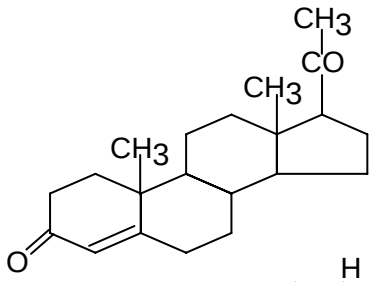
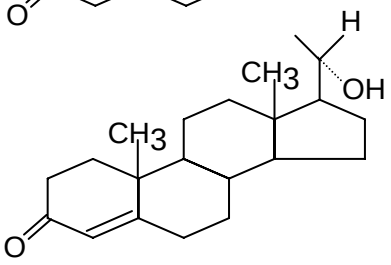


Figure 3. Possible pathways in the metabolism of progesterone (compiled from Gower 1984a,b; Langton & Armstrong 1994)

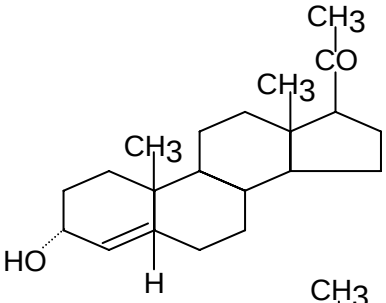
1. Progesterone
(4-pregnene-3,20-dione)



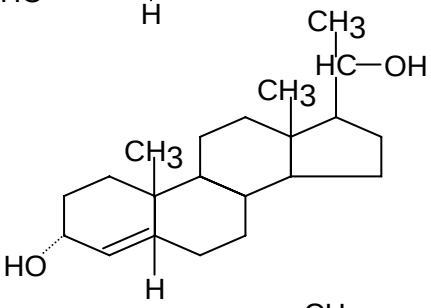
2. 20α-dihydroprogesterone
(4-pregnene-20α-ol-3-one)



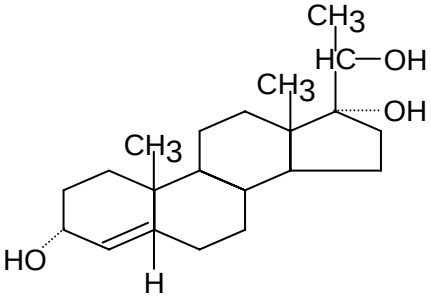
3. Pregnanolone
(3α-hydroxy-5β-pregnane-20-one)



4. Pregnanediol
(5β-pregnane-3α,20α-diol)



5. Pregnanetriol
(5β-pregnane-3α,17α,20α-triol)



6. Androsterone
(3α-hydroxy-5α-androstan-17-one)

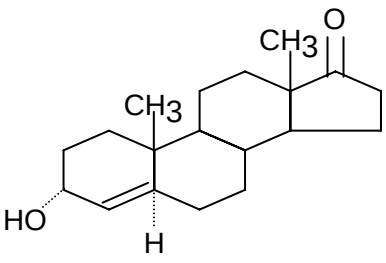


Figure 4. Structure of the major progesterone metabolites

see chapter III in detail). The possible pathways in metabolism of progesterone are shown in Figure 3, and the structures of the major metabolites of progesterone are shown in Figure 4.

Oestrogens are produced as the final step in steroidogenesis in the mature ovarian follicle and are derived from androgen precursors. There are two androgens (i.e., androstenedione and testosterone) as source of oestrogens (see Figure 3). Aromatase, induced by follicle stimulating hormone (FSH) in the developing granulosa cells, acts to convert these two androgens to the C-18 oestrone or oestradiol. Usually, no further reductions occur and all oestrogens produced are subsequently excreted as C-18 steroid molecules and are recognizable as oestrogen (Lasley *et al*. 1989). Furthermore, oestrone can be metabolized to oestriol, a reaction that occurs predominantly in the liver. Metabolism of oetrogens via oxidation or conversion to glucuronide and sulphate conjugates also occurs predominantly in the liver. The resulting metabolites are excreted in the bile and reabsorbed into the bloodstream via the enterohepatic circulation, before the products are finally excreted into the urine or faeces. Possible pathways in the metabolism of oestrogen are described in Figure 5, and the structure of the major metabolites of oestrogen is shown in Figure 6.

As mentioned above, species can differ markedly in the metabolism of hormones, and thus may excrete largely different metabolites of the same parent compound. Table 2 shows the major urinary and faecal metabolites of oestrogen and progesterone during the oestrous cycle of the four species of rhinoceros and other mammals.

Moreover, species can also vary in terms of route of hormone excretion (see Table 3). To date, radiometabolism studies in particular have yielded much important data on the relative importance of the urinary and faecal pathway of steroid excretion (Hodges *et al*. in press). From these studies, it is clear that major differences exist, not only between species, but also between hormones within the same species

(Hindle & Hodges 1990; Heistermann *et al*. 1998; Brown 2006; Hodges *et al*. in press, see Table3).

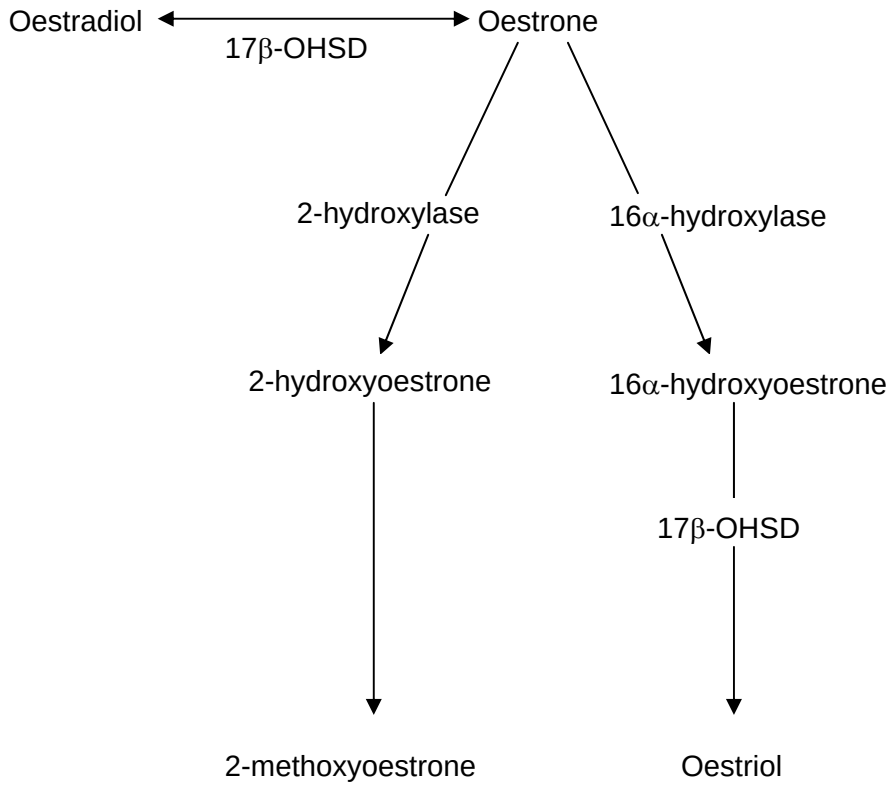
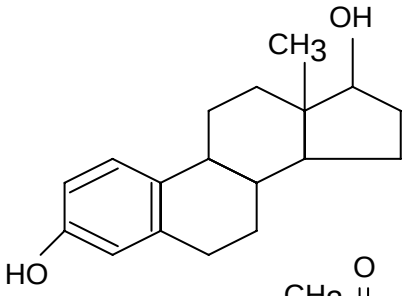


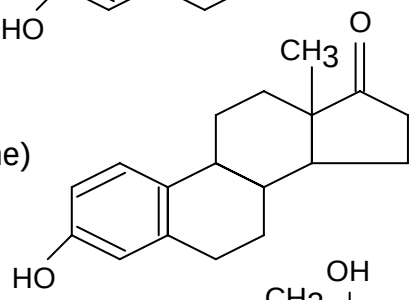
Figure 5. Possible pathways in the metabolism of oestrogens (compiled from Goldfien and Monroe 1991; Langton and Armstrong 1994)

There are also differences in the time course of hormone excretion, i.e., the time lag between the secretion of the parent compound into the bloodstream and the excretion of the corresponding metabolites. This time lag depends mainly on the route of excretion, the hormone in question and the species. Mostly, hormone excretion in urine is faster than that in faeces, due to gut transition time. Palme *et al*. (1997) also described the mean retention time of radioactively labelled hormones in faeces and suggested that the passage rate of digesta (duodenum to rectum) plays an important role in the time course of steroid excretion from his study in sheep, pig and pony. In this respect, radiometabolism

1. Oestradiol 17β
   (1,3,5(10)-Estratrien-3,17β-diol)



2. Oestrone
   (3-hydroxy-1,3,5(10)-Estratrien-17-one)



3. Oestriol
   (1,3,5(10)-Estratrien-3,16α,17β-triol)

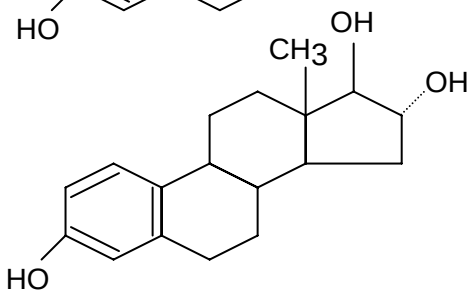


Figure 6. Structure of the major oestrogen metabolites

studies indicate that in most large-bodied mammals, steroids are excreted in faeces 24-48 h after appearance in circulation, while in domestic cat and small-bodied species (common marmoset), the lag-time is usually only 4-8 h (see Brown *et al*. 1994; Palme *et al*. 1996; 1997; Schwarzenberger *et al*. 1996; Bahr *et al*. 2000; Möhle *et al*. 2002). Table 4 shows the time course of hormone excretion in urine and faeces in different species. Thus, in practical terms, changes in the pattern of urinary and faecal hormone excretion are usually considered to reflect physiological events that happened several hours earlier, and this has to be taken into account when interpreting urinary and faecal hormone profiles (Hodges *et al*. in press).

Table 2. Major urinary and faecal metabolites of oestrogen and progesterone during the oestrous cycle in exotic species

Species/ Hormones	Hormone metabolites excreted into	
	Urine	Faeces
Indian rhinoceros[a)]		
Oestrogen	Oestrone sulphate	?
Progesterone	Pregnanediol glucuronide	?
Black rhinoceros[a)]		
Oestrogen	Oestrone glucuronide	?
Progesterone	20α-DHP glucuronide	?
White rhinoceros[a)]		
Oestrogen	Oestradiol 17β glucuronide	?
Progesterone	20α-DHP sulphate	?
Sumatran rhinoceros[b)]		
Oestrogen	Oestradiol 17β glucuronide	Oestrone
Progesterone	nd	Pregnanediol Pregnanolone
African wild dog[c)]		
Ostrogen	Oestrone glucuronide	Oestradiol 17β Oestrone
Progesterone	Pregnanediol glucuronide	Pregnanolone
Domestic cat[d)]		
Oestrogen	nd	Oestradiol 17β Unhydrolyze Oestrone
Progesterone	nd	Unhydrolyze Progestin

nd = not detectable

a) data adapted from Hodges (1992)

b) Heistermann et al (1998)

c) Monfort et al (1997)

d) Brown (2006)

Table 3. Route of hormone excretion in domestic and exotic animals

Species/ Hormones	Route of hormone excretion	
	Urine (%)	Faeces (%)
Cotton top tamarins[a)]		
Oestradiol	87	13
Oestrone	57	43
Progesterone	5.1	94.9
Yellow baboon[b)]		
Oestradiol	90	10
Progesterone	60	40
Rhesus monkey[c)]		
Oestradiol	55	45
Progesterone	42	58
Domestic cat[d)]		
Oestradiol	3	97
Progesterone	2.6	76.7
Sheep[e)]		
Oestrone	11.4	88.9
Progesterone	23.3	76.7
African wild dog[f)]		
Oestradiol	41.1	58.9
Progesterone	39.7	60.3
Sumatran rhinoceros[g)]		
Oestradiol	70	30
Progesterone	<1	>99

Data are compiled from [a)] Ziegler *et al*. (1989); [b)] Wasser *et al*. (1994); [c)] Shideler *et al*. (1993a,b); [d)] Brown *et al*. (1994); [e)] Palme *et al*. (1993); [f)] Monfort *et al*. (1997); [g)] Heistermann *et al*. (1998).

Table 4. The time course of hormone excretion in urine and in faeces

Species/ Hormones	Route of hormone excretion	
	Urine (hours)	Faeces (hours)
Cotton top tamarins[a)]		
Oestradiol	48	48
Oestrone	8	48
Progesterone	24	24
Yellow baboon[b)]		
Oestradiol	4.5	36
Progesterone	4.5	36
Rhesus monkey[c)]		
Oestradiol	8	24-56
Progesterone	8	32-56
Domestic cat[d)]		
Oestradiol	9-11	12-21
Progesterone	8-13	12-50
Sheep[e)]		
Oestrone	$\leq$ 4	6.5-20
Progesterone	$\leq$ 4	6.5-20
African wild dog[f)]		
Oestradiol	8	18
Progesterone	12	18
Sumatran rhinoceros[g)]		
Oestradiol	~24	~48-72
Progesterone	~24	~48

Data are compiled from [a)] Ziegler *et al*. (1989); [b)] Wasser *et al*. (1994); [c)] Shideler *et al*. (1993a,b); [d)] Brown *et al*. (1994); [e)] Palme *et al*. (1993); [f)] Monfort *et al*. (1997); [g)] Heistermann *et al*. (1998).

Given the extent of species variation as mentioned before, great care must be taken when applying non-invasive endocrine measurements to a new species. A careful validation of hormone measurement for each species is therefore required if the results are to be accurate and reliable with respect to reproductive assessment. In this regard, radiometabolism studies can clarify the metabolic fate and route of hormone excretion and thus provide valuable information for the development of appropriate assays (Heistermann *et al*. 1995b).

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HORMONE METABOLISM AND EXCRETION IN A FEMALE SUMATRAN RHINOCEROS

Introduction

Information on the reproductive biology and physiology of the Sumatran rhinoceros is extremely limited. This general lack of understanding of the reproductive biology of the species is probably one important factor contributing to the breeding failure of animals maintained in captivity. In this respect, the inability to assess female reproductive status and in particular to reliably detect a proper time for mating has been a major problem in breeding the species. This is because the Sumatran rhinoceros, shows a violent behaviour during courtship if the male and female are not put together in the right time of the cycle, and often, this violent behaviour results in injuries or occasionally death. It is therefore important to recognize oestrous properly in order to determine the right time for mating and thus increase the chances for successful breeding. In this respect, the establishment of reliable and practical methods for reproductive monitoring is important. As shown for other species of rhinoceros, the use of non-invasive techniques based on analysis of urinary and faecal reproductive hormone metabolites can provide reliable information on female reproductive status and, in particular, valuable basic data on the characteristics of the ovarian cycle and pregnancy (black African rhinoceros: Schwarzenberger *et al*. 1993; Berkeley *et al*. 1997; Radcliffe *et al*. 2001; Garnier *et al*. 2002; white African rhinoceros: Radcliffe *et al*. 1997; Schwarzenberger *et al*. 1998; Patton *et al*. 1999; Indian rhinoceros: Kassam & Lasley 1981; Kasman *et al*. 1986; Schwarzenberger *et al*. 2000; Stoops *et al*. 2004).

Since information on the identity and route of excretion of oestradiol and progesterone metabolites for the Sumatran rhinoceros does not exist, and, furthermore, given the high degree of species differences in metabolism and excretion of hormones (see Ziegler *et al*. 1989; Brown

et al. 1994; 2006; Wasser *et al*. 1994; Palme *et al*. 1996; Monfort *et al.* 1997; Möhle *et al.* 2002; Busso *et al*. 2007), basic studies on steroid metabolism in the Sumatran rhinoceros are essential for establishing a non-invasive reproductive monitoring methodology. This can best be accomplished by a radiometabolism study, by which information on the route of excretion and characteristics of excreted metabolites of the reproductive hormones can be generated. Based on this information, reliable methods for the measurement of urinary and faecal steroid metabolites can then be developed.

To this end, the aims of this part of the study were to: (1) determine the time course and distribution of ^{3}H-labelled oestradiol-17β and ^{14}C-labelled progesterone metabolites in urine and faeces of the Sumatran rhinoceros and (2) provide information on the identity of the major urinary and faecal metabolites of both hormones.

Materials and Methods

Animal and housing

The animal used in this study was an adult, female Sumatran rhinoceros (~15 years of age) maintained at Taman Safari, Bogor, Indonesia. During the experiment, the female was housed in an indoor enclosure with a concrete floor and fence, and was separated from its male partner, which helped to prevent contamination of female urine and faecal samples by those of males.

According to preliminary observations, the study animal showed regular cyclic changes in sexual behaviour (male-female interest, female whistling behaviour) and cyclic changes in vulval appearance (coloration and discharge). This suggested that the female was reproductively functional, although successful matings had not occurred.

Preparation and injection of radiolabelled steroids

An infusion mixture containing 50 μCi ^{14}C-progesterone (specific activity 48.9 mCi/mmol; NEN DuPont, Dreieich, Germany) and 100 μCi ^{3}H-oestradiol-17β (specific activity 40 mCi/mmol; NEN DuPont) together with 100 μg oestradiol and 1000 μg progesterone as carrier was prepared in 1.6 ml ethanol as shown in detail in Figure 7. An aliquot (10μl) of each radioactive steroid was counted during the preparation process to determine the total pre-injection radioactivity. Sterile propylene glycol (30% in water, v/v) was prepared in a separate tube and 3.4 ml was added immediately to the radiolabeled solution before injection. The final solution was gently poured and shaken and the total volume injected into an ear vein of the female.

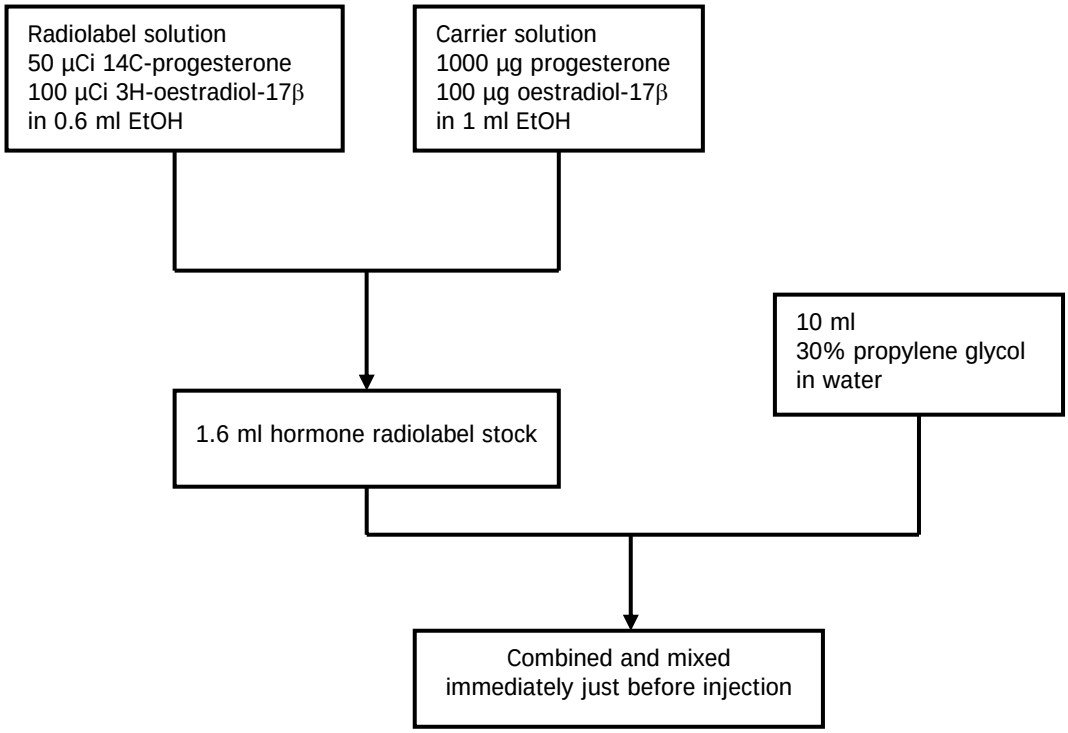

Figure 7. Preparation of hormone radiolabel mixture containing 14C-progesterone (50 µCi) and 3H-oestradiol-17β for the radiometabolism study in the Sumatran rhinoceros.

Before radiolabel administration, the animal was tranquilized following sedation with acepromazine maleat (6 mg/100 kg BW Acepromazine®; Aveco Co., Fort Dodge) and local anaesthesia (8 ml lidocaine hydrochloride 2%; Phoenix, Missouri) administrated sub-cuntaneously. The female was still standing and feeding while being injected with the radiolabel infusion mixture.

After isotope administration, the syringe and tube containing the radiolabel solution were each rinsed two times with 5 ml scintillation fluid (Quickszint 2000; Zinser Analytic. Frankfurt, Germany), the residual radioactivity counted and substracted from the pre-injection total to give the amount of radioactivity administered. All radioactive counting was conducted for 10 min in 20 ml scintillation fluid by running a dual $^{3}H/^{14}C$ quench compensation program on a Packard TriCarb CA 2000 liquid scintillation counter (Packard Instruments).

Collection and storage of samples

All urine and faeces were collected separately each day for four days following the radiolabel injection. To prevent cross-contamination between excreted urine and faeces, faecal material was removed within 5 minutes of voiding. The total amount of faeces excreted during each 24 hours was recorded and mixed, and two well-mixed aliquots (each 0.5 kg) were stored immediately at -20^{O}C until analysis. Urine was collected into 50 l plastic bags placed beneath the external outlet of the enclosure drain. The total volume of urine of each day was recorded and, and two well-mixed aliquots (each 0.5 l) were immediately stored at -20^{O}C with 0.1% sodium azide added as preservative.

All samples were delivered to the German Primate Centre in cooler box with dry ice to keep samples frozen during the 18 hours transfer period from Indonesia to Germany. After final sample collection on day 4 of the experiment, the enclosure was cleaned and wiped with alcohol (90%) in order to remove the remaining radiolabel. The radioactive disposal was sent to the Isotope Institute in Serpong, Tangerang for isotope waste treatment.

Sample analysis

a). Distribution of radioactivity

To determine the amount of radioactivity excreted into the urine, duplicate 1 ml aliquots of freshly thawed urine from each day of sampling were counted for 30 minutes using the Tri-Carb 2000 liquid scintillation counter (Packard Instruments) mentioned above. Quench correction was directly performed by a quench compensation program (see above) to ascertain dpm-values in samples (see Figure 8 in detail).

To determine the amount of radioactivity eliminated via the faecal route, faecal samples (duplicates) were analysed by subjecting a

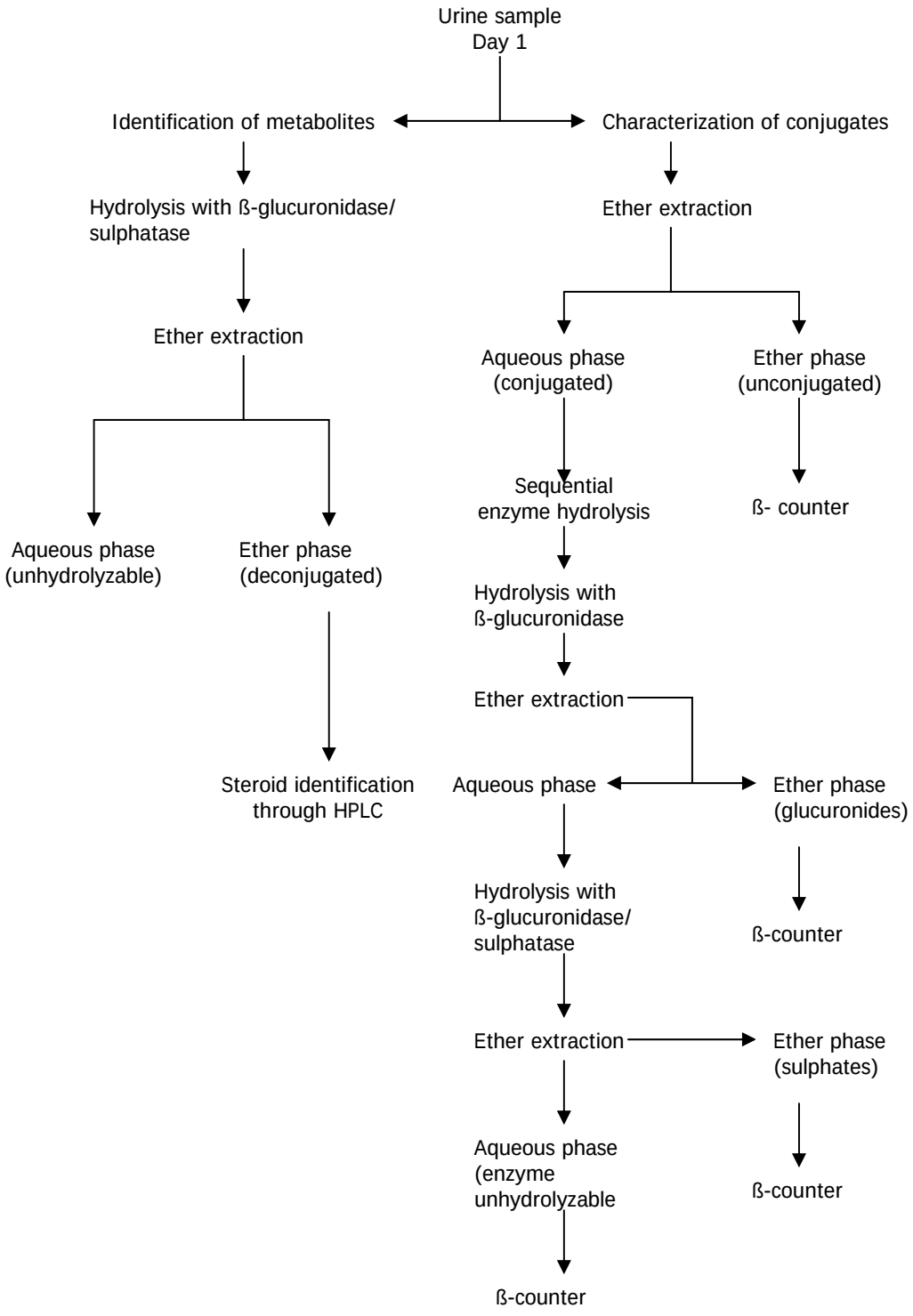

Figure 8. Scheme for separation and identification of oestrogen metabolites in urine. The analyses was conducted on material collected on day 1 after i.v. injection of ^{3}H-oestradiol-17β where the peak of radioactivity was excreted.

well-mixed portion of 0.5 g thawed material in polycarbonate capsules from each day of sampling to catalytic combustion in an oxygen stream (Oxymat®; Intertechnique, USA) according to the method of Peterson (1969). Following combustion of the faecal material, the resulting $^{3}H_2O$ and $^{14}CO_2$ were selectively absorbed in scintillation fluids and then directly counted for 30 minutes as described above to determine radioactivity (see Figure 9 in detail).

Values were multiplied by urinary volumes or faecal weights to determine total excreted radioactivity. All subsequent analyses were carried out on samples collected on the days of peak radioactivity excretion (i.e., day 1 for urine and day 2 for faeces; see Results).

b). Separation of conjugated and unconjugated steroids

The proportion of steroid metabolites excreted in urine as free or conjugated form and the proportion of conjugates excreted as glucuronides or sulphates was assessed by ether-water extraction and sequential enzyme hydrolysis, respectively. Quadruplicate 2 ml aliquots of urine (divided into 4 tubes of 0.5ml) were extracted with 15 volumes of diethylether by vortexing for 15 minutes. The aqueous phase was snap-frozen in a bath of methanol-dry ice, the ether phases decanted, pooled, evaporated to dryness and reconstituted in 300 μl absolute ethanol. Radioactivity of the radiolabelled free steroids was assessed in duplicate 50 μl aliquots.

The aqueous phase was subjected to sequential enzyme hydrolysis by incubation with 2500 U specific β-glucuronidase (without sulfatase actifity, No. G 7396; Sigma Chemie, Deisenhofen, Germany) in 0.5 M NaAc buffer (pH 6.8) overnight in a shaking

waterbath at 37^{O}C. Liberated steroids were then extracted with 10 volumes diethylether as described above, the ether phase evaporated to dryness, redissolved in 300 μl absolute ethanol and duplicate aliquots (100 μl) measured for radioactivity (glucuronide fraction). The aqueous residual was adjusted to pH 4.7 by adding 8 μl acetic acid (10%) before further hydrolysis with 2500 U β-glucuronidase/sulfatase (No. G 1512; Sigma Chemie, Deisenhofen, Germany) overnight at 37^{O}C. Following ether extraction, the radioactivity of the sulphate fraction was counted in duplicate 100 μl aliquots of the reconstituted ethanolic extract (see Figure 8 in detail).

The proportion of free and conjugated steroid metabolites in faeces was determined in quadruplicate 1 g aliquots (divided into 2 tubes of 0.5 g wet faeces). Samples were extracted three times with 10 ml diethylether by vortexing for 30 minutes. Following centrifugation (3000 rpm; 10 minutes), the ether phase (free steroid fraction) of each extraction step were decanted and pooled for each aliquot. The remaining faecal pellets were dried and subjected to catalytic oxygen combustion as described above and the amount of radioactivity counted to determine the proportion of conjugates in the faecal sample. The pooled ether phase was evaporated totally and the remaining was subjected to catalytic combustion. Radioactivity of free steroid fraction was counted to determine the proportion of both steroids (see Figure 9 in detail). Further analysis of the conjugate forms was not carried out, since the majority of both steroid metabolites in faeces were ether-extractable (free steroid; see Results).

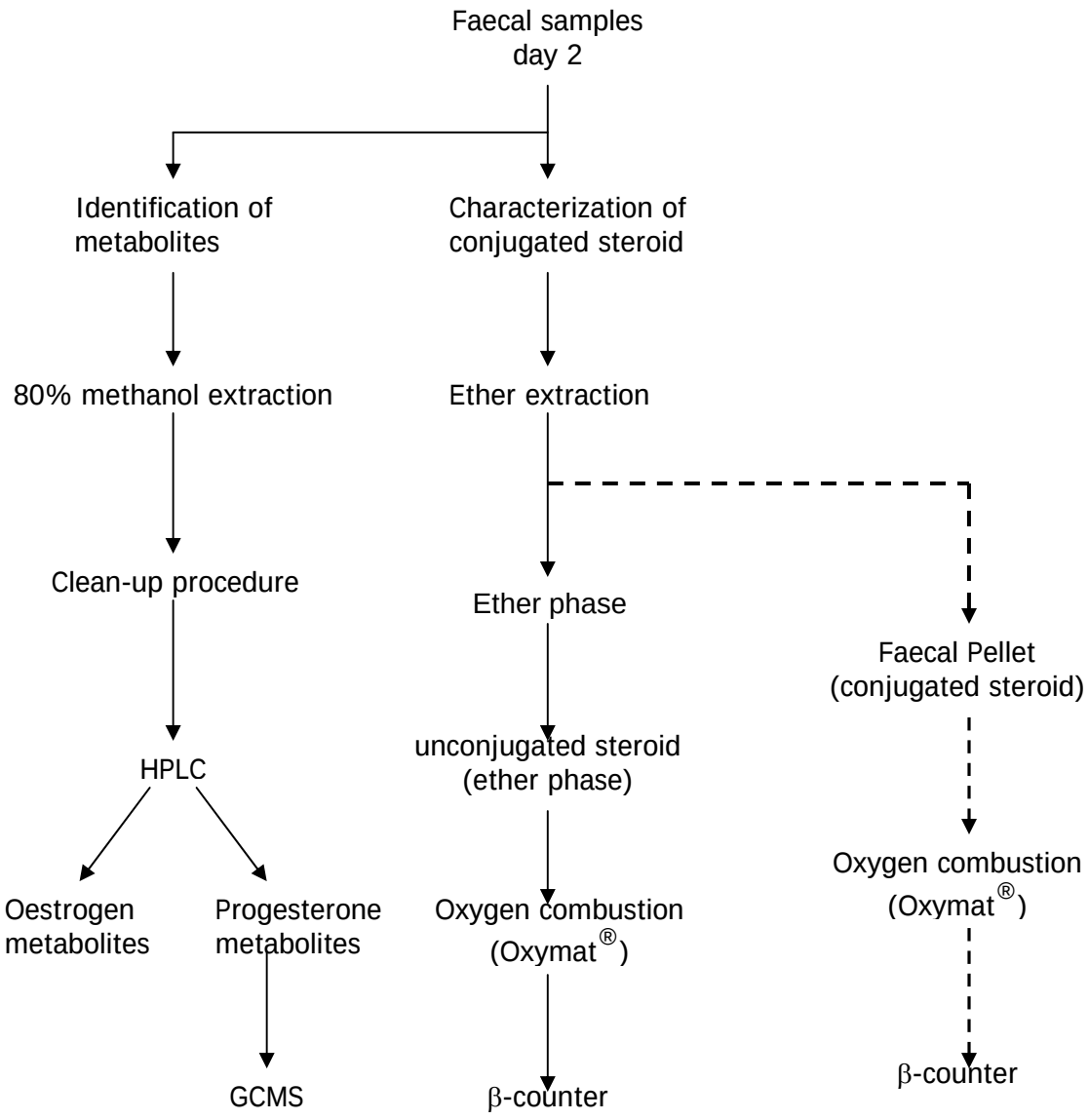

Figure 9. Scheme for the separation and identification of oestrogen and progesterone metabolites in faeces. The analyses was carried out on material collected on day 2 after i.v. injection of ^{3}H-oestradiol-17β and ^{14}C-progesterone where the peak of radioactivity was excreted.

c). High pressure liquid (HPLC) co-chromatography

In order to characterise the progesterone and oestradiol metabolites excreted into urine and faeces, HPLC was performed on peak radioactive samples. ^{3}H-oestradiol metabolites were

separated on a reverse-phase NovaPak C-18 column (3.9 X 150 mm, Millipore Milford, MA, USA) using an isocratic solvent system of acetonitrile (ACN)/water (H_2O) (30/70, v/v) at flow rate of 1 ml/minute (Heistermann *et al*. 1993). Prior to HPLC, 1 ml urine (divided into 2 tubes of 0.5 ml each) was extracted with 15 volumes diethylether to remove free steroids and the aqueous phase hydrolysed with 2500 U β-glucuronidase/sulphatase as describe above. The subsequent pooled ether extract was reconstituted in 130 µl ACN/H_2O (50/50, v/v), 100 µl injected onto the HPLC (Waters 600E-system controller, Millipore, Massachusetts) and 30 fractions (1 ml each) collected. Each fraction was reduced in volume in a vacuum oven (Vacutherm VT 6060P; Heraus) at 50° C to ~0.2 ml and the radioactivity in the total volume was subsequently counted for 30 minutes.

For separation of oestrogens from faeces, an aliquot of 10 g wet faeces was extracted three times with 25 ml 50% methanol (MeOH). Combined extracts were subjected to a clean up procedure as described by Palme *et al*. (1997). For this, 2.5 volumes of sodium acetate buffer (0.2 M, pH 4.7) was added to the pooled extract and the total volume passed through a Sep-Pak®Plus C18 environmental cartridge (WAT023635; Waters, Milford, MA, USA) with a flow rate of 2.5 ml/minutes. Steroids were eluted with 2 X 5 ml dichloromethane (flow rate 2.5 ml/minute), reduced in volume to ~1ml under a stream of nitrogen at 40°C, transferred into a conical glass vial and then totally evaporated. The residual was redissolved in 200 µl 2-propanol and centrifuged at 4000 rpm for 10 minutes. The supernatant was transferred to a small glass tube, evaporated to dryness, reconstituted in ACN/H_2O (130 µl, 50/50, v/v). 100 µl were then

separated on HPLC as described above and radioactivity determined in each fraction (see above).

As the majority of progesterone metabolites were excreted into faeces (see Results), characterisation of ^{14}C-progesterone metabolites in urine was not performed. Progesterone metabolites in faeces were separated on a reverse-phase NovaPak C-18 column (3.9 X 300, Millipore, Millford, MA, USA) with ACN/H_2O (40/60, v/v) as eluent at a flow rate 1 ml/minute (Heistermann *et al*. 1993). From each of the 140 1ml-fractions collected, 850 μl were reduced in volume using the vacuum oven as described above to ~0.2 ml and counted to generate the profile of ^{14}C-radioactivity. Following this HPLC run, in addition a gradient solvent system of acetonitrile (ACN)/water (H_2O) at flow rate of 1 ml/minute was used to evaluate the native of the radioactivity in the extended peak eluting in fractions 3-11, in the isocratic HPLC run (see Figure 13B). For this, during the first 60 minutes HPLC was run with ACN/H2O (40/60, v/v), which was changed to ACN/H2O (30/70, v/v) for the remaining 80 minutes of the run. Fractions were counted to determined radioactivity as described for isocratic run.

The remaining 150 μl of the fractions from the isocratic run were evaporated to dryness and reconstituted in assay buffer to determine profiles of pregnanediol (Pd) and 5α-pregnane-3α-ol-20-one (pregnanolone/5α-P-3OH)immunoreactivity.

d). Gas chromatography mass spectrometry (GCMS)

GCMS analysis was conducted to identify the steroids present in the major peaks of radioactivity in faeces and the principal immunoreactive faecal progestin detected in the 5α-pregnane-3α-

ol-20-one assay. Faecal samples (0.1 g dry weight) were extracted and subjected to HPLC as described above and fractions corresponding to the peaks from three HPLC runs were collected, pooled and evaporated to dryness. The final samples were reconstituted in 50 μl toluene of which 1 μl was subjected to GCMS analysis at the Department of Analytical Chemistry and Endocrinology of the Hannover Veterinary School under conditions described previously by Hodges *et al*. (1994) as follows: The identification of was achieved by GCMS analyses with a SSQ-710 mass spectrometer coupled to a varian 3400 gas chromatograph, using spitless injection (Finnigan MAT Instruments, Bremen). The GC column (30 m fused silica capillary column, inner diameter of 0.25 mm, with DB-5 MS as the stationary phase; J and W Scientific, Folson, CA) operated at 25 psi helium, a temperature programme of 100-270^{O}C, an injector temperature of 260^{O}C and a transfer line temperature of 250^{O}C. Electron impact positive ionization was used at 70eV. Mass spectrometry profiles were scanned and identification of unknown peak achieved by GC retention times, computer MS library search (Finnigan MAT Wiley Library, V5.0) and comparison of fragmentation patterns and retention times with those of steroid reference standards.

e). Hormone assays

All measurements were carried out by competitive, double-antibody, enzymeimmunoassay procedures (EIA) as already described in detail by Hodges *et al*. (1997) for 5α-P-3OH and Heistermann *et al*. (1993) for Pd. The enzymeimmunoassays were performed on microtitreplates coated with a goat anti rabbit IgG (1µg/well; Quartett Immunodiagnostika und Biotechnologie GmbH, Berlin) and

using hormone-specific antibody as second antibody and enzyme- or biotin-labelled hormones as competitive tracers.

Pregnanolone (5-P-3OH) assay

5-P-3OH was determined using the streptavidin-biotin technique. The antiserum used was raised in rabbit against 5α-pregnane-3β-ol-20-one-3HS-BSA (supplied by E. Möstl, Institute of Biochemistry, University of Veterinary Medicine, Vienna, Austria), 5α-pregnane-3β-ol-20-one-3HS coupled to biotin was used as label (supplied by E. Möstl, Vienna), and 5α-pregnane3α-ol-20-one as standard. The 5α-pregnane-3β-ol-20-one antiserum cross-reacts 100% with 5-P-3OH, 66.7% with 5α-dihydroprogesterone, 47.3% with progesterone, <1% with 5α-pregnane-20α-ol-3-one, and <1% with 5α-pregnane-20β-ol-3-one. For determination of 5-P-3OH, 50 μl aliquots of each HPLC fraction or standard (range 4.9 – 1250 pg/50 μl-well) were combined with label (50 μl, dilution 1:800,000) and antiserum (50 μl, dilution 1:30,000) and incubated overnight at 4°C. After incubation, the plates were washed four times and blotted dry before 150 μl (20ng) of streptavidin-peroxidase (S-5512; Sigma Chemie, Deisenhofen, Germany) in assay buffer was added to each well. The plates were incubated at room temperature in the dark for 30 minutes, after which they were washed again four times. Substrate solution (150 μl, including 0.025% tetramethyl-benzidine and 0.05% H_2O_2) was added to each well and the plates incubated at room temperature in the darkness for another 45 minutes. The enzyme reaction was stopped by the addition of 50 μl 2 mol H_2SO_4 and absorbance measured at 450 nm on an automatic plate reader. Sensitivity of the assay was 9.8 pg/well at 90% binding. Intra- and interassay coefficient of variation were determined by replicate determinations of high- and low value quality controls (QC's). Intra-assay coefficient of variation (CV) of high and low

concentrated quality control (QC) was 7.9% (n=16) and 10.9% (n=17), respectively. Inter-assay CV was 9.8% (n=6) and 11.8% (n=6), for QC high and low, respectively.

Pregnanediol (Pd) assay

Pregnanediol in faeces was determined as described by Heistermann *et al*. (1993) as follow: The assay used an antiserum against pregnanediol-3-glucuronide-BSA. Crossreactivity of the antiserum with the free steroid was 22%. Crossreactivity values of the assay with other steroids are 45.1% with 5β-pregnanediol, 4.1% with 5β-pregnanedione, 0.11% with 5β-pregnanetriol, 0.10% with 17α-hydroxyprogesterone, 12.1% with 20α-hydoxyprogesterone, and 0.03% with progesterone (Hodges and Green, 1989). Biotinylated pregnanediol-3-glucuronide (prepared by E. Möstl, Vienna) in conjunction with peroxidase (POD) labelled streptavidin (No. S-5512, Sigma chemie) was used as conjugate, and pregnanediol was used as standard, although the assay was originally designed to measure the glucuronide. 50 µl aliquots of each HPLC fraction were taken to measure Pd immunoreactivity. Samples or pregnanediol standards (50 µl, 6.25-1600 pg per well) were combined with labelled pregnanediol glucuronide (50 µl) and antiserum (50 µl), mixed thoroughly, sealed with plastic film, and incubated and processed as described for the 5-P-3OH assay. Assay sensitivity at 90% binding was 6.25 pg per well. Intraassay coefficient of variation was 8.1% (QC high, n=16) and 10.8% (QC low, n=17). Interassay coefficient of variation for QC high and QC low were 9.6% (n=14) and 11.5% (n=14), respectively.

Results

Radiolabel recovery

The amounts of urine and faeces excreted over the 4-day radiometabolism study and the distribution of radioactivity during the observation are shown in Table 5. Total radiolabel recovered in urine and faeces was 68% and 89% for ^{3}H-oestradiol and ^{14}C-progesterone, respectively.

Table 5. Volume of urine and mass of faeces excreted over the radiometabolism study period, and distribution of 3H-oestradiol-17β and 14C-progesterone radioactivity shown as percentage of total administered

Day	Urine			Faeces		
	Volume excreted (l)	Percentage of radioactivity		Mass excreted (kg)	Percentage of radioactivity	
		^{3}H	^{14}C		^{3}H	^{14}C
Day 1	15.4	38.7	0.36	16.1	3.1	11.9
Day 2	18.7	5.5	0.18	23.7	8.3	44.1
Day 3	28.4	1.6	n.d.	22.0	8.5	17.3
Day 4	15.0	0.34	n.d.	31.5	2.0	15.2
Total Radioactivity Recovered (%)		46.14	0.54		21.9	88.5

n.d. = Not detectable

Route and time course of excretion

3H-oestradiol metabolites were predominantly (67.8%) excreted into the urine, whereas 14C-progesterone metabolites were almost exclusively (>99%) eliminated via the faeces (Table 6). Peak excretion of radioactivity in urine was found within the first 24 h following the radiolabel injection, and >95% of combined radioactivity had been excreted by the end of day 2 (Figure 10A). In contrast, the peak of radiolabel excreted into faeces occurred on day 2 for 14C-progesterone and days 2 and 3 for 3H-oestradiol, with relatively high amounts of radioactivity being still present at the end of day 4, particularly for 14C-progesterone (Figure 10B).

Table 6. Excretory fate of injected 3H-oestradiol-17β and 14C-progesterone in a female Sumatran rhinoceros

Radiolabel	% of total radioactivity recovered in[a]		
	Total recovery	Urine	Faeces
3H-oestradiol-17β	68.04	67.8	32.2
14C-progesterone	89.05	0.6	99.4

a) urine and faeces were collected over a total period of 4 days

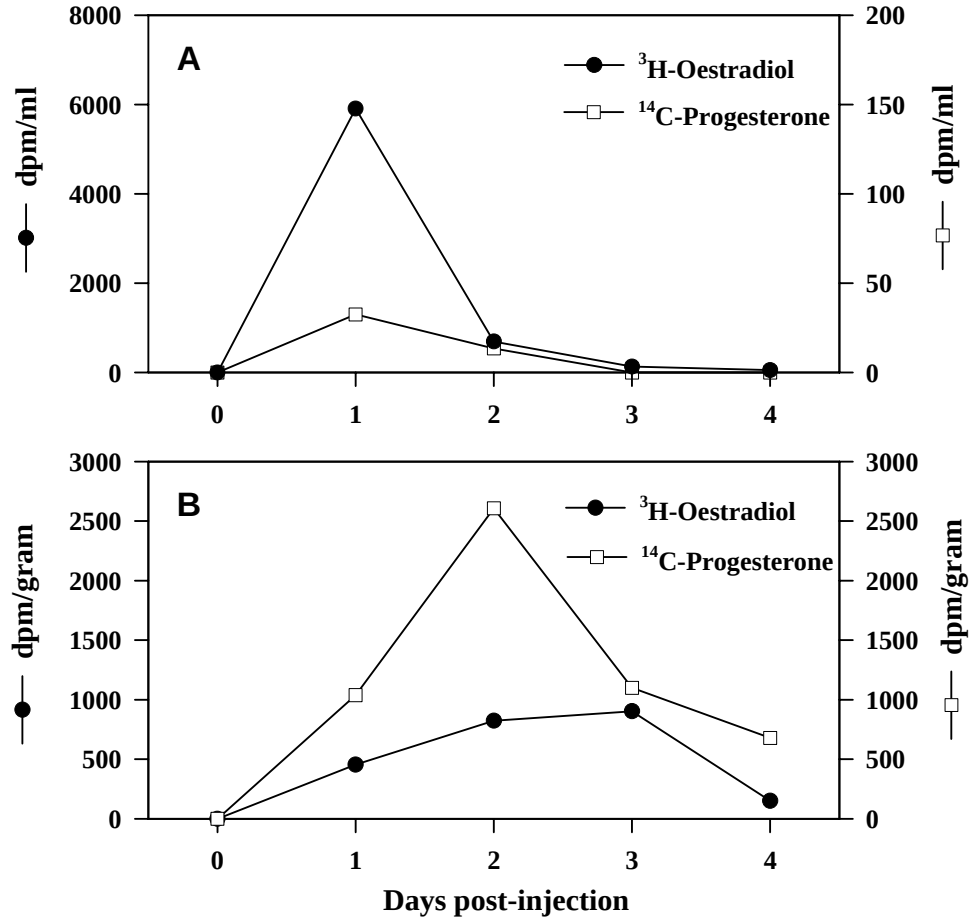

Figure 10. Profiles of the excretory time course of 3H-oestradiol-17β and 14C-progesterone in (A) urine and (B) faeces after i.v. administration at day 0 in a female Sumatran rhinoceros

Characterization of steroid metabolites

a). Proportion of conjugated and unconjugated steroids

The majority (> 70 %) of 3H-oestradiol-17β recovered from urine was associated with water-soluble (presumably conjugated) forms. In contrast, faecal metabolites of both steroids were primarily (>75%) ether-soluble (presumably unconjugated) forms (Table 7).

Enzyme hydrolysis of the conjugated oestradiol metabolites in urine revealed that almost 95 were enzyme-hydrolysable, with ~ 90 % of these conjugates representing glucuronides and ~ 10 % sulphates (see Figure 11).

Table 7. Proportion (%) of conjugated and unconjugated metabolites of urinary and faecal 3H-oestradiol-17β and faecal 14C-progesterone

Radilabel	Urine (%)		Faeces (%)	
	Ether soluble (unconjugated)	Water soluble (Conjugated)	Ether soluble (unconjugated)	Water soluble (conjugated)
3H-estradiol 17β	28.7	71.3	75.4	24.6
14C-progesterone	n.d.	n.d.	79.8	20.2

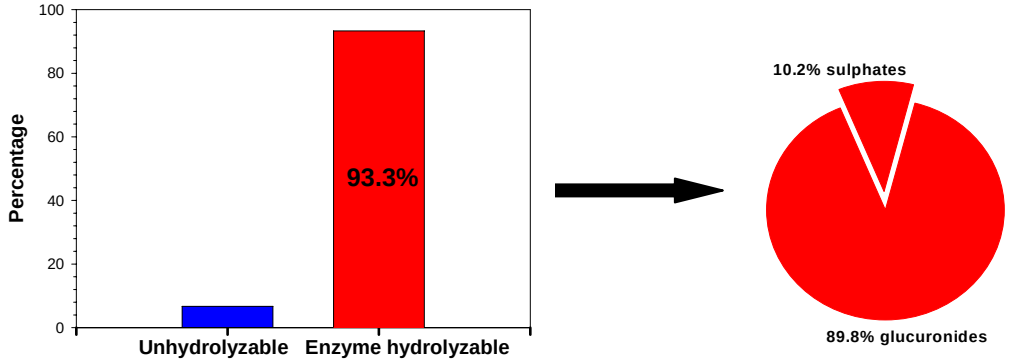

Figure 11. Proportion (%) of enzyme hydrolysable and enzyme unhydrolisable, and proportion of glucuronides and sulphates in urine

b). HPLC profiles of radioactive progesterone and oestrogen metabolites

A single major peak of radioactivity (fraction 14-16), co-eluting with authentic oestradiol-17ß, was detected by HPLC co-chromatography of 3H-oestradiol-17β metabolites in urine In contrast, two peaks of radioactivity were found following HPLC of oestradiol metabolites in faeces. The predominant one (representing 72% of combined radioactivity) co-eluted with authentic oestrone, while the second peak (28%) was detected at the elution position of oestradiol-17β (Figure 12).

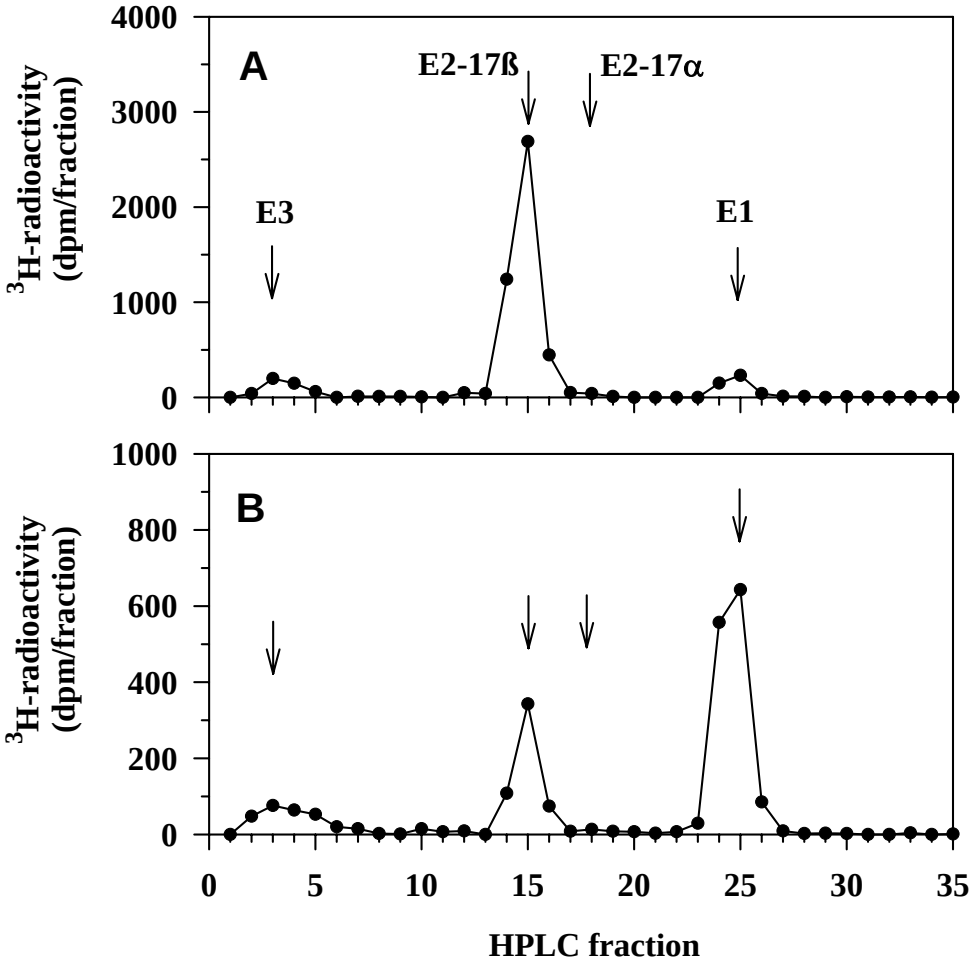

Figure 12. HPLC profiles of metabolised oestrogens in (A) urine and (B) faeces after i.v. injection of 3H-oestradiol-17β in a female Sumatran rhinoceros. Retention times of radioactive peaks are compared to those of authentic oestriol (E3), oestradiol-17β (E2-17β), oestradiol-17α (E2-17α) and oestrone (E1) reference tracers.

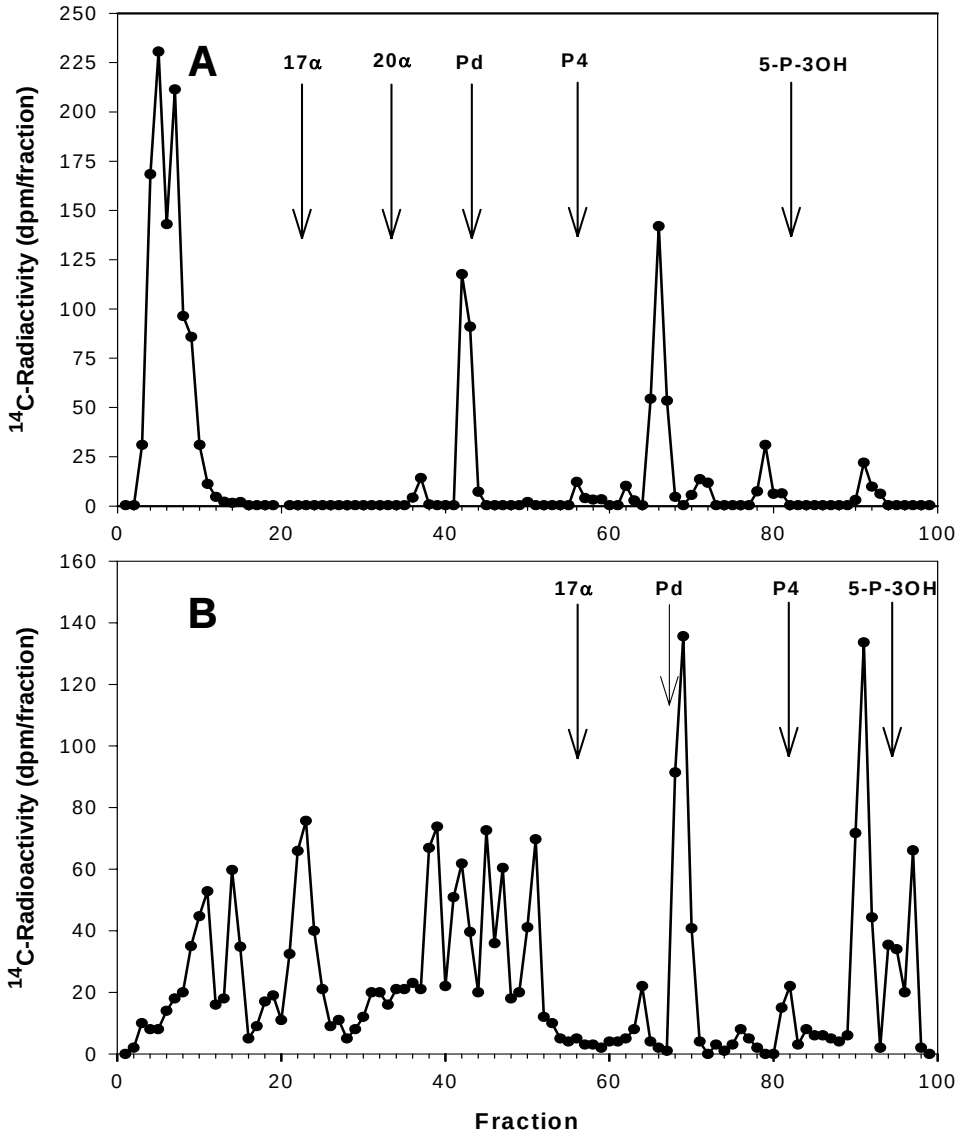

Figure 13. HPLC profiles of (A) ^{14}C-progesterone metabolites using the isocratic HPLC system (see Methods) and (B) ^{14}C-progesterone metabolites separated by the gradient HPLC system (see Methods). Retention times of ^{14}C-radioactive peaks are compared to those of authentic 17α-hydroxyprogesterone (17α), 20α-dihydroprogesterone (20α), 5β-pregnane-3α,20α-diol (Pd), progesterone (P4), 5α-pregnane-3α-ol-20-one (5-P-3OH) reference tracers. Radioactivity in fractions 101-140 was < 5 dpm (not show).

Isocratic HPLC of faecal 14C-progesterone metabolites indicated four substantial peaks of radioactivity (Figure 13A). Subsequent use of gradient HPLC, however, indicated that the radioactivity eluting in fractions 3-11 represented multiple small and quantitatively less significant peaks, whereas the radioactivity in fractions 42-44 and 65-67 remained as single prominent peaks (Figure 13B, peaks in fractions 68-70 and 70-92, respectively). The peak in fractions 42-44 co-eluted with authentic 5β-pregnane-3α, 20α-diol (pregnanediol, Pd) and its presence was confirmed by high levels of Pd immunoreactivity in the same fraction (see Figure 14B). In contrast, the elution position of the radioactive peak in fraction 65-67 (Figure 13A) did not correspond to that of any of the progestin reference tracers tested. Moreover, almost no immunoreactivity was detected in these fractions by application of the Pd and 5-P-3OH assay (see Figure 14B and 14C). No significant peak of radioactivity was found at the elution position of authentic progesterone.

c). Steroid characterisation GC/MS

In order to provide more detailed information on the identity of the two prominent faecal metabolites of 14C-progesterone detected by HPLC (see above), GC/MS analysis was conducted.

This analysis confirmed the presence of large amounts of 5β-pregnane-3α, 20α-diol in fraction 42-44 (Figure 14A) as suggested by the HPLC results (see above). Moreover, a second steroid with a molecular mass (320 kDa) and fragmentation pattern of a pregnanediol was also detected in these fractions, however, in the absence of a complete range of reference standards, its exact structure could not be deduced. In contrast, the radioactive peak

in fractions 65-67 contained only one major steroid with a molecular mass (318 kDa), fragmentation pattern and GC retention time identical to those of 5β-pregnane-3α-ol-20-one. The major peak of immunoreactivity detected by the 5-P-3OH assay (see Figure 14C) which eluted at the position of a small peak of radioactivity was identified by GC/MS as 5α-pregnane-3β-ol-20-one.

d). Immunoreactivity of the metabolites

In order to assess whether the two prominent peaks of 14C-progesterone metabolites in faeces could be measured by group-specific assays against pregnanediol (Pd) and 5-reduced 20-oxo pregnanes (5-P-3OH), HPLC fractions were measured in both assays. As shown in Figure 14 (and mentioned above), the radioactive peak in fraction 42-44 which, according to HPLC and GCMS analysis, contained authentic Pd was associated with high level of Pd immunoreactivity (Figure 14B). Additional significant amounts of Pd immunoreactivity were found in fractions 56-57 at the position of a small radioactive peak and some minor peaks of immunoreactivity corresponding to minor metabolites were also detected (Figure 14B).

In contrast, application of the 5-P-3OH assay showed only one major peak of immunoreactivity in fractions 79-81, corresponding to a minor radioactive metabolite which according to GCMS was identified as 5α-pregnane-3β-ol-20-one. Cross-reactivity tests showed that this compound had a cross-reactivity of 295% in the 5-P-3OH assay. No significant immunorectivity was found with any other of the radioactive peaks using this assay (Figure 14C).

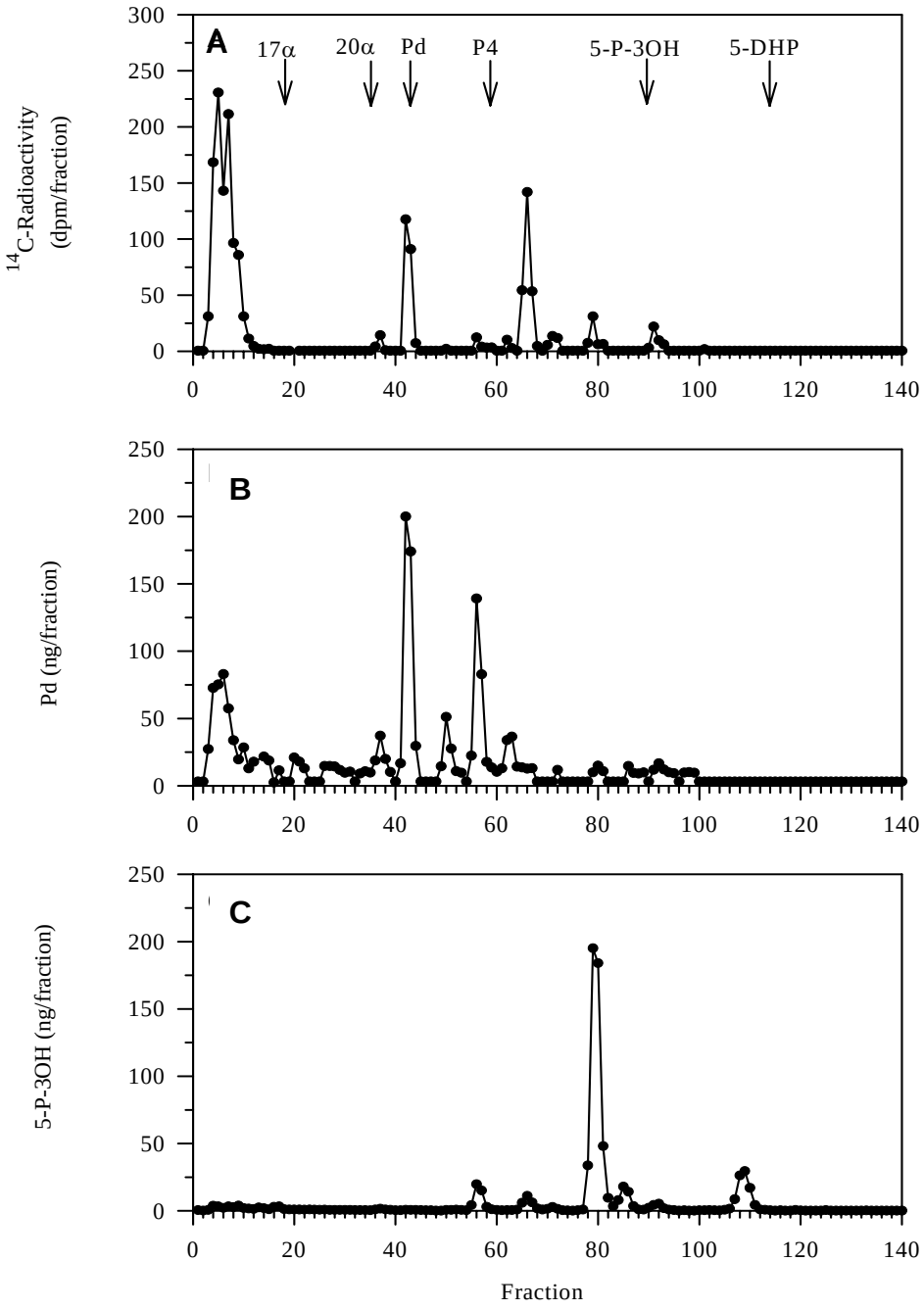

Figure 14. The HPLC profiles of (A) 14C-progesterone metabolites and (B) corresponding pregnanediol (Pd) and (C) 5α-pregnane- 3α-ol-20-one immunoreactivity in the faecal sample of peak radioactive excretion. Retention times of ^{14}C-radioactive peaks are compared to those authentic 17α-hydroxyprogesterone (17α), 20α-dihydroprogesterone (20α), 5β-pregnane-3α,20α-diol (Pd), progesterone (P4), 5α-pregnane-3α-ol-20-one (5-P-3OH) reference tracers.

Discussion

The study provides the first information on the *in vivo* metabolism and excretion of exogenously administered radiolabelled oestradiol-17β and progesterone in a female Sumatran rhinoceros. Of the total radioactivity injected into the female, the majority was recovered from urine and faeces within the 4 days of sample collection, with recovery values of 68% for 3H-oestradiol and 89% of 14C-progesterone. The figures were comparable to those obtained from radiometabolism studies in other mammalian species, such as slow loris, baboon, cotton top tamarin, rhesus monkey, yellow baboon, sheep, ponies, pig, cat, African wild dog, and African elephant (e.g. Perez *et al*. 1988; Brown *et al*. 1994; 1996; Wasser *et al*. 1994; 1996; Palme *et al*. 1996; Monfort *et al*. 1997; Ganswindt *et al*. 2003), and the white rhinoceros (Hindle & Hodges 1990). The 20% lower recovery for ^{3}H-oestradiol-17β metabolites compared to that for ^{14}C-progesterone was most probably due to oestradiol being excreted primarily into the urine (in contrast to progesterone), some of which was absorbed into the substrate or remained in the enclosure and did not flow into the outlet of the cage.

The majority (~ 84%) of the radioactivity recovered in urine was detected in the first 24 hour, whereas peak excretion of radioactivity in faeces (~ 50%) were found in samples from day 2 (progesterone), and between days 2 (38%) and 3 (39%) for oestradiol-17ß. Since urine and faecal samples within each 24 hours period were pooled before analysis, unfortunately, a more precise timing of peak excretion within these periods could not be determined. Nevertheless, the data are generally consistent with those reported for the white rhinoceros (Hindle & Hodges 1990) and various other mammalian species (see Schwarzenberger *et al*. 1996a; Wasser *et al*. 1996), showing that excretion of steroid metabolites into urine occurs within hours, while excretion into faeces, due to gut

transition times, is much more delayed and can also vary between hormones and species (see Schwarzenberger *et al*. 1996a for review). Palme *et al.* (1996) also described the mean retention time of faecal radioactivity and suggested that the passage rate of digesta (duodenum to rectum) plays an important role in the time course of steroids excretion from his study in sheep, pig and pony.

The results that oestradiol metabolites are excreted predominantly into urine, whereas progesterone metabolites are almost exclusively excreted into faeces are remarkably contrasting to those in the white rhinoceros, in which the opposite was found, e.g. the majority of progesterone metabolites are excreted into urine while oestradiol is mainly eliminated via the bile into faeces (Hindle & Hodges 1990). The present data in the Sumatran rhinoceros are thus consistent with findings in other mammal species, such as the African elephant (Wasser *et al*. 1996), cotton-top tamarin (Ziegler *et al*. 1993) and various farm animal species (Palme *et al*. 1996; 1997) showing that the route of hormone metabolite excretion can differ markedly according to the hormone in question. Although faeces represents the preferred route of steroid excretion in various other mammals (see Brown *et al*. 1994; Schwarzenberger *et al*. 1996a), the virtually exclusive elimination of progestins into faeces as observed in the Sumatran rhinoceros is unusual, maybe with the exception of some carnivore species. Although the reason for the almost exclusive excretion of progestins into the faeces of the Sumatran rhinoceros is not clear, it would explain the low levels of progestins previously measured in urine and the lack of information on ovarian function provided by urinary progestin analysis in this species (Hindle, personal communication, own unpublished data). Significant amounts of progestins in faeces have nevertheless also been reported for the white and black rhinoceros (Schwarzenberger *et al*. 1993; 1996b; Schwarzenberger & Walzer 1995; Garnier *et al*. 2002), in which their measurement was reliably used to monitor ovarian function and pregnancy.

The majority of the radiolabelled oestrogen and progesterone metabolites in faeces of the Sumatran rhinoceros was determined to be ether-extractable, presumably indicating steroids in an unconjugated form, whereas, the majority of urinary metabolites was found to be water-soluble, thus presumably representing conjugated forms. The excretion of predominantly conjugated steroids in urine and free hormone in faeces is similar to findings reported for the majority of other mammal species, including the Indian, white and black rhinoceros (Hindle & Hodges 1990; Hindle *et al*. 1992; Schwarzenberger *et al.* 1996a; 2000; Wasser *et al.* 1996; Fieß *et al.* 1999; Stoops *et al.* 2004). Sequential enzyme hydrolysis showed that the majority of the conjugates excreted into the urine were accounted for by glucuronides. Combined with HPLC co-chromatography results, the data in particular indicate that oestradiol-17β glucuronide is the only abundant oestrogen metabolite in the urine of the Sumatran rhinoceros, a finding similar to that reported for the white rhinoceros (Hindle *et al*. 1992). In contrast, in most other ungulate species, including the black (Hindle *et al*. 1992) and Indian (Kassam & Lasley 1981; Kasman *et al*. 1986) rhinoceros, urinary excretion of conjugated oestrone predominates, which moreover, in the case of the Indian rhinoceros is excreted as a sulphate (Kasman *et al.* 1986). The available data on oestrogen metabolism and urinary excretion within the Rhinocerotidae thus clearly show a high degree of species differences, indicating that the nature of metabolites excreted should not be presupposed on the taxonomic relatedness between species.

Both oestrogen metabolites, oestrone and oestradiol-17β, were excreted into faeces as free steroid, with oestrone being more abundant. Comparable information on the identity of faecal oestrogens in rhinos is only available for the white rhinoceros, in which oestrone was not found and both epimers of oestradiol accounted for all oestrogens detected (Hindle & Hodges 1990). Thus, as for oestrogen excretion into the urine, the data suggest that also with respect to faecal oestrogen excretion,

considerable species differences within the Rhinocerotidae do exist, although data are still limited to two of the four rhino species.

The HPLC separation of faecal ^{14}C-progesterone followed by GCMS analysis of the radioactive peaks revealed the presence of three abundant metabolites. In contrast to faecal ^{3}H-oestradiol-17ß excretion, however, ^{14}C-progesterone was not excreted in its native form. In general, these results are comparable to those reported for the black rhinoceros (Schwarzenberger *et al*. 1993; 1996b) and many other species (e.g., Heistermann *et al*. 1993; 1997; Shideler *et al*. 1993; Brown *et al*. 1994; Wasser *et al*. 1994; Palme *et al*. 1997) in which the metabolism of progesterone results in the excretion of a range of faecal progestins and progesterone itself being either absent or quantitatively of very little importance. Interestingly, and in contrast to the present findings in the Sumatran rhinoceros and those of most other mammals (see above), Hindle & Hodges (1990) reported that the white rhinoceros excretes native progesterone into faeces as the only significant faecal progestin.

According to the HPLC and GCMS data, the major faecal progesterone metabolites in the Sumatran rhinoceros were identified as two pregnandiols, and one 5-reduced 20-oxo pregnane, 5β-pregnane-3α-ol-20-one. Of the two pregnanediols, one could be finally identified as being 5ß-pregnane-3α, 20α-diol (Pd), and its presence was confirmed by high levels of immunoreactivity when applied to a Pd assay. Excretion of 5-reduced mono and/or dihydroxylated faecal pregnanes have also been reported for a number of other mammalian species (Brown *et al.* 1994; Wasser *et al.* 1994; Schwarzenberger *et al.* 1996a,b) and, in this respect, the findings for the Sumatran rhinoceros are consistent with these other studies. In contrast, the presence of pregnanediols as abundant faecal progestins, however differs to findings in the white (Hindle & Hodges 1990) and black (Schwarzenberger *et al*. 1993) rhinoceros. In these two African rhinoceros species, pregnanediol is either not present in faeces

(white rhinoceros) or only found during pregnancy (black rhinoceros). With respect to progesterone metabolism, the present data indicate that the Sumatran rhinoceros further differs from the black rhinoceros in that it predominantly excretes pregnanes of the 5β-series, whereas in the black rhinoceros only 5α-reduced pregnanes seem to be present in faeces (Schwarzenberger *et al*. 1996b). Moreover, the Sumatran rhinoceros does not excrete 5α-dihydroprogesterone, which has been determined as an abundant faecal progestin in the black rhinoceros (Schwarzenberger *et al.* 1996b).

In summary, the study provided first information on metabolism and excretion of reproductive hormones in the highly endangered Sumatran rhino. Using a radiometabolism study in combination with methods of chromatography and analytical chemistry, the route of oestrogen and progesterone excretion could be identified and, furthermore, information on the characteristics and identity of urinary and faecal metabolites of both reproductive hormones could be provided. Based on these findings, it can be concluded that the measurement of oestrogens, particularly oestradiol-17ß, in urine and progestins, specifically 5-reduced mono and/or dihydroxylated pregnanes in faeces should be most appropriate for a non-invasive assessment of ovarian function and pregnancy in the Sumatran rhinoceros.

Conclusions

(1) Oestradiol-17β metabolites are mainly excreted as conjugated forms in urine, whereas those of progesterone are almost exclusively excreted as unconjugated forms into faeces.

(2) Oestradiol-17β glucuronide is the only abundant oestrogen in urine, whereas oestrone is the major oestrogen in faeces, with oestradiol-17β being less abundant

(3) Metabolism of progesterone is more complex and resulted in the excretion of three major metabolites, two pregnanediols, one of which was 5β-pregnane-3α, 20α-diol, and a 5-reduced 20-oxo pregnane, being identified as 5β-pregnane-3α-ol-20-one.

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ENDOCRINE CHARACTERISATION OF REPRODUCTIVE STATUS IN THE FEMALE SUMATRAN RHINOCEROS

Introduction

The results of the radiometabolism study (chapter III) provide the basis for the development of non-invasive methods for assessing reproductive status in the female Sumatran rhinoceros through urinary and faecal hormone analysis. A longitudinal study on the female Sumatran rhinoceros reproductive physiology is highly important in order to characterize ovarian function and to recognize reproductive disorders, both essential components to evaluate the reproductive potential of the few animals in captivity.

Information on reproductive function of female Sumatran rhinos was not available at the onset of this study in 1996, until Roth *et al.* (2001) first described the ovarian cycle and early pregnancy through endocrine evaluation from serum and faeces. This study showed that faecal progestagen measurements matched those of circulating progesterone, indicating the reliability of non-invasive hormone analysis for monitoring ovarian activity in the species. Furthermore, based on these measurements, Roth *et al*. reported the cycle length in the Sumatran rhino to be about 21 days. The information generated was, however, limited to only six oestrous cycles from a single animal. Unfortunately, Roth *et al*. did not examine the relation between changes in genital morphology and sexual behaviour with endocrine patterns. This, however, could help to evaluate the reliability of oestrous signs in this species for predicting ovulation, which in turn might be used as a basis to improve timing of pairing of the sexes for breeding as this is often accompanied by violent courtship behaviour when timing is inappropriate. Knowledge on this relationship may therefore provide a practical tool for supporting breeding programs in all facilities, particularly when endocrine monitoring is difficult or impossible.

As a basis to assist the breeding management of the Sumatran rhinoceros in captivity, the overall aim of this part of the study was to provide information on the fertility status of individual female Sumatran rhinoceros using non-invasive endocrine assessment. To this end, the specific objectives were to: (a) establish and biologically validate reliable methods of urinary and faecal hormone analysis for monitoring female endocrine function, (b) to apply these methods to assess ovarian function in individual females, (c) to describe changes in oestrogen and progestin excretion during an individual pregnancy, (d) to provide information on ovarian cycle length, and (e) to examine the temporal relationships between changes in genital morphology, sexual behaviour and follicle development and endocrine patterns in a single female. It is hoped that this information provides an initial basis to assist and improve the breeding management of the Sumatran rhinoceros in captivity.

Materials and Methods

Animals and housing conditions

Four adult female Sumatran rhinoceros (ages: 9-20 years) served as subjects in this study. The females were maintained at different places in Indonesia, Malaysia and USA. All subjects were housed with a male partner, from which they were, however, separated for most of the study period except when they showed signs of oestrus. Whilst none of the females had bred successfully prior to the study, one female (EMI) conceived and gave birth to a healthy offspring during the study period. Table 8 provides information on demographic data and reproductive history of the individual females

Table 8. Demographic data and reproductive history of study subjects

Female and Studbook No.	Age * (years)	Location	Study period	Reproductive History
Bina (# 32)	18	SRS Way Kambas National Park	1996, and 1999-2005	Regular oestrus
Dusun (# 12)	20	SRS Way Kambas National Park	1999-2000	Not cycling
Gelogob (# 40)	9	Sepilok Rhino Center, Sabah	1999	Irregular cycle
Emi (# 29)	13	Cincinnati Zoo, USA	1999-2001	Regular oestrus

* Age at the beginning of study

Sample collection

From each of the females, regular faecal and, if possible, also urine samples were collected during the different study periods. Sample collection was performed according to the protocol reported by Hindle *et al*. (1992) and Heistermann *et al*. (1993). It was tried to collect samples on an almost daily basis, although, particularly for collection of urine samples, this could not always be achieved and in some animals no urine samples could be collected at all. The complete sample collection period was from 1996-2005. As is shown in Table 8, however, the period of

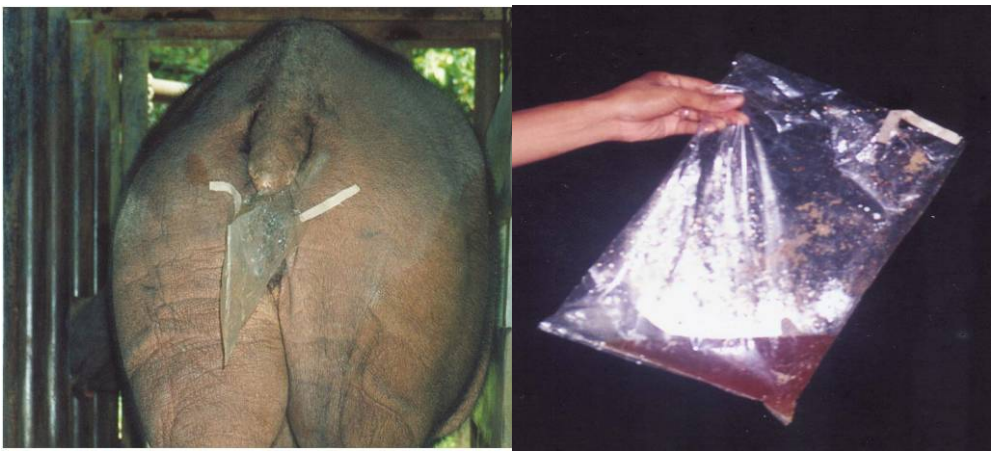
sample collection differed between females, depending on the possibility of getting access to the animal. For instance, faecal and urine samples from Emi were collected continuously from June 1999 till April 2000, and a second collection period started after a pregnancy was diagnosed during July 2000 until several months after birth in November 2001. Sample collection for Gelogob was conducted for a period of 11 months from February until December 1999, while for Dusun´s samples were collected between May and August 1999, after which she became ill and died in early 2000. Most frequent samples were collected from Bina. Firstly, in order to test the biological validity of the urinary oestrogen and faecal progestin measurements, samples were collected for a four months period from January till March 1996 when Bina was housed at Safari Park Indonesia, Bogor. A second longitudinal period of sample collection commenced from 1999-2005 following the animal´s transfer to the sanctuary in Way Kambas National Park.

In general, faecal samples were collected directly after voiding. Specifically, each sample was homogenized by mixing a bulk of faeces by hand (manually) before an aliquot of about 20 g was placed in a film tube and stored frozen at –20°C (see Figure 15). Urine was collected immediately when the animals were urinating by using a waterspade, bucket, or plastic bag that was placed in the back of the vulva (see Figure 16). An aliquot of about 5 ml was then placed in a plastic tube and immediately stored at -20^{o}C without addition of any preservative. Since collection of urine was more difficult compared to faeces, regular urine samples could only be obtained from female Bina when she was kept in an individual cage with a concrete floor in Safari Park Indonesia, Bogor. Due to the difficulties in providing regular sampling, urine samples were not collected from the animals in SRS anymore.

(a) (b)

(c)

Figure 15. Feacal sample collection in the Sumatran rhino. (a) faeces was collected directly or immediately after voiding into (b) a bucket, from which (c) an aliquot was placed in film tube for storage



(a) (b)

Figure 16. Urine collection from female in SRS. (a) a plastic bag was placed in the back of the vulva, (b) urine was collected immediately after urinating

Sample preparation

Sample preparation included several processes for preparing the samples before hormone analysis for both faecal and urine samples. These processes included a) drying and pulverization of feces, b) hydrolysis of urine and c) sample extraction (both faeces and urine).

a). Drying and pulverization of faeces

Faecal samples were dried according to the procedure described by Heistermann *et al*. (1993). In brief, faecal samples were lyophilised for 3-4 days using a freeze dryer machine (Christ®, Gamma 1-20) at a temperature of -20°C and a vacuum pressure 1.030 – 0.630 mbar. Dried faecal samples were then pulverized by hand using a pestle and mortar and sieved through a stainless steel strainer to separate the faecal powder from the fibrous material. The faecal powder was finally stored in a plastic tube at -20°C until analyzed.

b). Hydrolysis of urine

Hydrolysis of urine was conducted according to the protocol described by Heistermann *et al*. (1997). Urine samples were thawed, vortexed and centrifuged for 5 minutes (3000 rpm), and a 25 µl aliquot was hydrolyzed with 375 U β-glucuronidase/sulfatase (No. G 1512; Sigma Chemie, Stainheim, Germany) in 300 µl hydrolysis buffer (NaAc 0.5 M, pH 4.7). The hydrolysis process was carried out over night by incubation at 37°C in a shaking waterbath (Kottermann®, Germany). In order to determine combined efficiency of the hydrolysis and following extraction step (see c.), 4000 cpm [3H]-oetrone-3-glucuronide or PdG was added into each tube prior to hydrolysis.

c) Sample extraction

1. Urine samples

Hydrolyzed urine was adjusted to pH 7 by adding 110 μl NaOH (1 M) and subsequently extracted with 7 ml diethylether by vortexing for 15 minutes (Heidolph®, REAX 2000). The ether phase was then separated from the aqueous phase by snap-freezing in methanol-dry ice. The ether phase was decanted into a glass tube (100 x 16 mm) and evaporated totally at 35°C under a stream of nitrogen using a sample concentrator (Dri-Block® DB-3, sample concentrator SC-3, Techne, England). Following evaporation, the extract was reconstituted in 150 μl absolute ethanol (EtOH) by vortexing for 5 minutes. The ethanolic extract was then decanted into smaller glass tube (75x12 mm), sealed with a plastic cap and stored at -20^{o}C until hormone analysis which was performed within 6 hours of extraction. For determination of combined hydrolysis and extraction efficiency, radioactivity was counted for 5 minutes in a 25 μl aliquot of the urinary extract after reconstitution in ethanol. Extraction efficiency was 89.8 ± 7.9 % (n= 364). All urinary hormone data are expressed on a per mg creatinine (mg Cr).

2. Faecal samples

An aliquot of the faecal powder of each sample, representing approximately 50 mg (exact weight noted), was extracted with 3 ml of 80% MeOH by vortexing for 15 minute in a 15 ml plastic tube using a multi-tube vortexer (Multi-Tube Vortexer, SMI®, USA). Prior to vortexing, approximately 20,000 cpm [3H]-progesterone was added randomly to a subset of samples (n= 195) to determine the efficiency of the whole extraction procedure. After vortexing, tubes were centrifuged at 4000 rpm for 10 minutes. The extract of each sample was decanted into glass tubes (100x16 mm), sealed with a plastic cap and stored immediately at -20^{o}C until analyzed

For determination of extraction efficiency, 25µl of samples containing [3H]-progesterone were counted for radioactivity as described above and gave a mean value of 89.5 ± 2.7 %. All faecal data are expressed on a per gram dry weight basis.

Hormone assays

Based on the results of the radiometabolism study presented in chapter III, and for validation of the different progestin measurements for monitoring female ovarian function, urine samples of Bina during the study period 1996 were measured for immunoreactive oestradiol-17ß (E2), while her faecal samples were measured for 5α-pregnane-3α-ol-20-one (5-P-3OH) and pregnanediol (Pd) immunoreactivity. According to the results of these measurements, the remaining samples collected during the study were only measured for urinary E2 and, concerning progestin, for that progestin that best reflected luteal function. All measurements were carried out by competitive, double-antibody, enzymeimmunoassay procedures (EIA) as already described in Chapter III.

a) Oestradiol-17β (E2) assay

The EIA for measurement of oestradiol 17-β was carried out as described by Heistermann *et al*. (1998) as follows: An antiserum against oestradiol-6-CMO-BSA (Ab No. 1001, Biocline, Cardiff, UK) was used as second antibody in the assay together with oestradiol-alkaline phosphatase (Dept. of Reprod. Biol, DPZ) as label and oestradiol-17β as the standard. The E2 antiserum crossreacts 100% with oestradiol-17β and 2% with oestrone. Urine extrats were diluted 1:50 to 1:100 in assay buffer. For oestradiol analysis, duplicate 50µl aliquots of diluted urine extracts or standard (range 0.49-125 pg/50µl) in 0.04 mol phosphate-buffered saline containing 0.1% BSA (PBS, pH 7.0) were combined with label (50µl) and antiserum (50µl) and incubated overnight at 4°C. After incubation,

the plates were washed four times with distilled water containing 0.005% Tween 20, blotted dry and 150μl of phosphatase substrate (Sigma No. 104, 20 mg in 15 ml substrate buffer) was added to each well. The plates were incubated while shaking for further 2-3 hours before absorbance was measured at 405 nm. The amount of oestradiol-17β in the sample was calculated automatically by a reader linked computer program (MikroTek Laborsysteme GmbH, Overath). Assay sensitivity at 90% binding was 0.49 pg/well. Intra- and interassay coefficient of variation were determined by replicate determinations of high- and low value quality controls (QC's). Intraassay coefficient of variation was 8.7% (QC high, n= 16) and 10.5% (QC low, n= 18). Interassay coefficient of variation for QC high and QC low were 10.1% (n= 22) and 11.6% (n= 22), respectively. Serial dilutions of samples from the follicular and luteal phase of the ovarian cycle ran parallel to the oestradiol-17β standard curve.

b) Pregnanolone (5-P-3OH) assay

5-P-3OH immunoreactivity was determined in faecal extracts using the assay procedure already described in detail in chapter III. Extracts were diluted 1:20 to 1:90 (during oestrous cycle) and 1:200 to 1:10000 (during pregnancy) in assay buffer and duplicate 50 µl aliquots were taken to assay. Sensitivity of the assay was 9.8 pg/well at 90% binding. Serial dilutions of faecal extracts from the follicular and luteal phase of the ovarian cycle gave displacement curves parallel to that of the 5-P-3OH standard. Intraassay coefficient of variation for QC high and QC low were 7.0% (n=16) and 9.1% (n=17), while, interassay coefficient of variation for QC high and QC low were 7.8% (n= 42) and 9.39% (n= 42), respectively.

c) Pregnanediol (Pd) assay

Faecal extracts were measured for Pd immunoreactivity as described in detail in chapter III. Exctracts were diluted 1:10 in assay buffer and duplicate 50 µl aliquots were taken to assay. Assay sensitivity at 90% binding was 6.3 pg per well. Intraassay coefficient of variation was 7.5% (QC high, n=16) and 8.8% (QC low, n=17). Interassay coefficient of variation for QC high and QC low were 7.6% (n=14) and 11.7% (n=14), respectively. Serial dilutions of samples from the follicular and luteal phase of the ovarian cycle were parallel to the pregnanediol standard curve.

d) Creatinine measurement

In order to control for variation in fluid intake and output and variable periods of urine collection, the creatinine content of each urine sample was measured using a creatinine analyzer (Beckmann Instruments, Brea, CA, USA). Urine was diluted 1:10 in 0.9% NaCl solution before analysis and 25µl was pipetted into the machine. Urine samples with a creatinine concentration of less than 0.05 mg/ml were rejected from calculation of hormone concentration and only samples with values of creatinine $\geq$0.05 mg/ml were used for generating urinary hormone profiles.

e) Determination of assay specificity

As an additional criterion for evaluating the relative usefulness of the Pd and 5-P-3OH assay for monitoring ovarian activity, the specificity of the two faecal progestins measurements was assessed using HPLC analysis of a sample collected during the mid-luteal phase of the ovarian cycle of Bina. HPLC analysis was performed according to the method described in detail in chapter III. In brief, 0.1 g lyophilised powder was extracted twice with 1.5 ml methanol (80%), which was subsequently subjected to the clean-up

procedure as described in chapter III, reconstituted in ACN/H2O (50/50, v/v) and run by the same HPLC system as mentioned previously for the separation of radioactive progestins (see chapter III). The presence of immunoreactivity peaks was compared to the elution positions of authentic ^{3}H-progestin tracers determined in separate runs immediately prior to each sample run.

Since oestrogens were excreted predominantly in urine and oestradiol-17β was found to be the only major metabolite (see chapter III), a specificity test of the E2 assay was not conducted.

Observations of genital morphology, sexual behaviour, and follicle development

In order to examine the presence of external signs of female reproductive status and the temporal relationship between endocrine changes and changes in vulval morphology and sexual behaviour in the female Sumatran rhinoceros, longitudinal data on vulval coloration and different parameters of sexual behaviour were collected. Since this approach required daily access to the animal, it could only be conducted on female BINA from the Sumatran rhino sanctuary, Way Kambas National Park, Indonesia. Observations have been carried out from August 1999 to November 2005, thus covering a total period of 5 years. In parallel, if possible, urine and faecal samples were collected throughout the same period. Due to difficulties in collecting urine samples, urine was, however, not collected after early 2001. In addition, ultrasound scanning was performed to monitor follicle growth and the presence of a corpus luteum in the same female since early 2005. Since then, monitoring on the reproductive status could be inquired through comparing hormonal profile, vulval changes, sexual behaviour and follicle development.

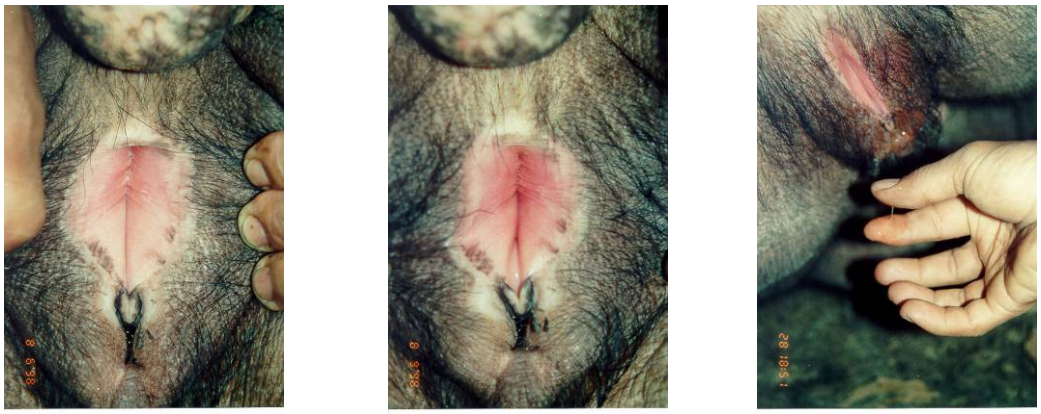
a) Ultrasonography

Female Sumatran rhinoceros in SRS Way Kambas was taught to cooperate for ultrasound examination, which allowed routine and repeatable examinations possible. The rhino was trained to enter chute and to fix her in the chute with one entire side of the chute that could move laterally to minimize lateral movement of the rhino during the examination. The ultrasound machine was placed at the back of the rhino that allowed scanning through per rectal palpation. Prior to ultrasound scanning, faeces were removed from entire distal rectum through exploration rectally. Examinations were performed with an Aloka SSD 500 (Aloka Co. Ltd, Tokyo, Japan) using a 5 MHz curvilinear tranducer, and connected to a Hi-8 video recorder. All images were recorded during the examination. During examination, the bladder, cervix, uterine body, uterine horns, and both ovaries were examined. Follicles > 10 mm in diameter were measured and the presence of corpus luteum was recorded. Ultrasongraphy examinations were conducted two to three times each week. When follicle ~ 18 mm in diameter observed, examinations were done in daily basis until the day of sexual behaviour or mating occurred. Ultrasonography examinations were lasted 10 to 15 minutes for each monitoring.

B) Genital morphology

Observations on vulval coloration changes were carried out everyday in the early morning (07.00-09.00 a.m.) when the animal was in its cage for routine health monitoring. The vulva was inspected after the animal has being washed from mud that covered the body after wallowing. Vulval appearance was rated on a four point scoring system based on colouration, presence and density of small (perifer) blood vessel and surface appearance as follows: Score 1: pale colour, no blood vessels visible, surface dry;

score 2: pinkish colour, no blood vessels visible, surface dry; score 3: red colour, low density of small blood vessels, surface dry; and score 4: intensive red colour, high density of small blood vessels, surface wet and glossy (see Figure 17).



(a) (b) (c)

Figure 17. Changes in vulval morphology (a) score 2, (b) score 3, and (c) score 4 as described above.

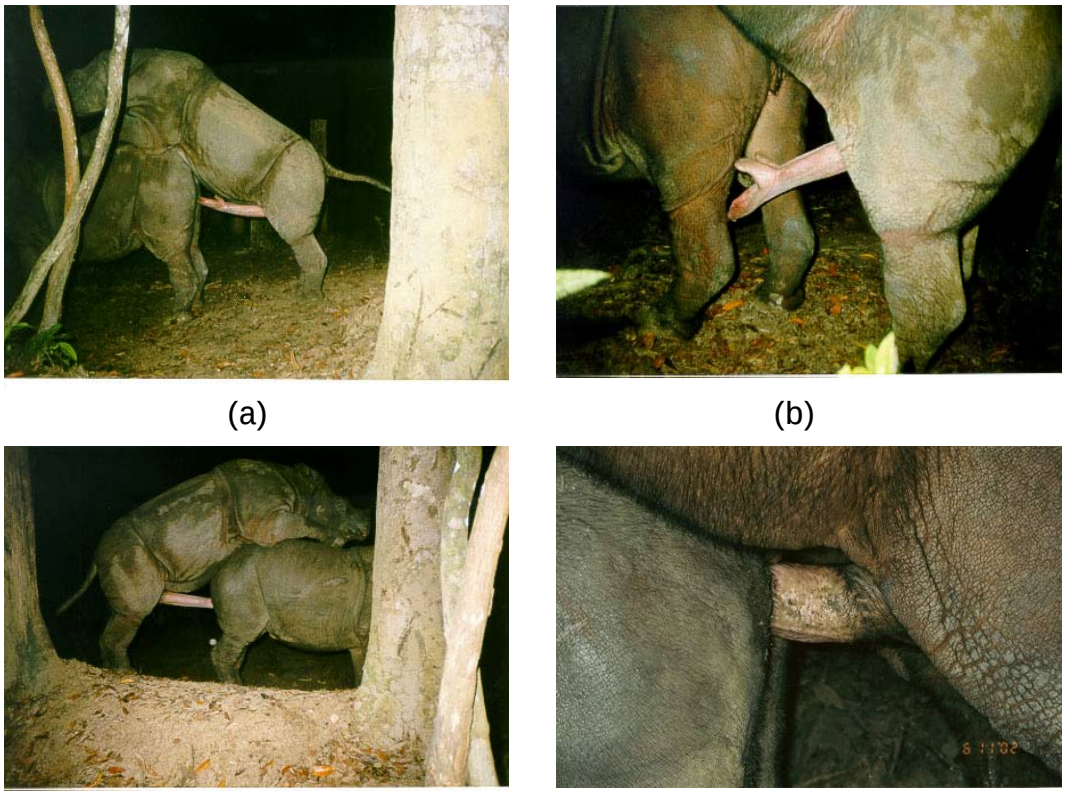
c) Sexual behaviour

Interest among the rhinos. "Interest" was initially defined as occurring when the male and female, separated by a fence, came into close proximity and stayed within a distance of 1-5 meter, often looking at each other and trying to get closer and approach each other for at least one hour. After having shown this behaviour for at least one hour, the gate in the fence was opened to allow animals to get together. If this was followed by the occurrence of immediate physical contact (e.g. sniffing, rubbing) between the male and female, the former behaviour was scored as indeed reflecting the occurrence of "interest" and was given a score of 1. In case animals moved away from each other after the gate was opened, the former behaviour was not scored as reflecting "interest" and was given a score of 0 (see Figure 18). The latter, however, occurred relatively rarely.

Figure 18. Sequence of interest behaviour between sexes was rated as zero or one as described in the text.

Courtship behaviour. Courtship behaviour was defined as occurring when, after letting animals together, the male and female maintained close proximity to each other and continuously displayed physical contact, such as sniffing, rubbing, chasing, following each other or chin rest.

Mating behaviour. Mating behaviour was defined as occurring when the male mounted the female and succeeded in partly or full intromission. If no intromission occurred, the behaviour was termed "mounting" (see Figure 19 in detail). The presence of ejaculation was assessed by the presence of semen drops immediately after copulation either directly from the penis or dribbling from the vulva.



(a) (b)

(c) (d)

Figure 19. Sequence of mating behaviour was defined as (a) early process of mounting, (b) mating behaviour without intromission "mounting", (c) mating behaviour with partly intromission, and (d) mating behaviour with full intromission

Analysis of data

The stages of the ovarian cycle were determined based on a defined rise of faecal progestin values. In this respect, a value exceeding a threshold of the mean + 2 SD of the preceding interluteal (presumed follicular) phase values, was taken to indicate the first day of the

postovulatory or luteal phase of the cycle (see Heistermann *et al*. 1993). A fall in hormone concentration below the threshold indicated the first day of the following follicular phase of the cycle. Determination of cycle stages and calculation of cycle length was solely based on progestin profiles, because urine samples were more difficult to obtain and therefore many values of oestradiol-17β were lacking in consecutive cycles. Cycle length was determined as the interval between successive progestin rises.

In addition, the interval between occurrences of oestrous behaviour (Inter-Oestrous-Interval/IOI) was also calculated and provided a second measure of cycle length. The length of the component cycle phases (follicular and luteal) in a given cycle were defined as follows: the follicular phase comprised all days in which faecal progestin values were below defined the threshold value (see above) while the luteal phase comprised the days in which the progestin values exceeded the threshold. For each cycle, 5-P-3OH values were calculated for the follicular and luteal phase and compared for statistical differences using the paired t-test.

Analysis of the relationships between hormonal profiles and behavioural and morphological parameters was done descriptively and statistically. Faecal 5-P-3OH levels were compared during periods with and without sexual behaviour. For this, mean hormone values were calculated for each period in each given cycle. Since, however, sexual behaviour occurred for only one or two days and since in some cases no hormone value was available on the day when sexual behaviour was shown, for calculation of mean 5-P-3OH levels for periods "with sexual behaviour" a three days window comprising the day of sexual behaviour $\pm$ one day was used. Progestin values on all days outside this window in any given cycle were then used to calculate average hormone values for the period "without sexual behaviour". 5-P-3OH levels between periods were tested for statistical differences using the paired t-test. In addition, 5-P-3OH levels recorded during the different stages of vulval score were tested for statistical differences using Repeated Measures (RM) ANOVA.

Post-hoc pairwise comparisons were performed using the Student-Newman-Keuls test. The temporal relationship between the occurrence of sexual behaviour and the rise of 5-P-3OH values was calculated as median value of days between the occurrence of first oestrous signs and the defined 5-P-3OH rise. The temporal relationship between scores of vulva changes and 5-P-3OH rise was determined accordingly. In addition, the temporal relationship between onset of the highest score of vulva change and onset of sexual behaviour was defined as median value of days between them. Due to limitation on the number of E2 profiles no assessment on the relationship between E2 values and sexual behaviour occurrence and vulva changes was done.

Results

Hormone profiles of the reproductive cycles

Female BINA (Taman Safari Indonesia and SRS Way Kambas)

a) Period 1996

Figure 20 shows the profiles of E2 and 5-P-3OH during the observation period January till March 1996. Excretion of oestradiol immunoreactivity in urine showed a cyclic pattern in which three periods of elevated concentrations (75-150 ng/mgCr) can be distinguished from periods of 3 – 6 times lower baseline values (15-30 ng/mgCr). The periods of elevated oestradiol were in all three cases associated with clear signs of oestrous behaviour as indicated by increases in proximity and interest between the male-female pair.

Concentrations of Pd in faeces were generally low (10-20 μg/g dry faeces) before the E2 elevations and increased to 30-60 μg/g during the presumed luteal phase. However, the Pd profile was less clear in the second cycle as compared to the first. HPLC analyses of Pd immunoreactivity in the luteal phase samples showed a similar profile as found in the radioactive sample, indicating that the measurement of Pd was non-specific (see insert, Figure 20). The more specific measurement of faecal 5-P-3OH immunoreactivity (see insert, Figure 20) revealed a clear cyclic pattern for both cycles with consistently low levels during the presumed follicular phase (3-8 μg/g dry faeces) and three- till fivefold elevated concentrations (15-25 μg/g dry faeces) during the presumed luteal phase. Based on the results, it appeared that measurement of 5α-P-3OH immunoreactivity gave clearer profiles compared to Pd and is thus superior for monitoring reproductive function in the female Sumatran rhinoceros. Therefore, for monitoring reproductive status in the other female rhinos, the 5-P-3OH only assay was applied.

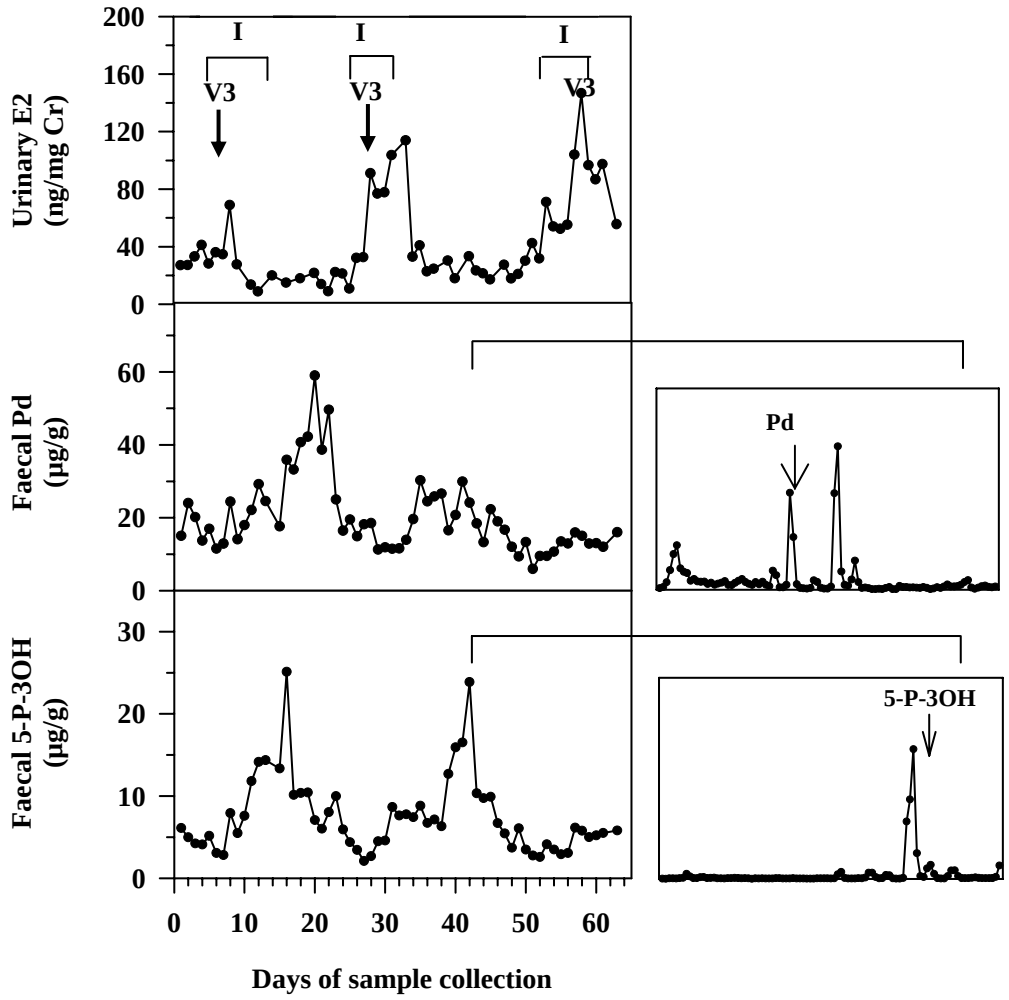


Figure 20. Profile of oestradiol-17β (E2) immunoreactivity in urine and pregnanediol (Pd) and 5α-pregnane-3α-ol-20-one (5-P-3OH) immunoreactivity in faeces are compared to periods of oestrous during two consecutive ovarian cycles in Bina. Insert on the right show respective HPLC profiles of Pd and 5-P-3OH immunoreactivity obtained from a mid-luteal phase sample. Arrow indicate elution positions of authentic 3H-Pd (Pd) and 5α-pregnane-3α-ol-20-one (5-P-3OH) tracers (I= interest, V3= score 3 vulva coloration change)

Oestrous cycle length was 23 days according to the interval between 5-P-3OH rises. Based on the occurrence of sexual behaviour, the cycle length was about 24 to 25 days. Sexual interest was displayed for 3 to 5 days, however, the most prominent of vulva colour change (score 3) was only observed for one day.

b) Period 1999-2000

Monitoring reproductive status in Bina has been continued after she arrived at the Sumatran rhino sanctuary (SRS), Way Kambas National Park, Lampung, Indonesia in 1998. Bina was kept at SRS under natural condition in the origin of the Sumatran rhino habitat together with another female (Dusun) and one male (Torgamba), although they were separated by electrical fences at the sanctuary facilities.

As can be seen from Figure 21, Bina showed six consecutive oestrous cycles during the observation time as indicated by the appearance of sexual interest and mounting. Due to difficulties to collect regular urine samples, the oestradiol profile is less complete and because of sample gaps, provided only partly a cyclic pattern during the monitoring period. Where regular oestradiol data were available, occurrence of oestrous signs were associated with elevated oestradiol levels, while low levels of E2 were not accompanied by oestrous behaviour. Apart from interest between the sexes, there was even mating behaviour which was never observed during time the animal was kept at the Zoo. In terms of concentrations, periods of E2 levels were three to four times higher during oestrous (228 – 335 ng/mg Cr) compared to inter oestrous periods (84 – 135 ng/mg Cr). In association with the occurrence of oestrous behaviour and high E2 levels, vulva appearance became prominent, reaching a score of 3 or 4.

The profile of 5-P-3OH immunoreactivity showed a clear cyclic pattern during the six oestrous cycles, from which follicular and luteal components could be clearly distinguished. However, there were two extended oestrous cycles from December 7th 1999 till January 17th 2000, and March 3rd till May 21st 2000, as indicated by prolongation of progestin excretion.

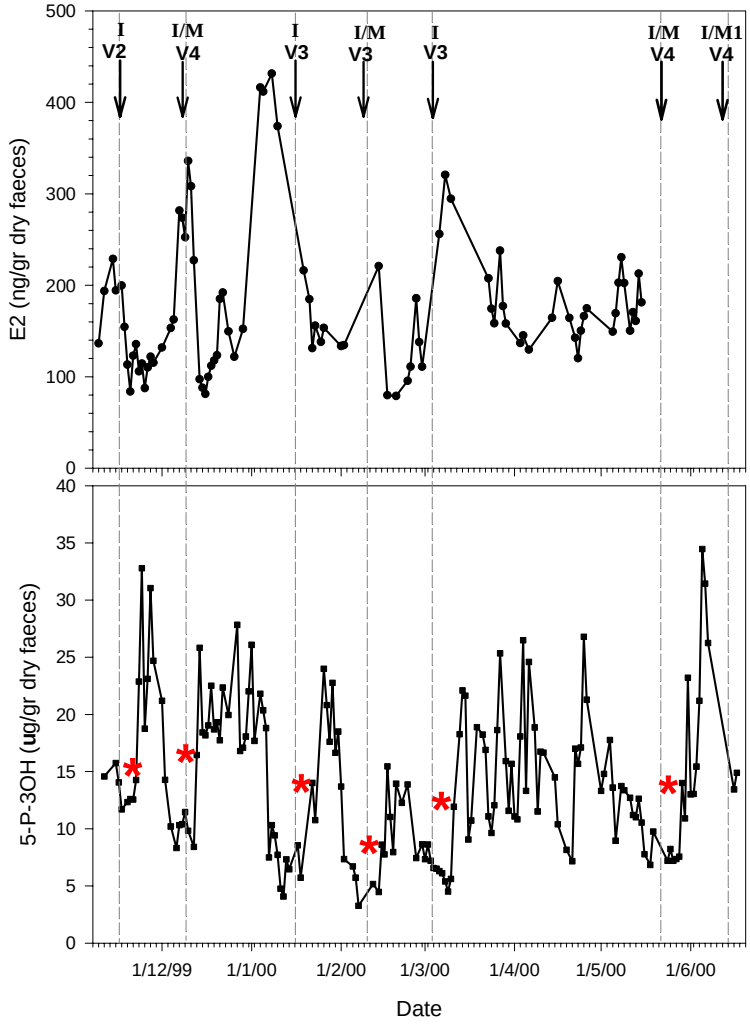


Figure 21. Profile of oestradiol-17β (E2) immunoreactivity in urine and 5α-pregnane-3α-ol-20-one (5-P-3OH) immunoreactivity in faeces are compared to periods of oestrous during five consecutive ovarian cycles in Bina from November 1999 until June 2000 (I=interest, M=mounting, V= vulval score, *= 5-P-3OH rise, – – – = timing of sexual behaviour)

5-P-3OH levels were constantly low (3.3 – 12.6 pg/µr dry faeces) during the presumed follicular phases and increased three- to five fold during the presumed luteal phases (10.7 - 34.5 µg/g dry faeces). When the cycle lasted for 40 days, 5-P-3OH values were consistently high for 29 days. On the second extended cycle of 79 days, pregnanolone values were elevated for the last 67 days, although there was a short-term (3 days) decrease at the end of April 2000. However, the levels during this

time were still above the threshold of this cycle and therefore, this period unlikely represented a follicular phase. This is supported by the finding that no oestrous behaviour occurred during these days.

Table 9 indicates the data on cycle lengths as determined from the progestin profile and occurrence of oestrous behaviour. Both data sets matched closely and provided similar results. Four cycles showed a length of about 20-25 days, while two cycles appeared to be extended, showing a length of 40 and 79 days. The interval between the occurrence of interest and mounting behaviour, and subsequent pregnanolone rises varied between 5 and 8 days (see Table 9).

Table 9. Oestrous cycle length in Bina from five consecutive oestrous cycles from November 1999 until June 2000

No. of oestrous cycle	Oestrous cycle length (days) based on		Interval between I/M/M1 and 5-P-3OH rise (days)
	IOI	5-P-3OH rise	
1.	20	21	5
2.	41	40	6
3.	24	24	5
4.	22	25	5
5.	79	79	8
6.	24		

c) Period 2001

Monitoring reproductive status in Bina was also conducted in 2001 for more then three months (August-November 2001). Bina showed three consecutive oestrous cycles, as clearly indicated by the presence of mating behaviour (see Figure 22). In each of the cycles, 5-P-3OH values were constantly low during the presumed follicular phases (2.12 – 6.24 µg/g dry faeces) and elevated two- to four fold during the presumed luteal phases (4.16 – 17.63 µg/g dry faeces).

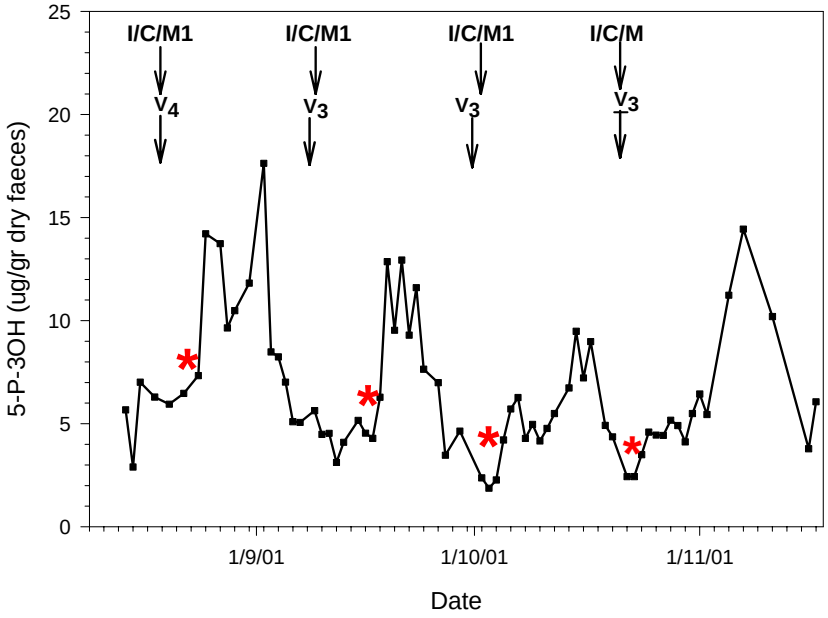


Figure 22. 5-P-3OH profile in Bina from three consecutive oestrous cycles during the period from August-November 2001 (I= interest, C= courtship, M=mounting, M1= mating, V= vulval score, and * = pregnanolone rise).

The oestrous cycle length was about 18 – 25 days for the three cycles recorded (Table 10). The interval between the occurrence of sexual behaviour and the pregnanolone rise was four to nine days.

Table 10. Oestrous cycle length from three consecutive oestrous cycle in BINA (August – November 2001)

No. of oestrous cycle	Oestrous cycle length (days) based on		Interval between I/M/M1 and 5-P-3OH rise(days)
	IOI	5-P-3OH rise	
1.	21	25	5
2.	23	19	9
3.	18	19	4

In the three of the four cycles, the appearance of the maximum vulva coloration changes was scored three, and in two of these cases this score was reached 1-2 days before sexual behaviour was displayed. In the fourth cycle, a vaginal score of four was observed at the same day of

sexual behaviour. As seen in the previous periods of data collection from Bina, oestrous behaviour was always associated with low progestin levels.

d) Period 2005

Five additional oestrous cycles were monitored during 2005. Three of these cycles were prolonged to 46, 50 and 54 days and associated with extended elevations (for 34-45 days) in progestin excretion during the presumed luteal phases. Based on the interval between 5α-P-3OH rises, the cycle length ranged from 20 – 55 days (Table 11), which fit well with the 21-54 day cycle length as indicated by the occurrence of sexual behaviour. The interval between the occurrence of sexual behaviour and the 5-P-3OH rise was between 5 to 10 days (see Table 11) and in each of the cycles, sexual behaviour was seen only during periods of low 5α-P-3OH concentrations.

Table 11. Oestrous cycle length from three consecutive oestrous cycle in BINA (April – November 2005)

No. of oestrous cycle	Oestrous cycle length (days) based on		Interval between I/M/M1 and 5-P-3OH rise (days)
	IOI	5-P-3OH rise	
1.	46	42	10
2.	22	20	6
3.	50	51	5
4.	21	21	5
5.	54	55	5

For these cycles, ultrasound examinations were performed to assess the presence of dominant follicles and corpus luteum (CL). In these cycles, a dominant follicle (∅ 19-25 mm) was found. These follicles were usually seen at the same day when mating behaviour occurred, although there was one evidence when a dominant follicle was observed but the behaviour was not displayed. In relation to progestin values,

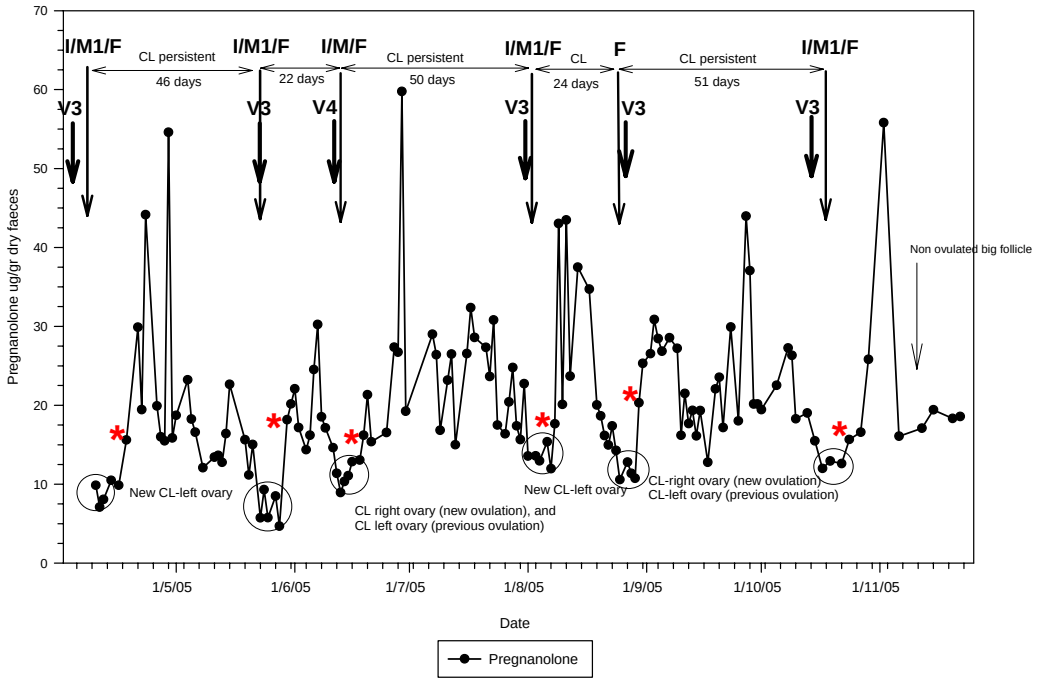


Figure 23. 5-P-3OH profile from five consecutive oestrous cycles in BINA during the year 2005 (V= vulval score, I= interest, M/M1= mounting/mating, F= follicle dominant, ⟶ = display of sexual behaviour, ⟶ = vulva coloration changes, *= pregnanolone rise.

dominant follicles were always seen when progestin values were low (Figure 23). In addition to follicles, ultrasound examinations revealed also the presence of a CL in each of the cycles. In every case, the presence of a CL was associated with elevated progestin levels which, according to definition, were indicative of a luteal phase.

In each of the cycles, there were clear changes in vulval coloration. With one exception, the maximum score was three and in the majority of cycles, this most prominent vulval appearance was seen 1-2 days before sexual behaviour occurred.

Female EM1 (Cincinnati Zoo, USA)

a) Period October 1999-May 2000

Although the Sumatran female rhino at Cincinnati Zoo has been regularly showing oestrous cycles since she was paired with the male (Ipuh) in 1995 (Roth, personal comm.), we had access to samples from her from October 1999 till May 2000, from which faecal progestin profiles could be generated (Figure 24). As indicated in the figure, there were two oestrous cycles, and one early pregnancy (confirmed by ultrasound) which was terminated by abortion after 55 days (Roth T.L, personal communication).

As in Bina, oestrous cycles were characterized by a cyclic pattern in 5-P-3OH excretion, with low levels in presumed follicular phases (5.6-13.6 µg/g dry faeces) and two to four fold elevated levels (10.4-36.6 µg/g dry faeces) in the presumed luteal phases. Interestingly, however, during mid February to mid March, 5-P-3OH concentrations significantly increased and showed a clear luteal phase elevation although no courtship and mating behaviour was reported. 5-P-3OH values were clearly staying high (10.38 – 41.91 µg/g dry faeces) over the period of early pregnancy and decreased after abortion.

According to the occurrence of mating behaviour the length of the cycle was 21 and 23 days (Table 12), which matched closely with the 19 and 24 days cycle length based on the interval between successive 5α-P-3OH rises. In the cycle where no sexual behaviour was observed, cycle length was 32 days as determined from successive progestin rises. The interval between the appearance of mating behaviour and the defined rise in 5α-P-3OH varied from 5 to 7 days.

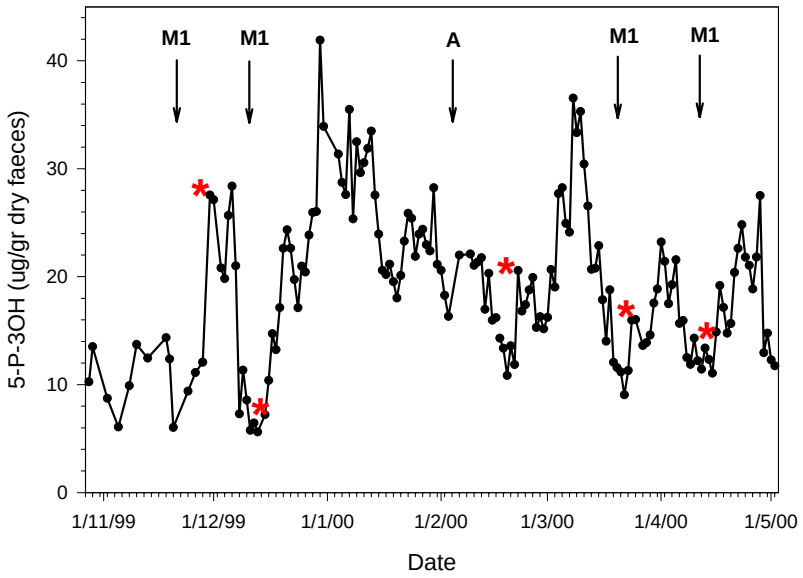


Figure 24. 5-P-3OH profile during oestrous cycle in EMI with three clear oestrous cycles and one pregnancy which resulted in abortion (M1= mating, A= abortion).

Table 12. Oestrous cycle length in EMI from three oestrous cycles from eight months observation (October 1999 until June 2000)

No. of oestrous cycle	Oestrous cycle length (days) based on		Interval between M1 and 5-P-3OH rise (days)
	IOI	5-P-3OH	
1.	21	19	5
2.		32	6
3.	23	24	7

b) Pregnancy period

In Emi, one full period of pregnancy could be followed during the study. Figure 25 shows the profile of faecal 5α-P-3OH during the almost complete gestation period until 23 days after parturition. No samples were available for the first 30 days after successful mating. Onset of a clear and steep rise in 5-P-3OH was detected by around day 60, when the progestin value (46.35 µg/g dry faeces) increased above the highest luteal phase values recorded for this female (42.0 µg/g dry faeces, see Figure

25). Progestin levels rose progressively for four to five months after which a plateau was reached and levels maintained until shortly before parturition.

Maximum 5-P-3OH values during pregnancy were ten- to twenty fold (344.2 – 787.07 µg/g dry faeces) higher compare to luteal phase values. 5-P-3OH concentrations declined rapidly four days before parturition and almost reached luteal phase values at the time of parturition. Levels further decreased following birth and remained constantly low thereafter (5.2 – 29.3 µg/g dry faeces). Gestation length, calculated from the interval between the day of successful mating and parturition, was 475 days.

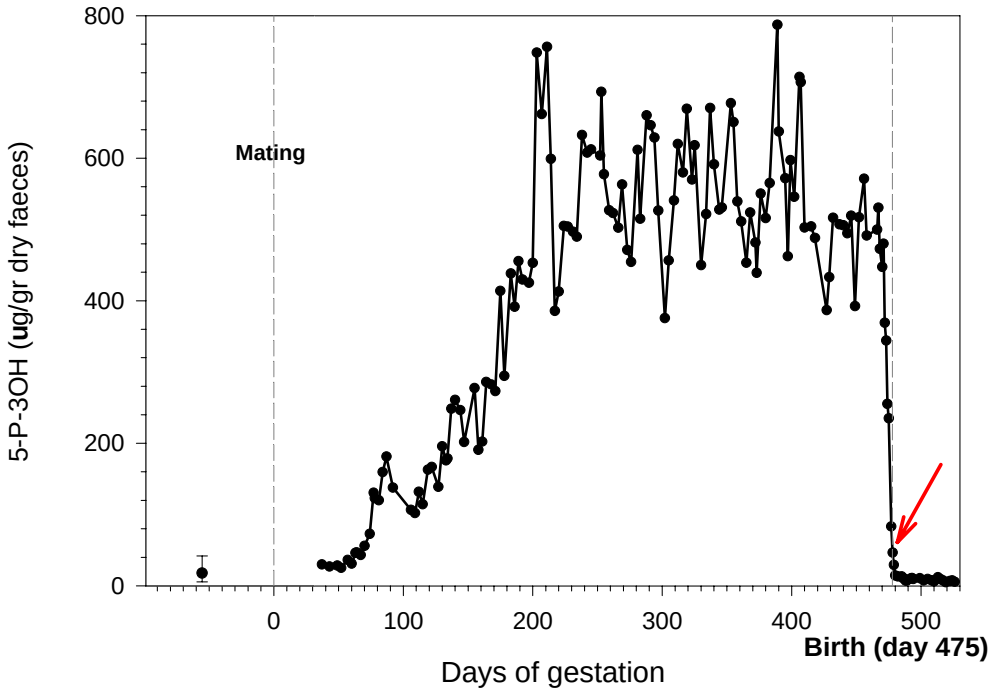


Figure 25. Proffile of faecal 5-P-3OH immunoreactivity during pregnancy in EMI (Cincinnati Zoo). Circle before day 0 represents mean and range 5-P-3OH concentrations of luteal phase values from this female. Arrow indicates day of parturition

Female GOLOGOB (Sumatran rhino center, Sepilok, Sabah)

The female Gologob was kept at the Sepilok Rhino Center, Sabah with one male in an adjacent enclosure. The animal showed very few and irregular breeding attempts. Figure 26 shows the faecal 5-P-3OH profile of Gologob during four months of monitoring. During the first two months, Gologob showed an erratic pattern of 5-P-3OH excretion, values being almost constantly low and there was no clear evidence for cyclic ovarian activity during thi period. By mid of April 1999, the profile indicated two clear periods of defined 5-P-3OH elevations, separated by a period of low levels. This suggests the presence of two ovarian cycles, although there was no report on oestrous behaviour. According to the rises of 5-P-3OH, cycle length was defined as being 23 days.

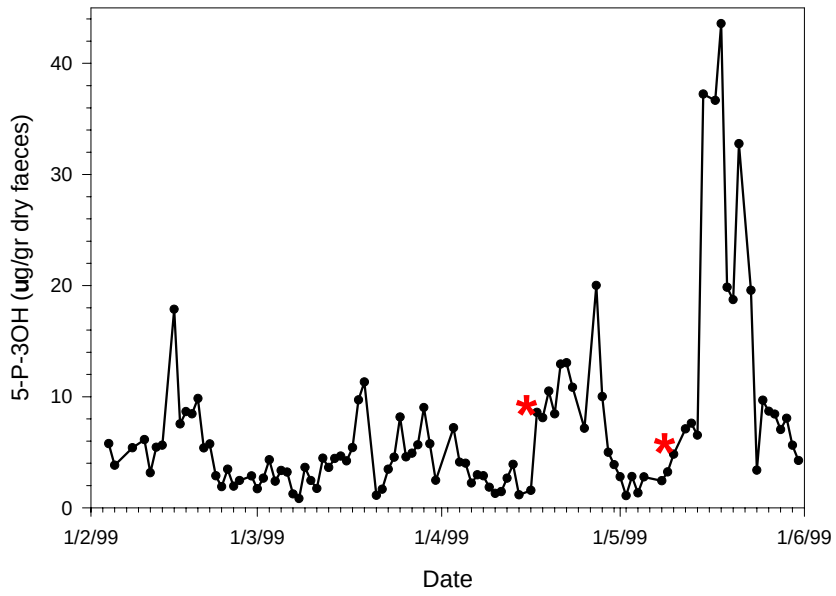


Figure 26. Faecal 5-P-3OH profile in female Gologob in Sepilok rhino center, Sabah (February-May 1999).

Female Dusun (SRS Way Kambas, Lampung, Indonesia)

The female Dusun was kept in SRS Way Kambas. Dusun never displayed sexual behaviour since she has been transferred to SRS in 1998. Her profile of E2 immunoreactivity in urine was flat and levels were

consistently low along the period of monitoring with a mean value of about 57.8 ng/mg Cr (Figure 27). Levels of faecal 5-P-3OH were also low during most of the observation period, although two short-term elevations occurred. However, these did not indicate a clear cyclic pattern. Dusun died in 2001 because of senility that was approved by necropsy results, and with no ovarian activities (see Figure 46 in Appendix I).

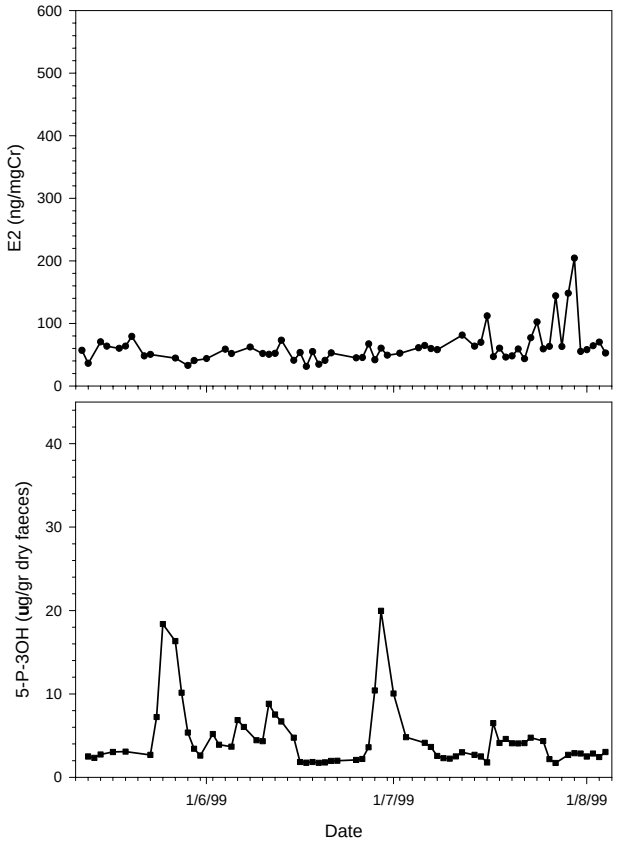


Figure 27. Urinary E2 and faecal 5-P-3OH profiles in female DUSUN from SRS Way Kambas, Indonesia (May-August 1999)

Summary of data on ovarian cycle characteristics

In total, 17 oestrous cycles from three females were observed during the study, i.e., 13 cycles from Bina, three cycles from Emi, and one cycle from Gologob. Based on the faecal 5-P-3OH profiles, five of these cycles showed clearly extended luteal phases and also a prolonged inter-oestrous interval (IOI). Based on the intervals between successive 5-P-3OH rises, a median cycle length of 24 days was determined (range 19-75

days, Table 13). Similarly, when the interval between occurrence of interest, mounting and/or mating behaviour was used, a median cycle length of 23 days, with a range of 18-76 days was found (Table 13).

Table 13. The length of oestrous cycle and cycle stages in Bina, Emi and Gologob during the period 1999-2000, 2001 and 2005

Cycle #	Cycle length		Length of cycle stages [b]	
	Inter oestrus Interval (IOI) [a]	5a-P-3OH rise	Follicular phase	Luteal phase
1.	20	21	7	14
2.	24	24	9	15
3.	22	25	6	19
4.	21	25	9	16
5.	23	19	5	14
6.	18	19	5	14
7.	22	20	5	15
8.	21	21	6	15
9.	21	19	6	13
10.	Nd	32	7	25
11.	23	24	7	17
12.	Nd	23	6	17
13.	41	40	9	31
14.	76	75	7	68
15.	46	42	7	35
16.	50	50	6	44
17.	54	55	6	49
Median	23	24	6	17

a) based on interval between the occurrence of sexual behaviour
b) based on interval between 5-P-3OH rise
extended oestrous cycle

Concerning the duration of component cycle phases, the data showed a relatively constant follicular phase length, with a median value of six days and a range of five to nine days (Table 13). In contrast, luteal phase length was much more variable, ranging from 13 – 68 days, with a median value of 17 days.

As shown in Figure 28, mean of 5-P-3OH levels during the presumed luteal phase (17.3 ± 6.4 µg/g dry faeces) were on average two to three fold higher compared to the presumed follicular phase (7.8 ± 3.8 µg/g dry faeces), the difference being highly statistically significant ($t = -9.879$, $p < 0.001$, detail statistical analysis in Appendix II).

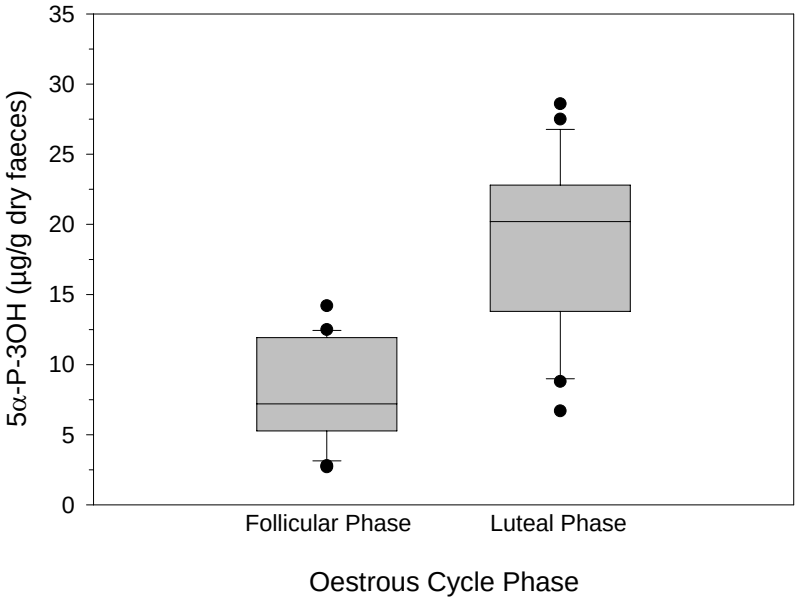


Figure 28. Box plot diagramme of 5-P-3OH values during the ovarian cycle stages of follicular and luteal phase. Values are from 17 cycles (three females).

Relationship between sexual behaviour, vulval changes, and endocrine pattern

Sequence of sexual behaviour display

Sexual behaviour initially started with interest between the male and the female. When both sexes were interested, they subsequently displayed physical contact, including sniffing, and rubbing, which was then followed by intensive courtship behaviour. During the courtship display, the animals showed intense physical contact and always maintained close proximity to each other. The courtship usually lasted for no longer than two hours before mating occurred. In the majority of cases (10 out of 16,

62.5%), sexual behaviour was displayed on two consecutive days and was associated with one or two mating events. On only two occasions, successful intromissions occurred on both days of the two days oestrous. Interestingly, two (50%) out of four pairings at the highest vulva score of 2 (V2) were not followed by mating behaviour.

Sometimes, aggressive behaviour was still seen during the courtship but in those occasions one of the animals always tried to run away for a while to avoid violation, but came back later to continue courtship. Severe violent behaviour never occurred during the animal's courtship at SRS Way Kambas. This is probably because the animals had enough space to hide or to avoid the violent behaviour when it is going to happen.

Relationship between sexual behaviour, vulva changes and endocrine pattern

In all oestrous cycles, sexual behaviour was always associated with low 5-P-3OH concentrations. Figure 29 shows 5-P-3OH values for periods with and without the presence of sexual behaviour. As can be seen, 5-P-3OH levels were on average two fold lower during the period when sexual behaviour occurred compared to when it not occurred and the difference in levels was highly statistically significant (paired t test: $t = -10.182$, $p < 0.001$).

As indicated in Table 14, onset of sexual behaviour or mating was recorded for on average five days (range 4-9 days) before 5-P-3OH levels showed the defined postovulatory rise. Due to the time lag in steroid excretion into faeces of 2-3 days (see chapter III), ovulation therefore presumably occurred 2-3 days after sexual behaviour was first displayed.

The temporal relationship between first appearance of the highest score of vulval coloration changes and the occurrence of sexual behaviour is shown in Table 15. The data show that if the highest score of vulva change was only 2 (V2), the onset of this vulva appearance takes place

Figure 29. Box plot diagramme of 5-P-3OH values during periods with and without sexual behaviour. Values are from 15 cycles (two females).

Table 14. Temporal relationship between the occurrence of sexual behaviour and the rise of 5-P-3OH values during the oestrous cycle in Bina and Emi

Cycle #	Interval between I/M/M1 and Pregnanolone rise (days) in	
	BINA	EMI
1.	5	5
2.	6	6
3.	5	7
4.	5	
5.	8	
6.	5	
7.	9	
8.	4	
9.	8	
10.	6	
11.	5	
12.	5	
13.	5	
Median value		5

2.5 days before the first display of sexual behaviour. On the other hand, when a vulva score of 3or 4 was recorded, this occurred on average 1 day prior to oestrous behaviour. However, onset of V3 or V4 could vary from 2 days prior until 1 day after onset of sexual behaviour, although in only two cases, the vulval change reached its maximum score after sexual behaviour was first recorded.

The duration of vulval coloration changes varied for each stage. The stage of Vulval score 2 (V2) lasted on average for 2.6 days, with a range of 2-4 days (Figure 30). In contrast, stage V3 and V4 showed a mean duration of five days (range V3: 4-6 days, range V4: 4-7 days).

Table 15. Temporal relationship between onset of the highest score of vulval change and occurrence of sexual behaviour

No.	$V_{changes-2}$ (V2)	$V_{changes-3}$ (V3)	$V_{changes-4}$ (V4)
1.	-3	0	-1
2.	-2	-2	0
3.	-3	0	-1
4.	-2	-1	+1
5.		-1	-2
6.		-1	-1
7.		-2	0
8.		-1	
9.		-2	
10.		-1	
11.		-1	
12.		+1	
13.		-2	
Median	-2,5	-1	-1

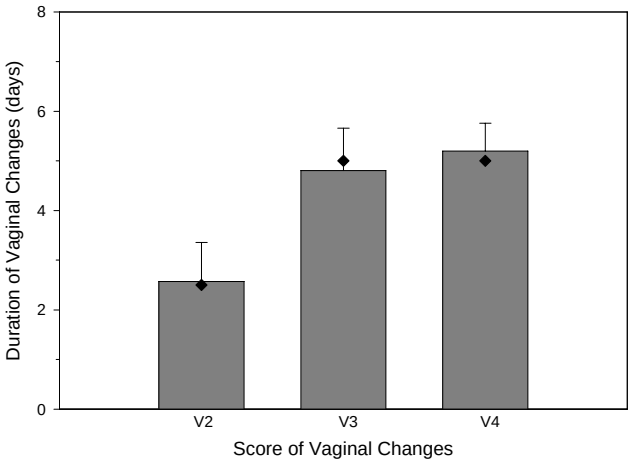


Figure 30. Duration of vulval coloration changes during the oestrous cycle (n= 17) in female BINA (Sumatran rhino sanctuary, Way Kambas National Park, Lampung Indonesia).

5-P-3OH values were still high (11.5 – 25.3 µg/g dry faeces) when vulval stage V2 was observed (Figure 31). In contrast, progestin concentrations were significantly lower when stage V3 and V4 were recorded (V3: range 4.4-13.6 µg/g dry faeces, and V4: range 5.9-10.3 µg/g dry faeces; RM ANOVA, $p< 0.001$, V2 vs V3: $p< 0.001$, V2 vs V4: $p= 0.003$, V3 vs V4: $p= 0.564$/n.s.). The average values of 5-P-3OH did not differ between V3 and V4.

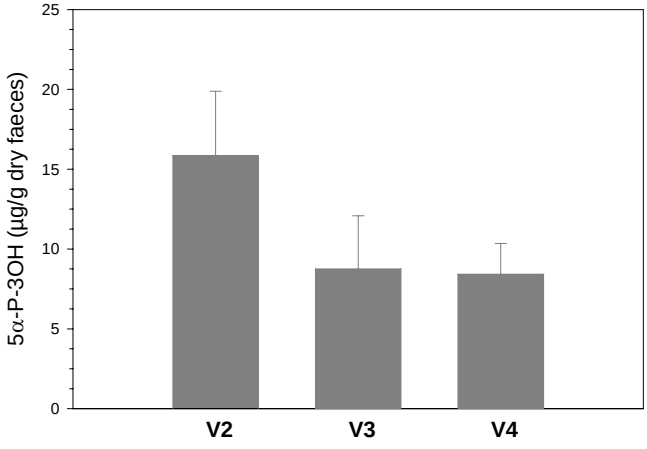


Figure 31. Relationship between 5-P-3OH values (n= 17) and vulval coloration changes during oestrous cycle in BINA (SRS Way Kambas National Park, Lampung Indonesia).

When animals were paired at a vulval score of 2 (V2), in about 50% (n=4) of cases, the animals did not show any sexual interest and in no occasion mounting or mating was observed. In contrast, when V3 and V4 was recorded, the animals were almost 100% interested by each other, and in many of the cases mounting or mating behaviour was observed. Particularly at vulval score 4, in more than 70% of pairings successful mating with intromission occurred and most of these mating were accompanied by ejaculation (see Figure 32 in detail).

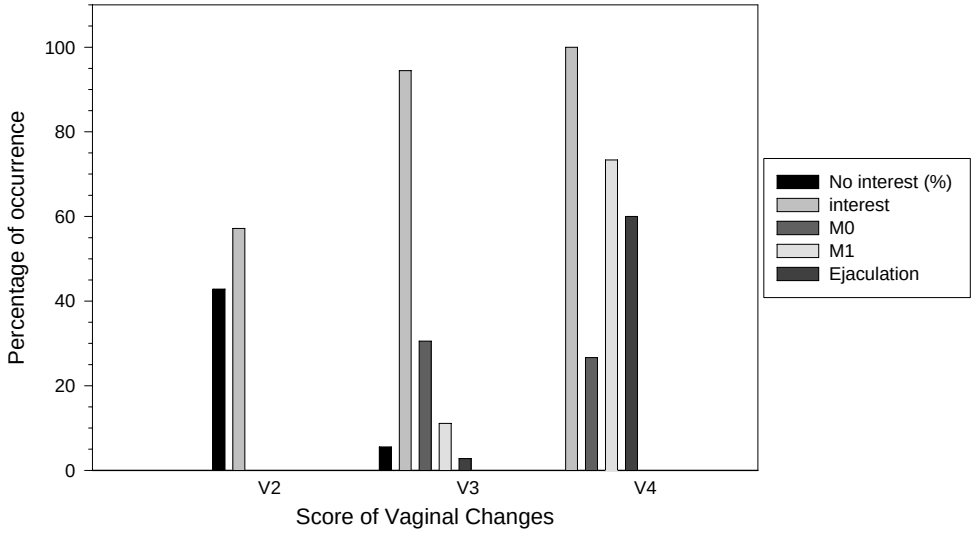


Figure 32. Relationship between vulval appearance and percentage of the occurrence of sexual behaviours during the observation period.

Discussion

The results clearly demonstrate that analysis of 5-P-3OH in faeces in the Sumatran rhinoceros can be used to characterize the major reproductive events in this species. Specifically, as in other species of rhino (African black rhino: Schwarzenberger *et al*. 1993; 1996b; African white rhino: Schwarzenberger & Walzer 1995; Schwarzenberger *et al*. 1998; Patton *et al*. 1999; Indian rhino: Schwarzenberger *et al*. 2000) and other mammals (farm animals: Schwarzenberger *et al*. 1996a; bitch: Möstl & Brunner 1997; maned wolves: Velloso *et al*. 1998; Asian and African elephant: Hodges 1998; Fieβ *et al.* 1999; Hanuman langur: Heistermann *et al*. 1995a; Ziegler *et al.* 2000; mare: Palme *et al*. 2001), the measurement of 20-oxo-pregnanes in faeces provides reliable information on ovarian activity, and enables to clearly distinguish follicular from luteal stages. Furthermore, the results showed that application of this technique can be used to detect pregnancy and to predict parturition, and provide information on reproductive disorder.

The study also shows that if urine can be collected regularly, it can provide additional information on ovarian function through the measurement of oestradiol, which revealed a cyclic pattern with a defined peak preceding the post-ovulatory faecal progestin rise by 5 days. The reliability of these non-invasive endocrine measures is clearly supported and biologically validated by the ultrasound findings. In this respect, the data showed that the presence of a dominant follicle was always associated with low faecal progestin levels and elevated urinary oestradiol concentrations, while the presence of a CL was always associated with high progestin levels. Thus, the events of follicular growth and formation of a corpus luteum appear to be well reflected in the excretory profiles of urinary oestradiol and faecal 5-P-3OH, indicating the value of these measures for assessing fertility status in the Sumatran rhinoceros.

Using these non-invasive endocrine measurements in the four study females enabled the generation of data on cycle length and its component follicular and luteal phase. Two-thirds of the oestrous cycles showed a duration between 18 and 25 days with a mean of 23-24 days. This finding is comparable to the 21 day cycle length reported by the study of Roth *et al.* (2001) in a single female based on both faecal progestin and serum progesterone measurements. Taking this as the normal cycle length, the Sumatran rhinoceroces thus exhibits the shortest cycle among all rhinoceros species. In the African black rhinoceros, an oestrous cycle length of 22-28 days was reported (see Hindle *et al.* 1992; Schwarzenberger *et al.* 1993; Brown *et al*. 2001; Radcliffe *et al.* 2001, and Garnier *et al.* 2002). On the other hand, Schwarzenberger *et al.* (1998) and Brown *et al*. (2001) reported for the African white rhinoceros two types of cycles, one with duration of about 4-10 weeks, the other with duration of about 10 or more weeks. This was confirmed by Patton *et al.* (1999) who found also cycles of about 5 weeks and 9 weeks in length in the same species. Cycle length in the Indian rhinoceros was reported to be 43-61 days (Kassam & Lasley 1981; Kasman *et al.* 1986; Schwarzenberger *et al*. 2000).

In the present study, five cycles in Bina were found to be extended due to a prolongation of the luteal phase. A similar situation was also reported for African black rhinoceros by Brown *et al*. (2001) and Garnier *et al*. (2002), where some cycles were characterized by luteal phases twice as long as in the majority of the other cycles. The reason for the extended luteal phases in Bina is not clear, but ultrasound findings indicated the presence of a persistent corpus luteum, suggesting that in these cycles difficulties in luteolysis occurred. Whether this also accounted for the prolonged cycles seen in other rhinoceros species (see above) is not known, but may provide an explanation.

Based on the defined changes in faecal progestin levels, this study provided the first information on the length of the follicular and luteal

phases of the oestrous cycle in the Sumatran rhinoceros. The results indicated a follicular and luteal phase length of on average 6 and 17 days, with the follicular phase being relatively consistent in length, while the luteal phase showed much higher variation, presumably due to the presence of persistent CLs in some cycles (see above). The length of the follicular phase in the Sumatran rhino is in the range of that of the African black rhinoceros for which a follicular phase length of 3-9 days (Hindle *et al.* 1992; Garnier *et al*. 2002) was reported. Compared to the Indian rhinoceros, for which a follicular phase of 13-19 days was recorded (see Kasman *et al*. 1986; Schwarzenberger *et al*. 2000; and Stoops *et al*. 2004), the follicular phase in the Sumatran rhinoceros is markedly shorter. The follicular phase length of 10-17 days in the African white rhinoceros is considerably more variable and longer than in the Sumatran rhinoceros, presumably due to the different types of oestrous cycle length recorded (Schwarzenberger *et al*. 1998; Patton *et al*. 1999). In terms of luteal phase length, the value of 17 days found for the Sumatran rhinoceros is in the range of that reported for the other rhino species (African black rhinoceros: 18 days, Hindle *et al*. 1992; Garnier *et al*. 2002; African white rhinoceros (type I): 17-25 days, Schwarzenberger *et al*. 1998; Patton *et al*. 1999; Indian rhinoceros: 17 – 21 days, Kasman *et al*. 1986; Stoops *et al.* 2004), provided that the luteal phase length of 55-59 days in the type II cycles of the African white rhinoceros (Schwarzenberger *et al*. 1998; Patton *et al*. 1999) indicated extended cycles. It thus appears, that in terms of component cycle phases, rhinoceros species are more similar with respect to luteal phase length than with respect to follicular phase length, the latter showing a marked interspecies variation.

A single pregnancy could be followed during the study from the female Sumatran rhinoceros held in Cincinnati Zoo. Based on 5-P-3OH values, the length of gestation in this female was 475 days, which fits to the information from mating and parturition date (Roth T.L. personal communication, Cincinnati Zoo) and from endocrine and ultrasonography

reports (Roth *et al.* 2004). The gestation period in the Sumatran rhinoceros is thus similar to that of other rhinoceros species, in which gestation also lasts for about 14-16 months (see Hindle *et al*. 1993; Schwarzenberger *et al*. 1993; Berkeley *et al*. 1997; and Garnier *et al*., 1998 for African black rhinoceros; Hindle *et al*. 1993; Schwarzenberger *et al*. 1998; and Patton *et al*. 1999 for the African white rhinoceros; Kasman *et al*. 1986; Hodges & Green 1989; and Schwarzenberger *et al*. 2000 for the Indian rhinoceros). During pregnancy, the concentration of faecal 5α-P-3OH increased steeply from day 60 onwards to reach maximum levels which were 10-20 folds higher than luteal phase concentrations by about six months of gestation. This finding is inline with Roth *et al.* (2004) who reports that serum progesterone concentrations remain at luteal levels during the first 60 days of gestation and then start to gradually increase to reach maximum levels around day 210. A similar pattern in progestin excretion during pregnancy has been described for the African black rhinoceros (Hindle *et al*. 1993; Schwarzenberger *et al*. 1993; Berkeley *et al*. 1997), African white rhinoceros (Schawrzenberger *et al*. 1998; *Patton et al*. 1999), and Indian rhinoceros (Kasman *et al*. 1986; Schwarzenberger *et al*. 2000) indicating that gestation in rhinos is generally characterized by a marked production of progesterone, although the present finding for the Sumatran rhinoceros has to be confirmed in the future. In the present Sumatran rhino pregnancy, 5-P-3OH decreased rapidly three days prior to parturition, and within 10 days post partum had reached follicular phase values. In contrast, a rapid decrease prior to birth was not found in the Southern white rhinoceros by Patton *et al*. 1999. However, Kasman *et al.* (1986) and Schwarzenberger *et al.* (1993) reported a similar rapid pre-parturition decline in progestin levels in the African black rhinoceros and Indian rhinoceros, respectively.

The data on the Sumatran rhinoceros show that pregnancy can presumably be diagnosed earlier (after about two months) compared to other rhinoceros species in which the marked progestin rise did not occur

before the 3rd - 5th month of gestation (African white rhinoceros: Schwarzenberger *et al*. 1998; Patton *et al*. 1999; African black rhinoceros: Schwarzenberger *et al*. 1993; Berkeley *et al*. 1997; Brown *et al*. 2001; Indian rhinoceros: Kasman *et al*. 1986; Schwarzenberger *et al*. 2000). In practical terms, the marked increase in faecal progestin levels allows to diagnose pregnancy using a single sample in the Sumatran rhinoceros as already described in other species (elk: Garrot *et al*. 1998; tule elk: Stoops *et al*, 1999; bighorn sheep: Schoenecker *et al*. 2004). Furthermore, the rapid decline in progestin levels some days before birth may be of predictive value in providing information on parturition time. However, since data are limited to one pregnancy, this has to be confirmed.

The present study is the first to report on the characteristics of sexual behaviour and courtship display and its temporal relationship with endocrine patterns in the Sumatran rhinoceros. The data clearly show that the occurrence of sexual behaviour is restricted to one or two days and is associated with low progestin levels and elevated oestrogen concentrations. These results are similar to findings in other rhino species (Patton *et al*. 1999; Schwarzenberger *et al.* 2000; Stoops *et al*. 2004) and other mammals (Blanvillain *et al*. 1997; Hodges 1998; Morrow & Monfort 1998; Fieß *et al*. 1999; Asa *et al*. 2001; Heistermann *et al*. 2001; Pickard *et al*. 2001; Ishikawa *et al*. 2002) suggesting that oestrous behaviour in the Sumatran rhinoceros is also highly dependent on the endocrine milieu, with oestrogen presumably facilitating and progesterone presumably inhibiting the expression of sexual activity. As shown in the Indian rhinoceros (Stoops *et al*. 2004), the Sumatran rhinoceros predominantly displayed sexual behaviour on two consecutive days. However, mating was usually observed to occur only on one day, either in the first or the second day when oestrous was displayed, and in only a few cases (20%, two occasions out of ten) mating was observed on both days, suggesting that there is usually only one day when the female is receptive.

Although the temporal relationship between occurrence of sexual behaviour and timing of ovulation varied to some extent, on average, first oestrous signs preceded ovulation by 2-3 days. This finding agrees well with those previously reported by Roth *et al*. (2001) for the female Emi and is also in line with findings in other rhino species in which ovulation was found to occur within 48-72 hours after observed oestrous (African black rhino: Radcliffe *et al*. 2001; Indian rhino: Stoops *et al*. 2004), although ovulation was observed within 24 hours in the African white rhinoceros (Radcliffe *et al*. 1997). The data suggest that daily monitoring of the incidence of sexual interest between the sexes may thus have a practical value in terms of providing rough information for predicting the timing of ovulation in the Sumatran rhinoceros and presumably also other rhinoceros.

Another important finding of the study was that vulval appearance clearly changed throughout the oestrous cycle with intense coloration, maximum turgidity and discharge occurring during the late follicular phase in association with low progestin level and elevated oestrogen concentrations. As inferred from studies in domestic animals (Hafez 2000), vulval coloration changes are presumably induced by oestrogen levels during oestrous, which lead to an increase in blood flow in the reproductive organ. Since occurrence of the most intense vulval appearance (score 3 or 4) usually preceded the onset of oestrous behaviour by 1 or 2 days, it may be used as an early indicator of forthcoming sexual interest between animals and the likelihood that ovulation will occur. In this respect, given the close relationship between the degree of vulval appearance and the intensity of oestrous behaviour, it appears that monitoring daily changes in vulval coloration and turgidity can provide a useful practical tool to predict the most appropriate time for pairing animals. By this, the risk that violent behaviour may occur during pairing might be substantially reduced. Since scoring of vulva appearance is, however, subjective by nature and since changes in vulval coloration or

turgidity are likely to differ between females, experience and training is necessary to ensure correct interpretation of the morphological signs. Nevertheless, as shown in this study, this is possible. In contrast to hormone measurements which, particularly when carried out in faeces, can provide only retrospective information on female reproductive status, monitoring of changes in vulval appearance and incidence of sexual interest is of predictive value in this respect. The results reported here are therefore presumably of significant practical value in assessing female fertility status and in improving the breeding management of the Sumatran rhinoceros in captivity, particularly when endocrine methodologies and ultrasound tools are unavailable.

Conclusions

1. Measurement of 5-P-3OH immunoreactivity in faeces (and E2 in urine) enables non-invasive monitoring of the oestrous cycle and pregnancy in the Sumatran rhinoceros.
2. The length of the oestrous cycle in the Sumatran rhinoceros is about 23-24 days, with a follicular phase of on average 6 days and a luteal phase of about 17 days.
3. Gestation length from single female the Sumatran rhinoceros was 475 days, and early pregnancy can be reliably diagnosed by a single faecal progestin measurement after about 60 days of gestation.
4. Ultrasound examinations of ovaries and observation on changes in vulval appearance provide useful information on female reproductive status which can be used as a tool for monitoring the oestrous cycle when endocrine measurement is not possible.
5. Monitoring daily changes in vulval appearance and occurrence of initial stages of sexual behaviour are useful to determine a proper

timing for pairing since intensive vulval coloration and interest between the sexes usually occurs a few days prior to ovulation.

6. These data provide valuable information to characterize important reproductive events in the Sumatran rhinocesros and should be useful for improving captive breeding efforts for the most critically endangered rhinoceros species.

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ASSESSMENT OF FERTILITY STATUS IN THE MALE SUMATRAN RHINO

Introduction

Despite many successful mating taken place since 2002, no pregnancy occurred in the female housed in SRS Way Kambas. Since the female showed a regular pattern of ovarian cyclicity, approved by hormone analysis and ultrasound examinations (see chapter IV), the fertility of the male Sumatran rhinoceros in this facility (Torgamba) has been questioned, and thought to represent a major factor contributing to the failure of the breeding program in SRS. Clinical examination of reproductive organs and assessment of semen quality have historically been used to predict potential fertilizing capability of stallion (Colenbrander *et al*. 1992; 2003). Application of this approach may also provide important information on male fertility status in the Sumatran rhinoceros.

In order to analyze semen quality of the male Torgamba, the development of a reliable semen collection method is required, since, to date, no such methods are available for the Sumatran rhinoceros. Semen collection has, however, become a standard practice for fertility assessment and assisted reproduction procedures in many domestic animal breeding programs (Penny 2005).

Therefore, this research was conducted with the objectives (1) to establish a reliable semen collection method for the Sumatran rhinoceros, (2) to assess semen parameters of the fresh ejaculates, and (3) to determine Torgamba's reproductive potency.

Material and Methods

Animal and housing conditions

For the study, only one male Sumatran rhino, Torgamba, a ~ 22 year old animal kept at the Sumatran Rhino Sanctuary (SRS), Way

Kambas National Park, Indonesia, was available. Torgamba has been moved from Port Lympne Zoo, England to SRS Way Kambas in early 1998. Generally, Torgamba is kept separately from the female rhino, Bina, but is allowed to have access to the female during times when Bina is showing oestrous signs and when there is an interest for mating (see Chapter IV). Semen collection procedures were conducted in an observation cage (6m x 6m), which is adjacent to the browsing enclosure (10 ha) area separated by a door and bars. Entry to or escape from the rhino was possible from along the side of the observation cage between the bars. Because Torgamba was very tolerant to new objects and personnel in his cage, semen was collected without physical restraint or sedation, except when eletroejaculation procedures were carried out. The study was conducted for two years from 2002 till 2004.

Semen collection methods

The study aimed to compare several combinations of semen collection techniques that might be applicable to the Sumatran rhinoceros. The first approach was to combine accessory gland massage (AGM), penile massage (PM) and use of an artificial vagina (AV); the second method combined only PM and AV, and the third was PM only. In addition, electroejaculation was performed, without combining it with any other technique.

Penile stimulation

Penile stimulation was conducted using penile massage and/or artificial vagina methods. The method was carried out according to the protocol described by Schaffer *et al*. (1990) for the Indian rhinoceros.

Penile massage (PM)

A penile massage was carried out with two operators kneeling at the rear of the male rhino and massaging the penis. Rubbing the medial

side of the rear legs and the penile sheath always induced let-down of the penis from the sheath, allowing a direct massage. After the penis was released from the sheath, it was first washed with warm water (30^{O}C-35^{O}C). Silicone gel (K-Y, Johnson & Johnson, New Brunswick, NJ) was then smeared on the distal of the penis, but the penile tip was kept free of lubricant. Complete erection was induced by rubbing back and forth on the top and bottom of this area with one hand. Gentle rubbing would become more vigorous as the penis became erect. With erection, the curve of the penis straightened, swinging the tip of the penis forward. A waterspade (dry and clean) was held down and in front of the tip of the penis to collect the ejaculate (Figure 33). Forceful ejaculations of seminal fluid occurred as the penis became fully erect. Shaking of the hind legs and thrusting of penis often occurred. Another operator would help support the penis as it became fully erect. The waterspade was held closed to the tip at all times during stimulation since semen could squirt or dripple out at any time. After forceful ejaculation, the penis relaxes and the animal remains in the cage for further manipulation. If ejaculation did not occur when the penis was fully erected, the penis was allowed to relax. The period to induce erection and ejaculation lasted from 20-45 minutes. The process was repeated until the rhino would no longer stand still, which usually occurred after two hours.

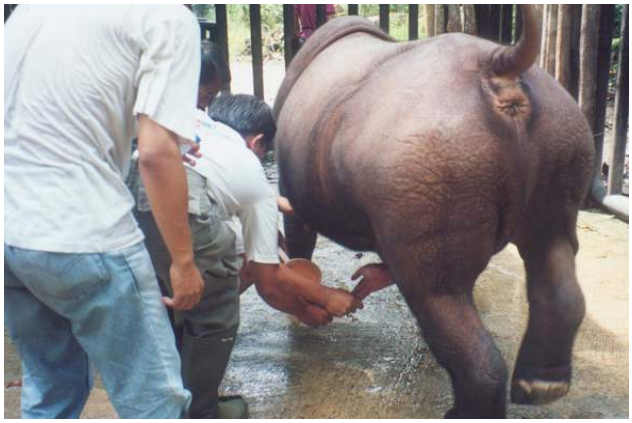


Figure 33. Penile massage and semen collection from the Sumatran rhinoceros.

Artificial vagina

A modified AV was provided and donated by N.E Schaffer (through SOS rhino) based on her experiences using the AV in the Indian rhinoceros. A 15 cm diameter latex liner was doubled over and vulcanized together with a 15 diameter rubber ring. The other end of the folder liner was also vulcanized close, except for an opening that could be stretched onto a 50 cc centrifuge tube (Figure 34). This AV was constructed to only cover the distal part (15-20 cm) of the penis. The cavity between both latex liners was then approximately half-filled with warm water (~40^{o}C-45^{o}C). Lubricant was then applied to the interior of the AV. Inducing a fully erect penis by penile massage was required before the AV could be fitted (Figure 35).

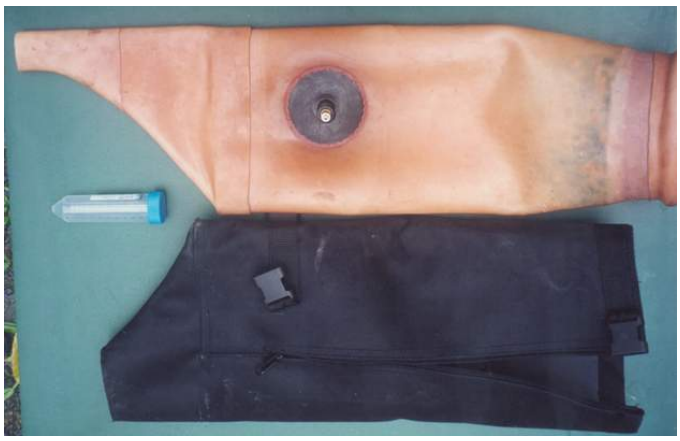


Figure 34. Water-filled latex AV for stimulation of distal tip of the penis

Rectal Stimulation

Stimulation inside the rectum was conducted according to the protocol described by Schaffer *et al*. (1990). Rectal stimulation was induced by either accessory glands massage or by an electrical probe.

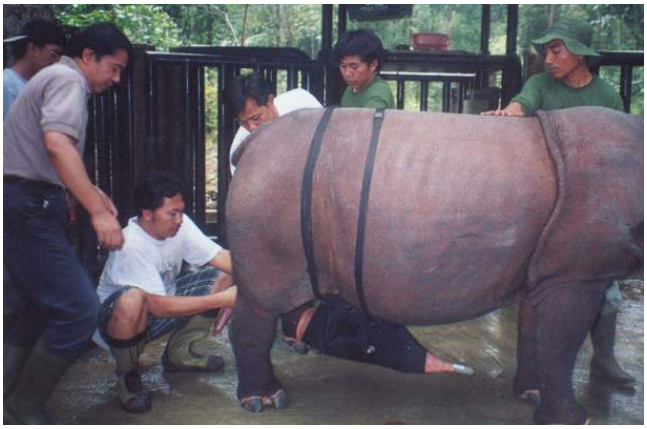


Figure 35. Penile massage was conducted to induce a fully erect penis before the AV could be fitted

Accessory glands massage

For massage of the accessory glands, an operator inserted a plastic-sleeved, lubricated hand through the anal sphincter and then manually massaged the accessory glands through the mucosa of the caudal rectum. The prostate and bulbourethral glands, which were about 10-15 cm proximally to the anal sphincter, were massage with rhythmic side-to-side downward pressure. Massage proceeded in 5 minute intervals with a 1-2 minute resting time between intervals. Duration of the whole procedures did not exceed 30 minutes.

Electroejaculation

Electroejaculation was conducted according to the protocol described by Roth (person. communication) and carried out under her supervision. The rhinoceros rectal probe (specifically designed for rhinoceros) and the electroejaculator (P-T Electronics, 11241 SE362nd, Boring, Oregon 97009, USA) were provided by T.L. Roth (through International Rhino Foundation/IRF, Figure 36).

The rhino was fasted 12 h prior to anesthesia preceding the electroejaculation process. Water was withdrawn from the cage in the morning of the procedure. The rhino was immobilized according to the anesthesia protocol described by Radcliffe *et al*. (2000) using a cocktail of

butorphanol (Tobugesic, Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA: 10 mg/ml, dosage: 50 mg/200 kg body weight) and azaperone (Stresnil, Janssen Pharmaceutical, Mississauga, Ontario L5N 5R9, Canada: 40 mg/ml, dosage: 100 mg/200 kg body weight). Immediately after electroejaculation was finished, the rhinoceros was reversed with antidote 10 mg naloxone (Narcan, DuPont Pharmaceuticals, Manati, Puerto Rico 007001; 1 mg/ml).

As soon as the rhinoceros was sedated, ropes were placed around the rear legs and fixed securely around the bars of the enclosure (Figure 37). Faeces were manually removed from the entire distal rectum. The penis was released from the sheath and cleaned with warm water (~30^{O}C-35^{O}C) and a towel.

Prior to use, the surface of each electrode of the probe required sanding to remove oxidation. Following each use, the properly sealed probe was immersed in a bath of disinfectant cleaner. Once inserted, the handle of the probe was lifted, forcing the head of the probe down within the rectum and the electrodes made contact with the rectal lining just over the reproductive accessory glands. Electrical stimulation was administered in several series of increasing voltage (+ 1 Volt each increase) with rest intervals of up to 5 minutes between each series. Each series consisted of 5-15 stimulations during which voltage was stepwise increased in three voltage ranges. Once the male started producing the fluid, the collection cups were exchanged frequently to avoid potential contamination of a good-quality fraction with one of poorer quality.

The position of the probe was changed frequently by shifting it slightly left, right, cranial, or caudal during the procedure in order to find the best results of penile stimulation and ejaculate production. Maintaining the probe in proper position with good rectal contact was often difficult, especially when the animal's muscles contacted hardly due to an increase of higher voltages.

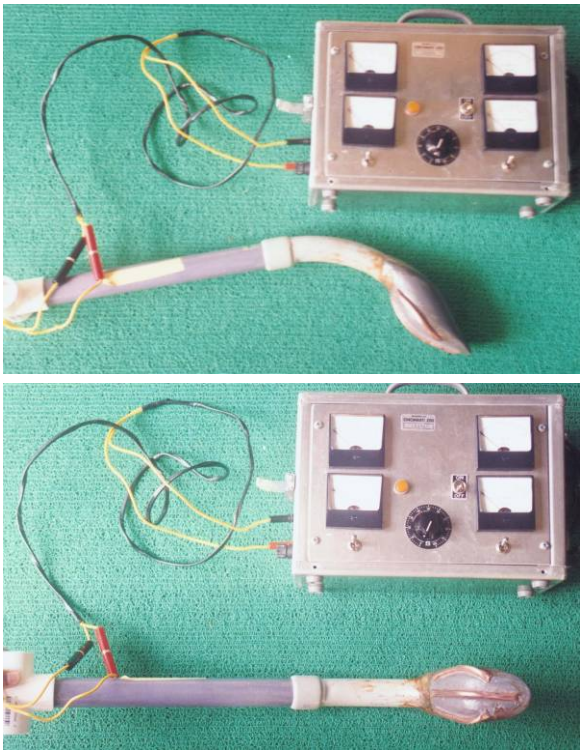


Figure 36. Electroejaculator and the probe for elctroejaculation procedure to collect semen in the Sumatran rhinoceros

Figure 37. Elctroejaculation procedure required a number of people, including one handling the probe, running the ejaculator, massaging the penis, collecting the samples, and delivering sample collection cups. The hind legs were roped to secure all operators.

Sample processing and evaluation

Semen in sample tubes were immediately protected from direct sunlight and analyzed in the field laboratory at SRS. Semen assessment included assessment of total volume, pH, sperm concentration, total sperm number, sperm motility and morphology. Specifically, sperm concentration was assessed in a 10 µl aliquot of the ejaculate using a haemocytometer (Neubauer). For sperm motility and morphology an aliquot (10 µl) from each sample was diluted 1:10 in pre-warmed (~35^{O}C) deionize water and evaluated for percent sperm move forward progression using a microscope equipped with a warm stage (Olympus, Japan). Ejaculate fractions were evaluated for pH by assessing the color change after adding 10 µl of samples to an indicator strip (Neutralit pH 5-10, Merck, Darmstadt, Germany).

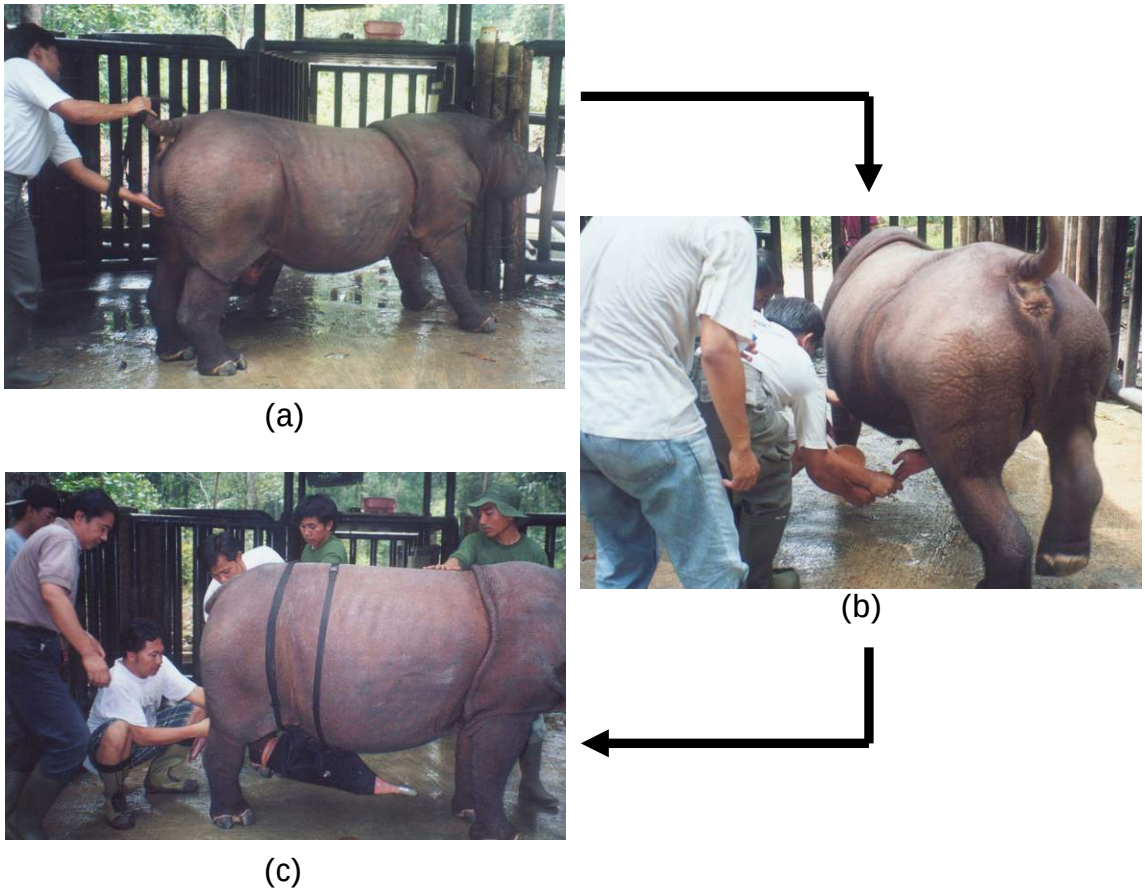
Sperm morphology was assessed under microscope from native smear and/or from stained smear using dip-quick staining procedure. Native smear was prepared using 10 µl of ejaculates and mixed with 10 µl of dionized water prior to the assessment. Dip-quick staining was used a commercial reagent, which has two different reagents (reagent #1, and reagent #2) for two steps of staining. Each step of staining procedure lasted for 2 minutes. A 10 µl aliquot of ejaculate was smeared on the object glass, and then dried on the air for 10 minutes prior to staining procedure. Native smear was observed under bright field microscopy at 400x magnification, while dip-quick slide was investigated at 1000x magnification. Sperm morphology was characterized as being normal or abnormal sperm. Abnormalities included defects of the head (micro- and macrochepalic), detachment of the tails and heads from the mid-piece flagellum, or the occurrence of cytoplasmic droplets.

Results

A two months training of the rhino was required to develop the various procedures and for habituation of the animals to the protocols. A total of 10 ejaculates were collected from 16 times the procedures were attempted, giving an overall success rate of about 62.5%.

There were, however, clear differences in the success rates between the different collection procedures applied (Table 16). In this respect, the combination of AGM, PM, and AV yielded the best results with 85.7% (6/7, n=7) of the attempts resulting in an ejaculate. The other collection methods were less successful, in that the combination of PM and AV yielded in only 50% of cases (2/4, n=4) an ejaculate, while stimulation by PM only resulted in only 25% of attempts (1/4, n=4) in an ejaculate. In all semen collection attempts using a combination of accessory gland massage, penile massage and artificial vagina, the rhino behaved very tolerant. This procedure basically needed several people to be involved. As shown in Figure 38, the procedure started by massaging the accessory glands until the penis drops from the sheath; soon after, another operator started to massage the penis until it was fully erect. It was then immediately inserted into the artificial vagina which was hold by another operator while the rhino was thrusting (Figure 38).

The ejaculate volumes obtained by the different collection procedures ranged between 1.2 - 34 ml (Figure 39). From the 10 ejaculates obtained, only four, however, contained spermatozoa. In each of these ejaculates, sperm concentration was very low ranging from 143 x 10^3 – 333 x 10^3 spermatozoa/ml ejaculate. In the ejaculates obtained by the procedures of PM only and electroejaculation, no sperm was found at all, although, the electroejaculation procedure produced the highest volume of ejaculate (34 ml).



(a)

(b)

(c)

Figure 38. A sequence of semen collection using a combination of (a) accessory glands massage, (b) penile massage, and (c) artificial vagina. By this procedure in 86% of attempts an ejaculate was obtained.

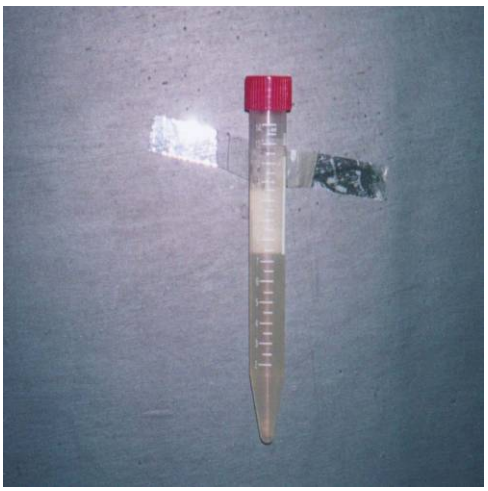


Figure 39. Semen collected from the Sumatran rhinoceros at SRS Way Kambas, Lampung, Indonesia.

Most of the sperms (85%) contained a proximal cytoplasmic droplet (Figure 40a and 42b), and some sperms (15%) were found with abnormal head so called, macro- and microcephalic, and detached tail (broken tail) (Figure 41). However, the presence of a proximal cytoplasmic droplet decreased gradually from 85% in the first ejaculate obtained to 5% at the last ejaculate collected about one year later.

In terms of sperm motility, all samples showed a very low degree of motile sperm (< 10%), and most of the sperms were daed. From these results, there were presumably only one percent of sperm that showed a progressive move forward.

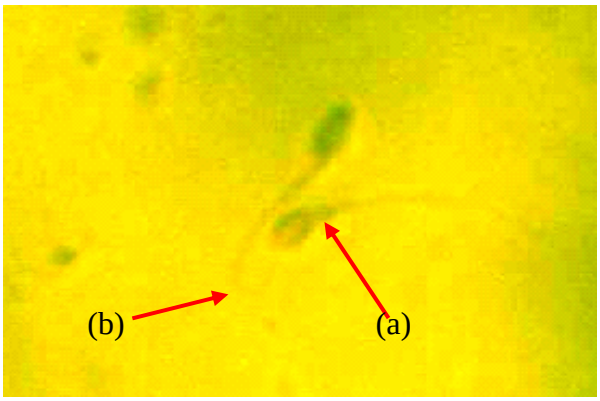


Figure 40. (a) sperm with a proximal cytoplasmic droplet, (b) sperm with bent tail (from native slide) (40x10)

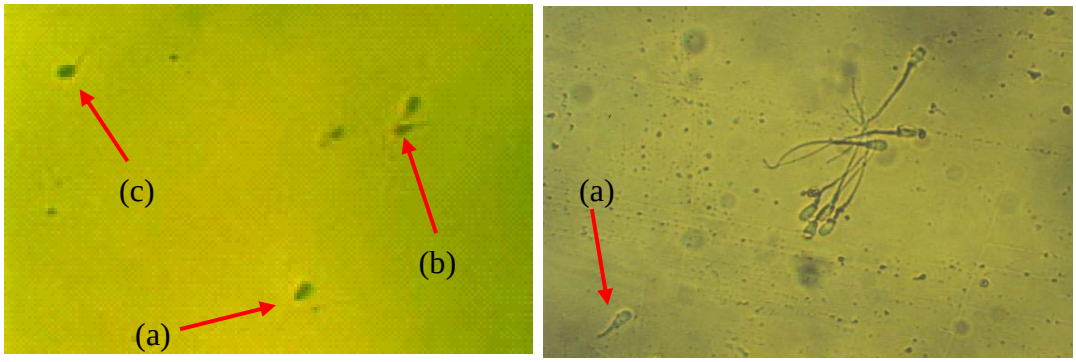


Figure 41. (a) sperm with detached tail in several forms, (b) microchepalic, (c) macrocephalic (from native smear) (10x10)

Electroejaculation produced an ejaculate with pinkish color, which was different from the creamy turbid ejaculates obtained by the other procedures. This sample was contaminated by red blood cells, as indicated by microscopic assessment. The pH value (6.9-7) was similar between the "non-invasive" semen collection procedures, while electroejaculation samples had a markedly higher pH of about 8.7.

(A)

(B)

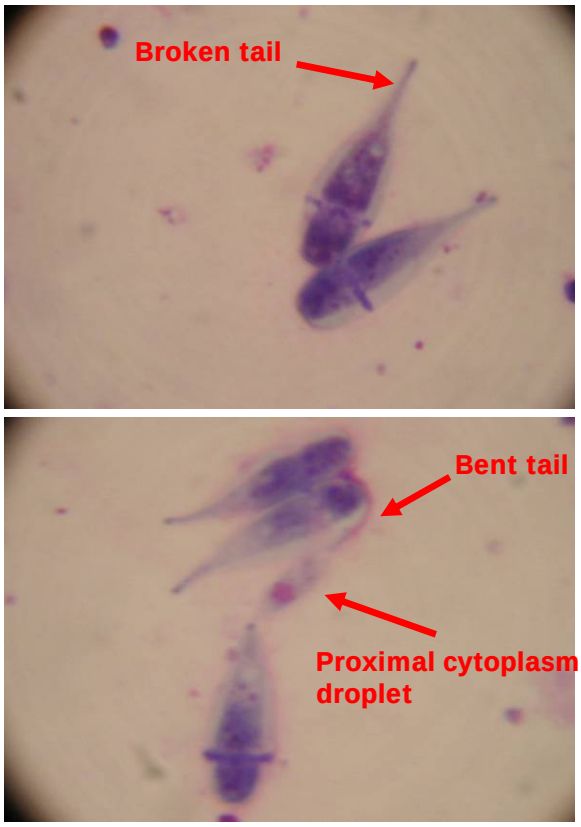


Figure 42. (A) spermatozoa with detached tail, (B) spermatozoa with proximal cytoplasmic droplet and bent flagellum (stained smear using dip-quick procedure, 100x10) (doc. SRS Way Kambas)

Table 16. Seminal parameters (range) for various collection methods from the Sumatran rhinoceros

Method	Σ ejaculates	Volume (ml)	pH	Color	Sperm conc. (x 10^3/ml)	Motility (%)	Abnormality (%)
AGM, PM and AV (n=7)	6	1.2-12.4	6.9-7.0	Whitish turbid	0-167	1	30-80
PM and AV (n=4)	2	1.5-2.1	6.9-7.0	Cream turbid	143-333	1	5-30
PM (n=4)	1	1.5	6.9	Whitish turbid	0	n/c	n/c
Electroejaculation (n=1)	1	34	8.7	Pink	0	n/c	n/c

n/c = data not collected

Discussion

Although only a limited number of ejaculates could be obtained, the study provided the first data on the characterization of fresh ejaculates obtained from varying semen collection methods in the Sumatran rhinoceros. More importantly, it provided the first comparative information on the value of different stimulation protocols for collecting semen in this species. Single stimulation using rectal massage (accessory glands massage, AGM) only was not used in the present study because Schaffer *et al*. (1990) reported that although this procedure could produce seminal emission, the stimulation was not sufficient to cause ejaculation in the Indian rhino. In line with this, for the beef bull, Palmer *et al*. (2004; 2005) reported that the rectal massage showed an inability to stimulate erection and contributed to poor semen sample quality. Although, it thus appears that AGM alone is not an effective method for stimulating ejaculation, many researchers used it as a priming procedure in combination with other stimulation techniques in wild animals, e.g. penile massage in Ceylon elephant (Jainudeen *et al*. 1971), or AV in Asiatic elephant (Heath *et al*. 1983).

In the present study, three "non-invasive" semen collection methods differing in stimulation (penile massage, PM; PM in combination with an artificial vagina, AV; and PM in combination with accessory gland massage, AGM and AV) and the invasive procedure of electroejaculation were tested for their usefulness in obtaining ejaculates in the Sumatran rhinoceros. The results indicated that the success rate in terms of obtaining an ejaculate progressively increased with the intensity of the stimulation, with the highest success rate (86%) being found when all three stimulation procedures (AGM, PM, AV) were combined. The combination of AGM, PM and AV resulted also in generally higher ejaculate volumes. The combination of these techniques was also reported to work well in terms of obtaining proper ejaculates in elephant (Heath *et al*.

1983), and domestic animals (Hafez 2000; Palmer *et al*. 2005). In contrast, Schaffer *et al*. (1990) reported that the AV procedure was not able to provide a proper ejaculate, in the Indian rhinoceros since sperm concentrations in the samples using AV were low. Similarly to this, sperm concentration was also generally lower in ejaculates obtained in the present study using AGM compared to when only PM in combination with AV was used. Since, furthermore, the proportion of abnormal sperm was lower in ejaculates obtained without AGM, these preliminary data may suggest that although success rate and ejaculate volumes are lower using PM and AV only, the ejaculates obtained by this procedure might be of better quality. In line with this result, Palmer *et al*. (2005) reported that the semen collected by AGM in beef bull may have contained more residual (dead) sperm and contributed to poor semen sample quality. The combination of AGM, PM and AV produced better results in terms of success rate of the ejaculation and the ejaculate volume, suggesting that the use of AV presumably increased stimulation of the animal to produce an ejaculate. More trials with other male Sumatran rhinoceros are, however, needed to investigate this further.

Electroejaculation is the most commonly used method for collecting semen from domestic species (Roth *et al*. 2005). However, Schaffer *et al*. (1990) reported that the method does not produce proper ejaculates in the rhinoceros that can be used for cryobanking or assisted reproduction. Nevertheless, in a recent report, Hermes *et al*. (2005) indicated that use of a specially designed rectal probe during electroejaculation can improve the effectiveness of semen collection in the African white rhinoceros species and this was confirmed by a study of Roth *et al*. (2005) in the African black, African white and Indian rhinoceros. In particular, the electroejaculator technique stimulates an optimal erection and ejaculation process, and, due to high stimulation of semen plasma secretion (Hafez 2000) the technique usually results in the production of higher ejaculate volumes compared to other techniques. The latter was also found in the

present study, however, despite a 3-20 fold higher ejaculate volume, no single sperm was found in the sample obtained by electroejaculation. In comparison to studies in other rhinos, it thus appears that the procedure of electroejaculation is presumably of only limited value for collecting semen in the Sumatran rhinoceros, although the present data are extremely limited and therefore more studies are required to clarify whether this is really the case.

The results showed that the male Sumatran rhinoceros at SRS produced very low ejaculate volumes (1.5-34 ml semen/ejaculate) and very low sperm concentrations (133-333 x 10^3 spermatozoa/ml ejaculate). In contrast, in the other rhinoceros species ejaculate volumes of 20 ml to 200 ml, and sperm concentrations of about 1.8-75.8 x 10^6 spermatozoa/ml ejaculate have been reported using different semen collection methods (see Schaffer *et al*. 1990; Hermes *et al*. 2005; Roth *et al*. 2005). Similarly, the present results for Torgamba are also different in comparison to those of the other male Sumatran rhinoceros housed in Cincinnati Zoo, for which an ejaculate recovered post-coitus was shown to be as large as 104 ml and containing a sperm concentration of 25 x 10^6 spermatozoa/ml (O'Brien & Roth 2000). Moreover, ejaculate volume and sperm concentration in Torgamba were also lower compared to the related horse, which can produce ejaculate volumes of about 20-100 ml and sperm concentrations of 120-300 x 10^6 spermatozoa/ml ejaculate (Janett *et al*. 2003a; 2003b; Turner & McDonnell 2003). It thus appears that the male Sumatran rhinoceros at SRS suffers presumably from an abnormality in semen production, so called oligospermia and oligozoospermia (Hafez 2000; Turner *et al*. 2003).

Moreover, analysis of sperm motility and morphology indicated that the male appeared to produce a high proportion of sperm less motile and dead sperm as weel as with primary abnormalities (a cytoplasmic droplet

and abnormal head), and small numbers with secondary abnormalities (detached tail). Interestingly, the abnormality of sperm decreased from 85% at the beginning of the study to 5% after one year. This may indicate that with time and repeated application of procedures, a noticeable improvement in sperm quality (reduced degree of abnormality) in the Sumatran rhino might be achieved, a finding also reported by Schaffer *et al*. (1990) for the Indian rhinoceros. Most of the abnormal sperm had *a proximal cytoplasmic droplet*, indicating that the sperm that was ejaculated, was immature (Hafez 2000, Pesch & Bergmann 2006). The presence of high amounts of immature sperm has also been described in other rhino species (Schaffer *et al*. 1990; O'Brien & Roth 2000). Cytoplasmic droplets, are the most common defect at the neck region, but they can also be found at the midpiece and the principal piece of the tail. They represent a failure of maturation, because normally the residual cytoplasm is released down the tail during spermiogenesis. Proximal droplets are thought to have a great impact on fertility and therefore are classified as major defects (Pesch & Bergmann 2006). Similarly, the defects found in the sperm head (i.e., micro- and macrocephalic) were presumably associated with abnormal spermiogenesis, furthermore indicating sub- or even infertility as often reported in stallions (Pesch *et al*. 2005) and ram (Ott *et al*. 1982). The reasons for this phenomenon are thought to be various, e.g., prolonged sexual rest (Blom 1945 in Pesch & Bergmann 2006), cell death (Austin & Bishop 1958), and the fixation for morphological evaluation (Hurtgen & Johnson 1982). For Torgamba, it is likely that the first explanation applies because the rhino has not mated since he was young when captured until at the age ~22 years when the first ejaculate was observed in 2002 during his first natural mating.

Collectively, the data provided strong evidence that the male Sumatran rhino in SRS has possibly a low fertility capacity and this would clearly explain the lack of successful breeding given that the female (Bina) appeared to have normal ovarian function (see chapter IV). It is

important to do further fertility assessment in order to identify the causes of the infertility in Torgamba and to estimate the chances that he can be used in a breeding program.

Conclusion

The following conclusions can be drawn from this study:

1. The study has provided the first data on the characterization of the fresh ejaculates obtaining from artificial semen collection methods
2. A combination of AGM, PM and AV yielded a higher success rate in stimulating ejaculate compared to the other collection methods, however semen quality appeared to be better when PM and AV without AGM was used.
3. In comparison to other rhino species using the same semen collection methods, the volume of ejaculates and sperm concentration were very low
4. The results indicate that the male Torgamba presumably has a low fertilization capacity as a result of low sperm concentrations, so called *oligozoospermia* and low ejaculate volume, so called *oligospermia*.

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GENERAL DISCUSSION AND CONCLUSIONS

Discussion

It is clear that the Sumatran rhinoceros is a critically endangered species, with probably fewer than 300 individuals left in the wild, and a declining rate which is very high. Moreover, very few animals have been kept in captivity, and of those which have, only one pair has bred successfully. Unknown fertility potency and limited knowledge of the reproductive physiology of the Sumatran rhinoceros are probably the major factors contributing to the poor breeding success of the species in captive breeding programs. The present study was undertaken to generate new and important information on aspects of both female and male reproductive physiology which should, on the one hand, help to improve our general knowledge of the species biology and, on the other hand, provide a basis to facilitate improved captive breeding in order to propagate the species. Since there were no reliable methods available for monitoring reproductive status non-invasively in the Sumatran rhinoceros, the study emphasis has been put on the establishment of non-invasive methods for evaluation of female and male fertility through characterizing reproductive status in the females using urinary and faecal hormone analysis, and evaluation of male fertility based on semen collection and assessment.

Basic reproductive studies, using non-invasive hormone and semen analysis, have been carried out in the rhinoceros genera to broaden our knowledge of species specific reproductive physiology and help to improve captive breeding success since the early 1980's (African black rhino: Platz *et al*. 1979; Ramsay *et al*. 1987; Brett *et al*. 1989; Hindle *et al*. 1992; African white rhino: Hodges & Hindle 1989; Indian rhino: Kassam & Lasley 1981; Kasman *et al*. 1986; Hodges & Green 1989; Schaffer *et al*. 1990). However, since correct interpretation of hormonal data requires at least

some knowledge of the physiology of the species in question, monitoring methods based on hormonal analysis first need to provide the basic physiological information (hormone metabolism, patterns of secretion and excretion) on which their subsequent application depends (Hodges *et al*., in press). In that respect, species variation in the types and levels of hormones secreted and/or excreted make extrapolation of results from one species to another difficult and potentially misleading (see Hodges & Green 1989; Hodges 1998; Brown 2006; Hodges *et al*. in press). One major part of this study was therefore to examine the metabolism and excretion pattern of the reproductive hormones in the Sumatran rhinoceros. As shown in chapter III, the Sumatran rhinoceros indeed metabolizes and excretes oestradiol and progesterone as different compounds and through a different route compared to the other rhinoceros species. Interestingly, different to the other rhino species, the Sumatran rhinoceros excretes progesterone metabolites almost exclusively into faeces, whereas oestradiol metabolites are excreted predominantly into urine, indicating that different to other rhino species measurement of progestins in urine are not useful to monitor female reproductive status. The Sumatran rhinoceros excretes progesterone metabolites predominantly as pregnanediols, and pregnanolones, with measurement of the latter, as shown in chapter IV, enabling a reliable assessment of ovarian function. On the other hand, oestradiol 17β is the major metabolite in urine whereas oestrone is predominating in faeces. In practical terms, since urine samples are usually difficult to collect, monitoring reproductive status in the Sumatran rhinoceros should preferably rely on faecal progestin analysis. However, if urine samples are available, urinary hormone analysis could provide additional valuable information on timing for breeding in the Sumatran rhinoceros since elevated levels of oestradiol are clearly associated with the occurrence of sexual behaviour and mating.

Based on the methodology established, this study provided basic information on the reproductive cycles and pregnancy and enabled recognizing reproductive disorders in females of the captive population. In this respect, the results shown in chapter IV provided valuable information in terms of recognizing individual female Sumatran rhinoceroses which show an acyclic and erratic pattern of ovarian activity, suggesting these animals to be infertile. Providing such information on fertility status of individual females is important for future breeding programs as infertile animals or those with low fertility potential can be excluded from the breeding program. Alternatively, it opens the possibility to decision makers to investigate such animals more deeply in order to find a solution for treatment or enhancing the reproductive potency of the individual and thus may help to save its potential genetic value. Apart from assessing the overall fertility status, endocrine measurements, can also help to provide information on the timing of ovulation, a prerequisite for application of assisted reproductive technologies (ART). In this respect, frequent urinary analysis may help to predict the presumed time of ovulation while faecal progestin can confirm the outcome of ART. Although ART have yet to be established for the Sumatran rhinoceros, the fundamental techniques for characterizing basal reproductive parameters in the Sumatran rhinoceros are now available. Historically, the same situation has been described for the successful ART development in other species (felids: Swanson 2006; black buck: Holt *et al*. 1988; Eld's deer: Monfort *et al*. 1993). Thus, in the long-term, ART may become also a valuable option in captive breeding programs of the Sumatran rhinoceros with the methods for assessing ovarian activity developed in this study presumably playing an important role.

Faecal progestin measurements enable to detect early pregnancy in the Sumatran rhinoceros after 60 days out of the 15 to 16 months of pregnancy. Since, as shown in chapter IV, it is possible to use single faecal samples to determine pregnancy in the species as also reported in

other species (elk: Garrot *et al*. 1998; tule elk: Stoops *et al*. 1999; bighorn sheep: Schoenecker *et al*. 2004), from a conservation point of view, this opens the possibility to evaluate the reproductive potential of the free ranging animals through identifying the number of pregnant females in the wild, provided that a fresh faecal sample can be collected. On the other hand, the possibility to predict parturition using faecal progestin analysis as shown in chapter IV can help the sanctuary or zoo manager to prepare for the prospecting birth. The ability to predict the time of ovulation, to diagnose early pregnancy, to monitor pregnancy and to predict the time of parturition may have important implications in improving the efficiency of natural breeding in the Sumatran rhinoceros (see Heistermann *et al*. 1995).

As shown in chapter IV, there are clear changes in morphological and behavioural parameters during a female's cycle which are closely associated with changes in endocrine levels. Specifically, it is shown that observation on changes in vulva morphology can provide valuable information for predicting the most appropriate time for pairing potential breeding animals because the onset of the highest value of vulval change is before the expected time of ovulation or the occurrence of mating behaviour. In practical terms, these results show that observation on vulval changes and sexual behaviour on a long term scale have the potential to provide reliable information on a female's ovarian activity. This can be useful for supporting the breeding management in certain circumstances, (e.g. when access to large area to allow animals display sexual behaviour independently), particularly, when endocrine assessment and ultrasound examinations are difficult or impossible.

Apart from information on female fertility status, knowledge on male reproductive fertility is also important for a successful breeding program. Therefore assessment on the male reproductive potency is needed. In chapter V, different methods for semen collection in the Sumatran rhinoceros have been evaluated for their use in obtaining a

proper ejaculate. The data suggested that use of "non-invasive" techniques based on AGM, and PM in combination with an AV appeared to be superior in this respect compared to the invasive procedure of electroejaculation. Irrespective of the methodology, the results for the breeding male Torgamba at SRS clearly showed that he has a low fertility with low volume of ejaculates (oligospermia), low sperm concentrations (oligozoospermia), and a high degree of abnormalities in sperm motility and morphology. The reason for low fertility in this male remains unknown, therefore it is necessary to assess the causes, i.e. whether there is an ejaculatory failure, or testicular failure to produce proper sperm and/or problems in sperm transport. This can be done using the alkaline phosphatase activity test as described by Turner *et al.* (2001; 2003). Alkaline phospatase (AP) activity in ejaculatory fluid is an accurate marker for true ejaculation in stallions with severe abnormalities of these testes and epididymides. Turner *et al*. (2001) reported that AP value > 1000 IU/l suggests true ejaculate; < 100 IU/l suggests ejaculatory failure or blockage; 100 – 1000 IU/l may indicate partial ejaculate or partial blockage in horse.

In summary, this study has established the methodologies for non-invasive assessment of the fertility status in both the female and male Sumatran rhinoceros. Moreover, by applying these newly established techniques, the study generated important new information on the reproductive physiology of the species and on the fertility status of a number of captive individuals, and thus provided important knowledge which can help to improve future breeding management. However, since all results are obtained from a limited number of animals, the reproducibility of the results in a wider range of individuals has to be confirmed in the future. For this, it is important to continue the study on monitoring reproductive status non-invasively in the different animals held in captivity. This will hopefully help to improve the long term breeding

program and conservation of this most critically endangered rhinoceros species.

Conclusions

1. Methods have been established for characterizing reproductive function in the Sumatran rhinoceros i.e., characterization of oestrous cycle, detection early pregnancy, monitoring pregnancy, and recognizing reproductive disorder.
2. Non-invasive urinary and faecal analysis can support the development of assisted reproductive technology in the Sumatran rhinoceros by assessing reproductive status in individual Sumatran rhinoceroses
3. In terms of conservation of the species, the method could serve and support field work on investigating fertility status of wild rhinoceros by means faecal analysis to detect pregnancy in the Sumatran rhinoceros
4. The study shows the information on sexual behaviour together with vulval morphological changes provide useful information for predicting the proper time to mix male and female Sumatran rhinoceros for mating.
5. Assessment of the male Torgamba has to be followed up to investigate the causes of low fertility using the alkaline phosphatase activity test.

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APPENDIX I

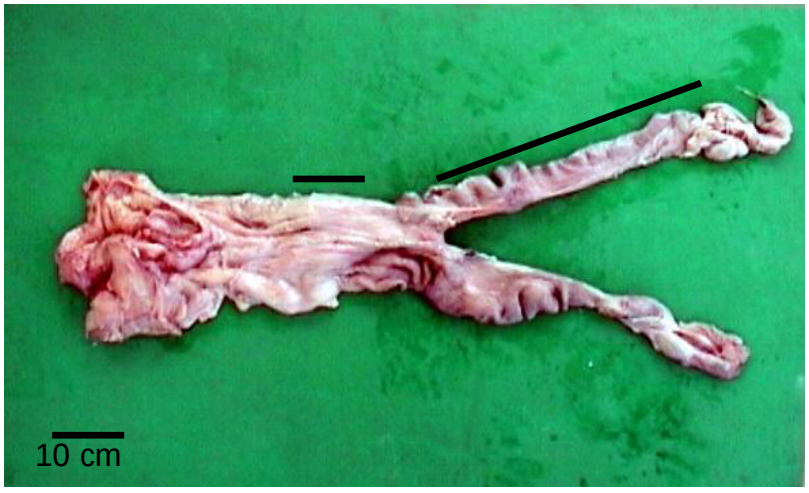
Reproductive Tract and Organs in the Sumatran Rhinoceros

The following series of pictures and tables demonstrate the gross anatomy and measurements of the reproductive tract and organs in the female and male Sumatran rhinoceros.

Female reproductive tracts and organ

Data are from two females; Rima died in 2003 from Sumatran Rhino Conservation Center, Sungai Dusun, Malaysia; and Dusun died in 2001 from SRS, Way Kambas National Park Lampung, Indonesia.

(A)



(B)

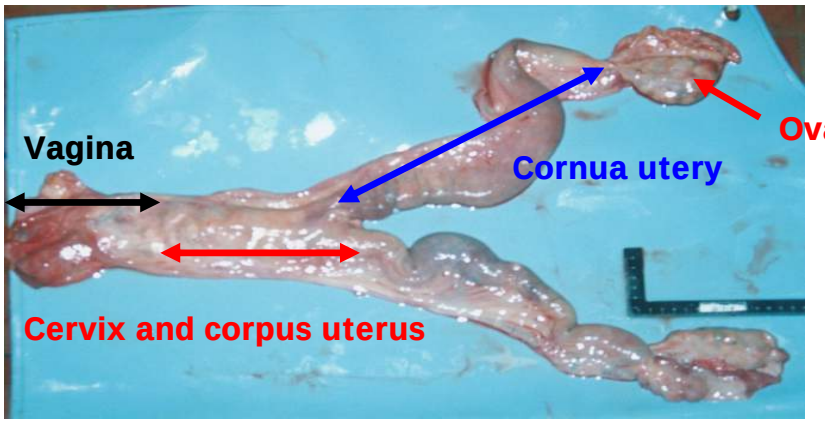
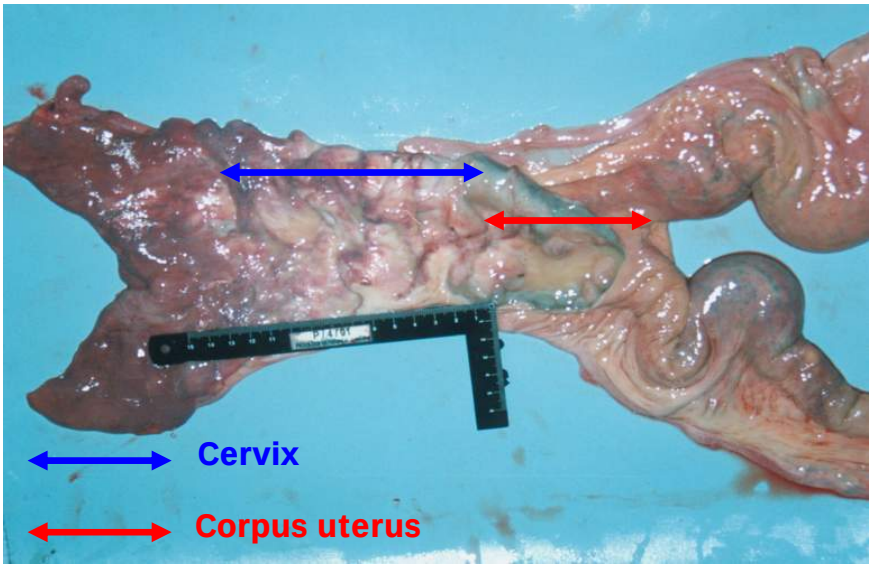


Figure 43. Gross anatomy of female reproductive tract of the Sumatran rhinoceros from two females (A) Rima, Sumatran Rhino Conservation Center, Sungai Dusun, Malaysia, (B) Dusun, Sumatran Rhino Sanctuary, Way Kambas National Park, Lampung, Indonesia

(A)



(B)

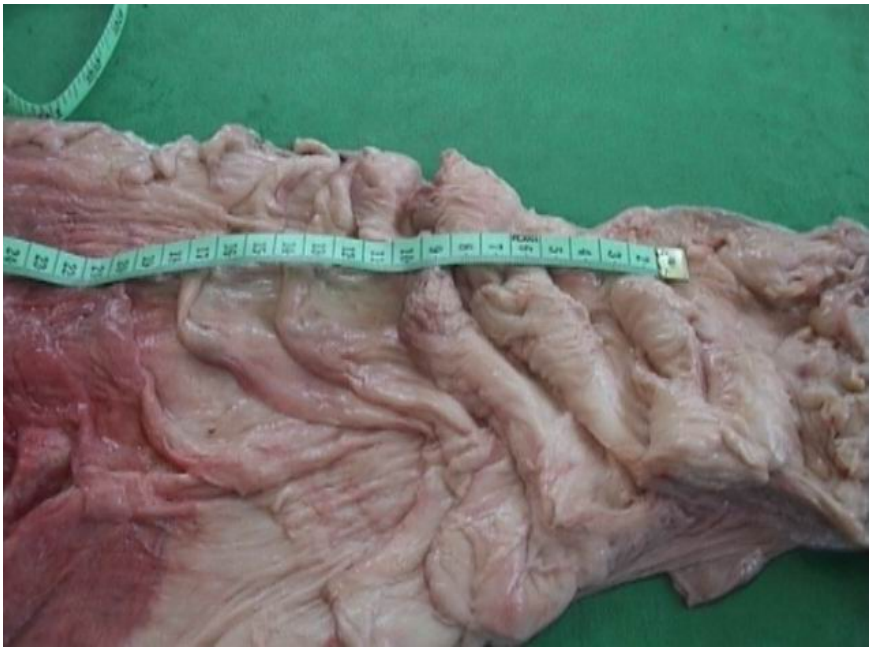


Figure 44. (A) Cervix and short corpus uterus, (B) Cervical fold clearly defined

The corpus uterus is very short, about seven centimetres, compared to the total length of the female reproductive tract (94-95 cm). Cervical folds vary between both females; Dusun has five cervical folds whereas Rima has six folds.

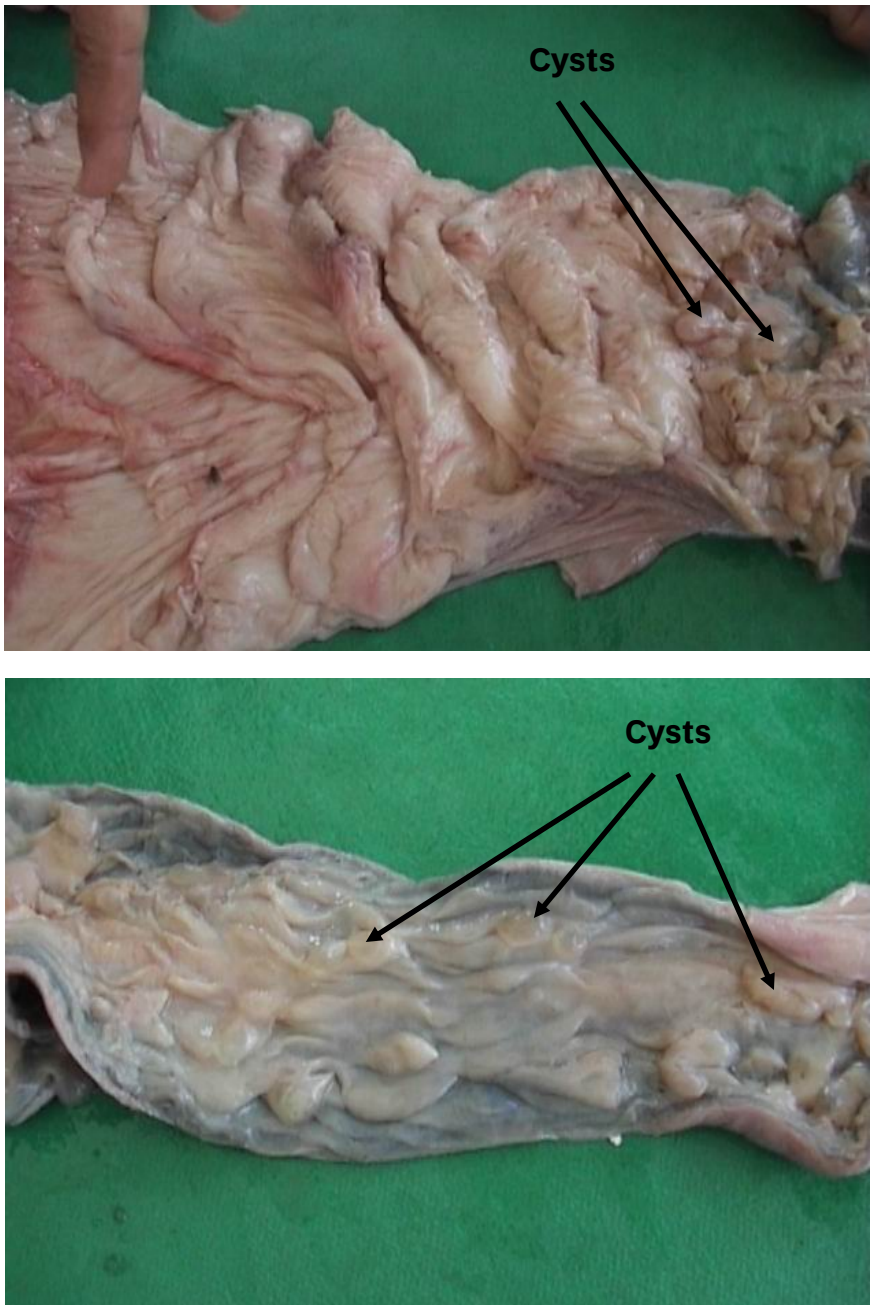
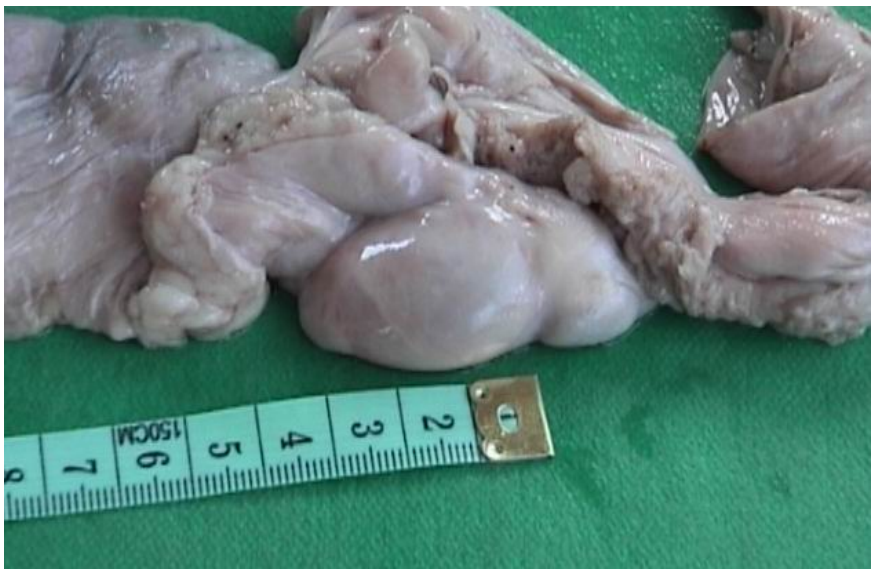


Figure 45. Cysts were found in both corpus and cornua uterus of Rima, there was no cyst found in the reproductive tract of Dusun.

Almost all female Sumatran rhinoceroses at Sungai Dusun had cysts in the uterus both along the corpus and cornua uterus (M. Aidi, personal comm.), and some of them had also tumors either in the uterus or in the distal part of the cervix.

(A)



(B)

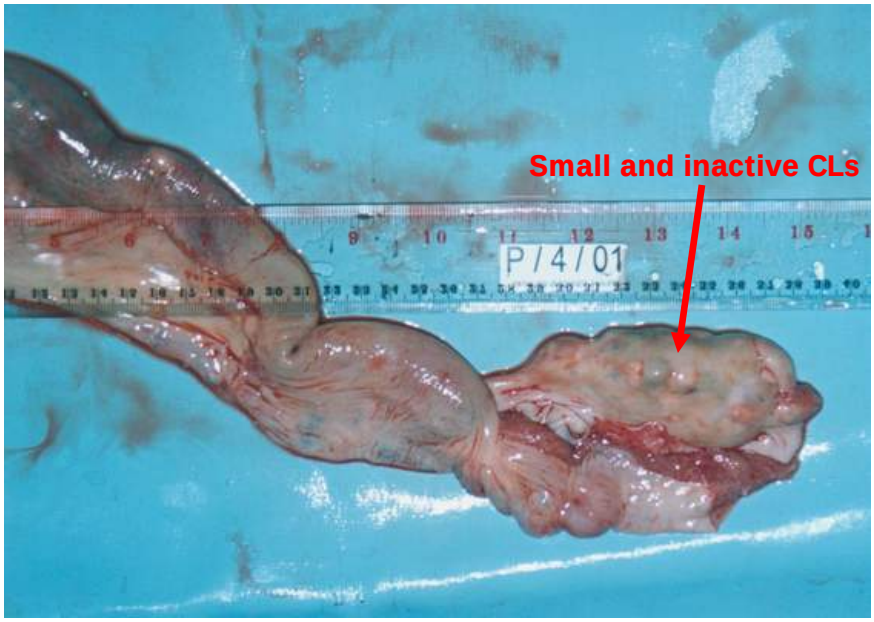


Figure 46. (A) no follicle and CL was found in the ovaries of Rima, (B) small and inactive CLs were found in the ovary of Dusun, but a follicle was not observed

The ovaries of Rima were smaller compared to those of Dusun. Both ovaries in Rima were not active; follicle and CL were not observed.

Table 17. Measurement of reproductive tract and organs from two female Sumatran rhinoceroses

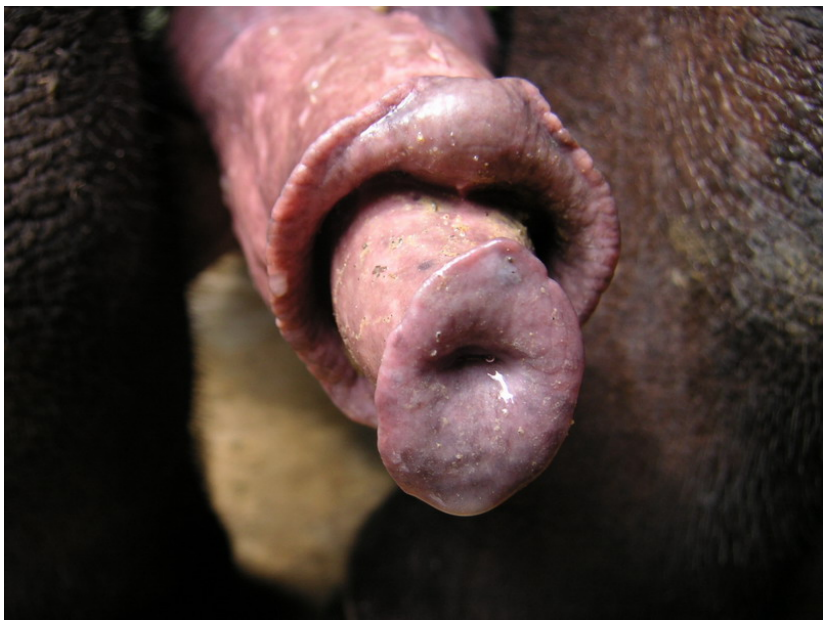
No.	Reproductive tract/organ	Measurement in cm	
		Rima	Dusun
1.	Vagina	50	56
2.	Cervix	12	10.5
3.	Cervical fold	6 folds	5 folds
4.	Corpus Uterus	7	7.5
6.	Cornua Uterus (right)	34.5	38.5
7.	Cornua Uterus (left)	34	44.5
8.	Ovary (L x W x T) right	3.5 x 1.3 x 1	7.0 x 4.3 x 2.5
9.	Ovary (L x W x T) left	4.8 x 2.2 x 2	7.5 x 4.4 x 2.5
10.	Tuba falopii	n.m	n.m
10.	Total length of reproductive tract	113-113.5	122-128

n.m. = not measured

Male reproductive tract

Data were obtained from the living Sumatran rhinoceros "Torgamba", which is kept in SRS, Way Kambas National Park Lampung, Indonesia; and one young male that was found dead in Bukit Barisan Selatan National Park Lampung, Indonesia in 2000.

(A)



(B)

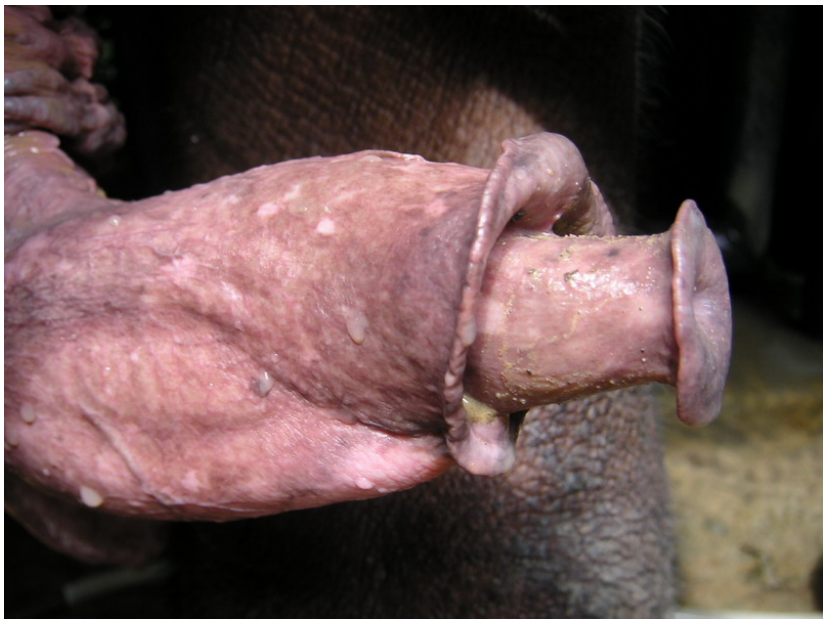
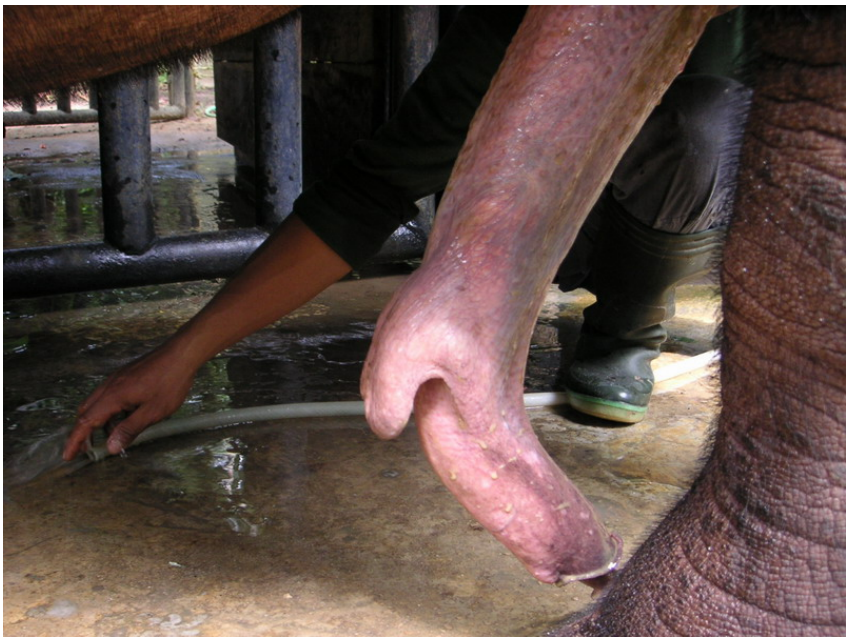


Figure 47. Glans penis of the Sumatran rhinoceros is very unique; it has commissura in front and in the base of penile processus.

(A)



(B)

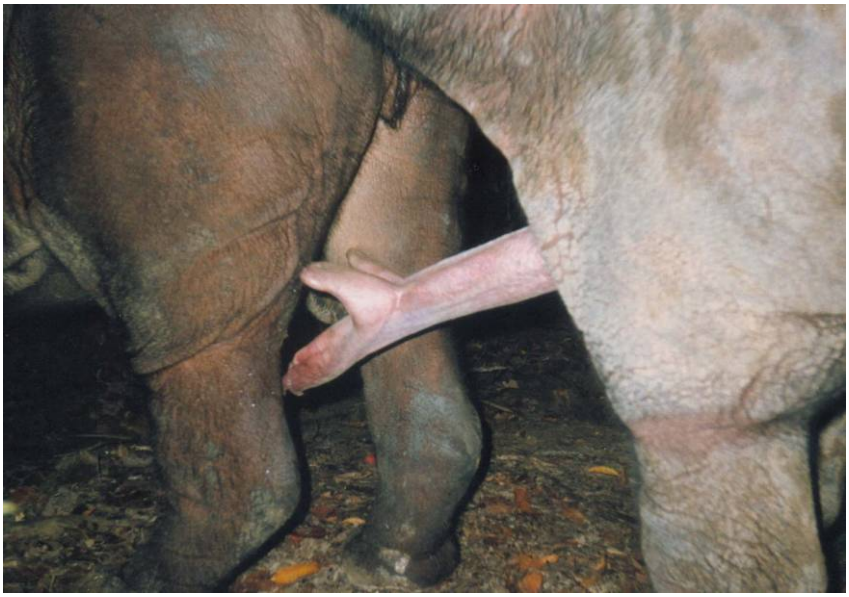


Figure 48. In the Sumatran rhino, the penis has two flaps in the distal of glans penis, (A) half erection, and (B) full erection.

(A)

(B)

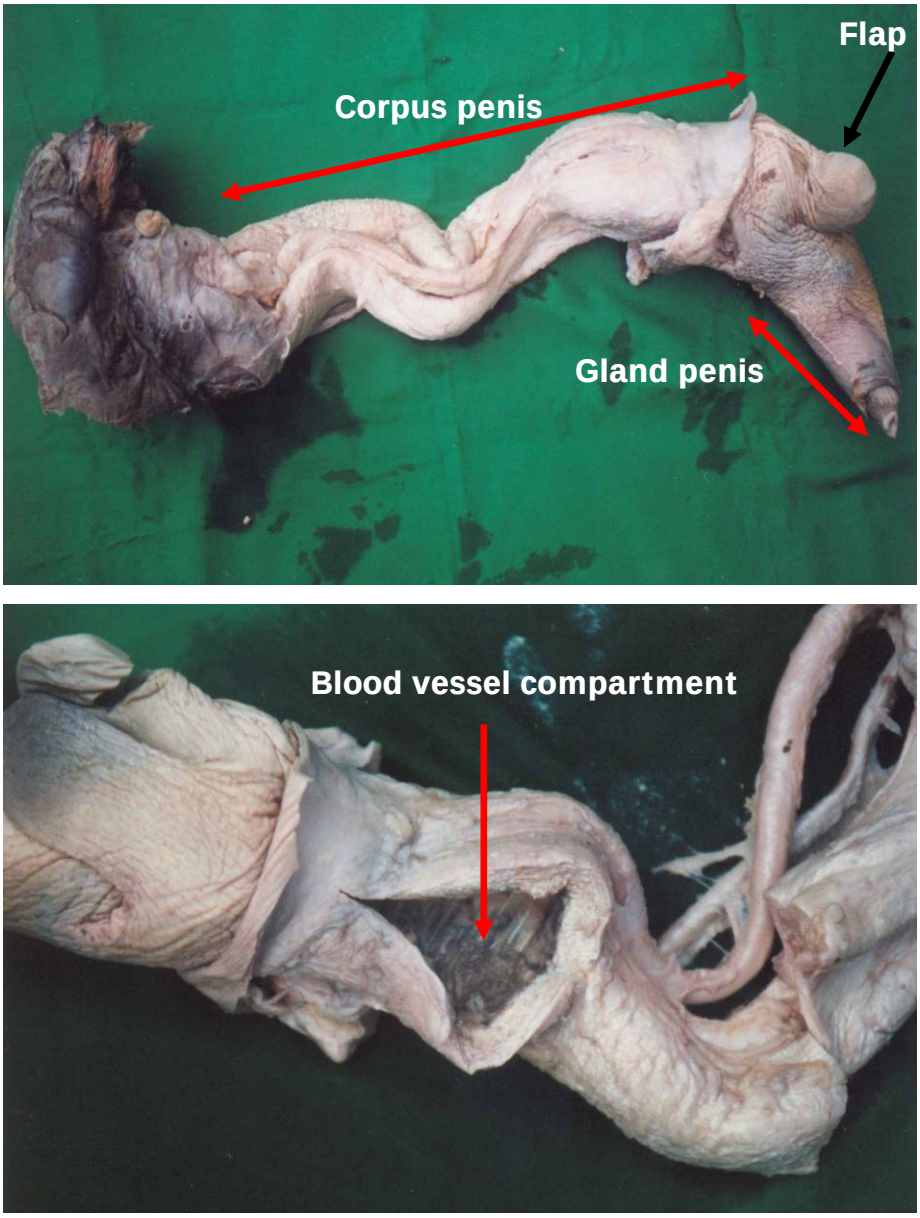
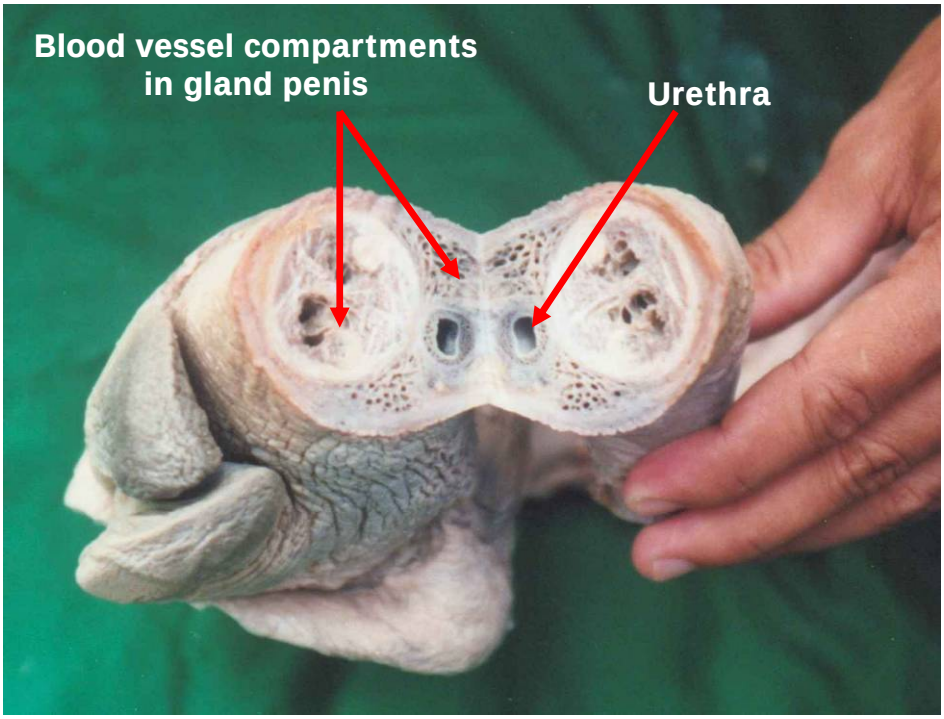


Figure 49. (A) a part of penis: corpus penis, glans penis, and flap; (B) blood vessel compartment along the corpus penis.

(A)



(B)

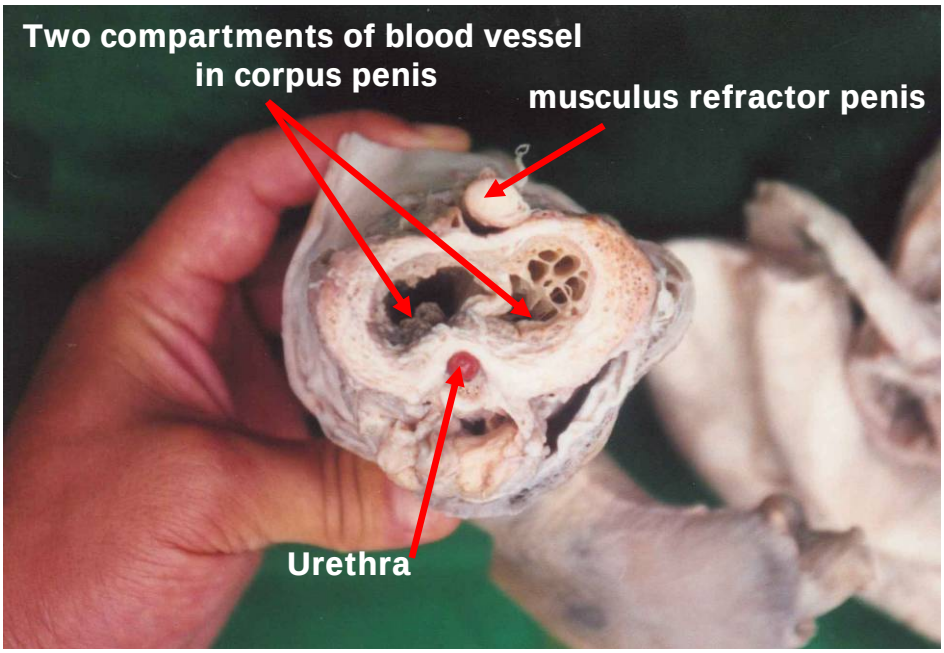
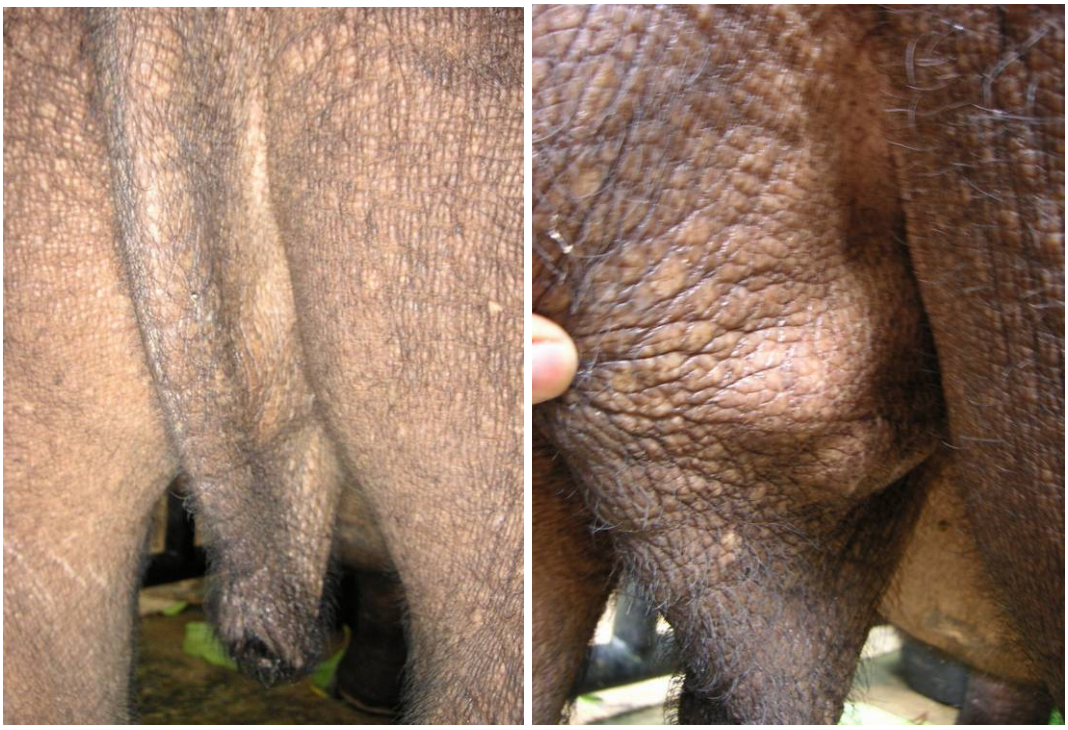


Figure 50. (A) cross section of glans penis; (B) corpus penis



(A) (B)

Figure 51. The Sumatran rhinoceros has no scrotum, testes are placed inside the penis sheath, (A) penis sheath and preputium, (B) testes inside the penis sheath.

APPENDIX II

Statistical Analysis

The difference of 5-P-3OH values between follicular and luteal phase

Paired t-test

Data source: Data 1 in Notebook

Normality Test: Passed (P > 0,200)

Treatment Name	N	Missing	Mean	Std Dev	SEM
Foll 5-P3OH	17	0	7,753	3,750	0,910
Lut 5-P3OH	17	0	17,326	6,387	1,549
	17	0	-9,573	3,995	0,969

Difference -9,573 3,995 0,969

t = -9,879 with 16 degrees of freedom. (P = <0,001)

95 percent confidence interval for difference of means: -11,627 to -7,519

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = <0,001)

Power of performed test with alpha = 0,050: 1,000

The difference of 5-P-3OH values between the period “with sexual behaviour” and “without sexual behaviour

Paired t-test

Data source: Data 1 in Notebook

Normality Test: Passed (P = 0,019)

Treatment Name	N	Missing	Mean	Std Dev	SEM
with sex	15	0	8,433	3,320	0,857
no sex	15	0	17,960	6,559	1,694
	15	0	-9,527	3,624	0,936

Difference -9,527 3,624 0,936

t = -10,182 with 14 degrees of freedom. (P = <0,001)

95 percent confidence interval for difference of means: -11,533 to -7,520

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = <0,001)

Power of performed test with alpha = 0,050: 1,000

The difference 5-P-3OH among the different stages of vulval score

One Way Repeated Measures Analysis of Variance

Data source: Data 1 in Notebook

Normality Test: Failed (P = <0,001)

Equal Variance Test: Passed (P = 0,654)

Treatment Name	N	Missing	Mean	Std Dev	SEM
Vulva score 2	10	0	15,847	4,029	1,274
Vulva score 3	14	0	8,752	3,333	0,891
Vulva score 4	5	0	8,420	1,929	0,863

Source of Variation	DF	SS	MS	F	P
Between Subjects	13	178,886	13,760		
Between Treatments	2	396,364	198,182	20,365	<0,001
Residual	13	126,507	9,731		
Total	28	643,770	22,992		

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001). To isolate the group or groups that differ from the others use a multiple comparison procedure.

Power of performed test with alpha = 0,050: 1,000

Expected Mean Squares:
Approximate DF Residual = 13,000
Expected MS (Subj) = var(res) + 2,000 var(Subj)
Expected MS (Treatment) = var(res) + var(Treatment)
Expected MS (Residual) = var(res)

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0,050
V2 vs. V3	8,520	3	8,637	<0,001	Yes
V2 vs. V4	7,426	2	5,276	0,003	Yes
V4 vs. V3	1,094	2	0,838	0,564	No

AUTOBIOGRAPHY

Author was born in Cirebon on 16th of August, 1963. Author is the eldest son of Mr. Muhammad Zein Ibrahim and Mrs. Entin Suarti.

In 1982, the author was graduated from SMA Negeri 1 Cirebon, and accepted at Bogor Agricultural University through "Program Perintis II" at the same year. Author was accepted at Faculty of Veterinary Medice, Bogor Agricultural University in 1993, and graduated as Bachelor in Veterinary Medicine in 1987. Author continued to follow two years internship program in 1987, and accomplished a doctor of veterinary medicine (Dokter hewan) in 1989. Author got a scholarship from DAAD to study Reproductive Physiology at the Georg-August University, Goettingen, Germany in 1993, and finished a Master degree in 1995. Doctoral scholarship has been provided by the Directorate General of Higher Education from 2000 until 2005, and registered at the Study Program of Reproductive Biology, Postgraduate School, Bogor Agricultural University

Author has been working at the Faculty of Veterinary Medicine, in the Division of Reproduction and Obstetrics since 1992 as a lecturer at the Obstetrics and Infertility laboratory. Since 1994, Author has been actively doing research on the reproductive biology of the Sumatran rhinoceros until now. Author is a member of the Indonesian Association of Veterinary Medicine, the Indonesian Association of Wildlife and Exotics Medicine, and a member of the South East Asian Rhino Specialist Group of Species Specialist Group-IUCN.

Scientific paper with the title of "A program of managed breeding for the Sumatran rhinoceros at the Sumatran rhino sanctuary, Way Kambas national park, Indonesia" was presented at the International Elephant and Rhino Research Symposium in Vienna on June 7-11, 2001. Second paper with the title of "The development of semen collection methods in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*, FISCHER 1814)" was presented at the International Asia Link Symposium in Bali on August 19-20, 2005. Third paper was presented at the 2nd Symposium of the Asian Zoo and Wildlife Medicine in Bangkok, Thailand on October 26-29, 2006, with the title of "Monitoring follicle size with ultrasound as an important tool to determine a proper time for mating in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*, FISCHER 1814) in the Sumatran Rhino Sanctuary (SRS) Way Kambas national park, Lampung". Scientific article with the title of "Semen collection in the Sumatran rhino (*Dicerorhinus sumatrensis*, FISCHER 1814) for breeding attempt to sustain biodiversity" was published in Advance in Ethology 38 (2004), supplement to Ethology. Moreover, two scientific articles will be published in 2007 and 2008. The article with the title of "Assessment of fertility status in the male sumatran rhino (Torgamba) at Suaka Rhino Sumatera (SRS) Way Kambas National Park, Lampung" will be published at HAYATI journal in 2007, and the second article with the title of "Characterization of oestrous

cycle in the female Sumatran rhinoceros (*Dicerorhinus sumatrensis*) through non-invasive hormone analysis, observation on sexual behaviour and morphological vulval changes" will be published in Pachyderm in 2008. Those scientific papers and articles are as part of the scientific publication produced during the postgraduate program of the author.