REPRODUCTIVE HORMONES AND BEHAVIOUR IN THE INDIAN (*Rhinoceros unicornis*) AND SUMATRAN (*Dicerorhinus sumatrensis*) RHINOCEROS

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ABSTRACT

This study had two main objectives. The first, described in Chapter 1, was to determine whether relationships exist between female estrous cycles, male testosterone levels and the behaviour of female and male Indian rhinos. Two captive male-female pairs of Indian rhinos were studied over the course of nine estrous cycles. The following seven behaviours were significantly positively correlated with female estrogen levels: Weaving, Pacing, Sniff/Taste Urine/Feces, Urine Spraying, Defecating, Blowing and Banging. In addition, Head Bobbing by one female and Blow-Snorting by one male were positively correlated with male testosterone levels.

Graphing behaviour and hormones over time revealed twenty-two behaviours which occurred only (or more frequently) around the time of high estrogen (or testosterone) levels, although the correlations were not statistically significant. These behaviours were: Walking, Trot/Canter, Weaving, Pacing, Charging, Side-to-Side, Sniff/Taste Urine/Feces, Flehmen, Urine Spraying, Penis Extended, Mooing, Blowing, Whistling, Whistle-Blowing, Whistle-Barking, Teeth Grinding, Banging, Bleating, Snorting, Bellowing and Barking.

The results of the first part of this study will assist animal managers in predicting the times during which rhinos are ready to breed, thus reducing the need for expensive, time-consuming and labour-intensive hormone analysis, lessening the chance of injury to the animals and, most importantly, potentially improving reproduction rates.

The second objective of the study, described in Chapter 2, was to develop an enzyme immunoassay (EIA) for use in monitoring fecal progestogens from female Sumatran rhinos, in order to simplify future research on the relationships between hormones and behaviour in Sumatran rhinos. Over a period of 182 days, one hundred and seventy fecal samples were collected from an 8-year-old, nulliparous female Sumatran rhino. These samples were analyzed using two assays: 1) a radioimmunoassay (RIA) previously described by Brown et al. (1994) for use in monitoring ovarian activity in felids, as well as by Roth et al. (2001) for use in monitoring the fecal progestogens of two female Sumatran rhinos, and 2) a new EIA, developed as part of this study, which used a protocol adapted from Schwarzenberger et al. (1996 and 1998) and the same antibody as that used in the above RIA. This antibody was produced against 4-pregnene-11-ol-3,20-dione hemisuccinate:BSA, and its major cross-reactivities were progesterone 100%, allopregnanolone 96% and 5α-pregnane-3α-ol-20-one 36%.

Results of fecal hormone analyses between the two assay systems were very similar ($r = 0.47$, $p < 0.001$), extraction efficiency was 115% and parallelism was established, so it was concluded that the new EIA can be used to monitor the reproductive cycle of the female Sumatran rhinoceros. The development of this EIA will not only greatly facilitate hormonal analysis of fecal samples collected from captive animals but will also allow field researchers to monitor the reproductive cycles of animals *in situ*, under conditions where it is impossible to gain access to laboratories equipped to handle radioactivity (i.e. RIA).

The data regarding the relationship of individual behaviours to particular hormonal events provide new insights into the reproductive biology of rhinos, and more specifically, the mechanisms by which these solitary, widely dispersed animals communicate information about their reproductive status and willingness to mate. Overall, results from both parts of the study serve to further elucidate the reproductive biology of these poorly understood mammals.

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LIST OF ABBREVIATIONS

Ab ..... Antibody

ABTS ..... 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)

AmS ..... Ammonium Sulfate $(NH_4)_2SO_4$

BL ..... Biotinylated Label or Biotin Labelled Steroid

BBS ..... Borate-Buffered Saline

BSA ..... Bovine Serum Albumin

C1 ..... "High Control" for assay quality control, ~ 30% binding

C2 ..... "Low Control" for assay quality control, ~ 70% binding

Cr ..... Creatinine

CRC ..... Smithsonian Institution's National Zoological Park/Conservation and Research Center (Front Royal, VA, USA)

DDW ..... Double-Distilled Water

DMSO ..... Dimethylsulfoxide

EC ..... Estrone Conjugates

E-HRP ..... Estrone-glucuronide-Horseradish Peroxidase

EIA ..... Enzyme Immunoassay

FSH ..... Follicle-Stimulating Hormone

FW Zoo ..... Fort Worth Zoo (Texas, USA)

LIST OF ABBREVIATIONS (Continued)

GnRH ..... Gonadotropin-Releasing Hormone

$[^3H]E_2$ ..... Tritiated Estradiol

IgG ..... Immunoglobulin G

LH ..... Luteinizing Hormone

M ..... Molar (mol/L)

N ..... Normal (describing solutions used for adjusting pH)

NSB ..... Non-Specific Binding wells

P ..... Progestogen

P-HRP ..... Pregnanediol-glucuronide-Horseradish Peroxidase

PBS ..... Phosphate-Buffered Solution

PdG ..... Pregnanediol-Glucuronide

RIA ..... Radioimmunoassay

T ..... Testosterone

T-HRP ..... Testosterone-Horseradish Peroxidase

TMB ..... 3,3',5,5'-tetramethylbenzedine

TO Zoo ..... Toronto Zoo (Ontario, Canada)

TRIS ("Trizma Base") ..... Tris[hydroxymethyl]aminomethane

LIST OF ABBREVIATIONS (Continued)

Tween 20 ..... Polyoxyethylenesorbitan Monolaurate

Tween 80 ..... Polyoxyethylenesorbitan Monooleate

GENERAL INTRODUCTION

Summary Statement of Purpose

This project was undertaken to explore potential relationships between female estrous cycles, male testosterone levels and behaviours of female and male Indian and Sumatran rhinoceros. The project was both theoretically and practically oriented, with the theoretical goal of exploring the reproductive biology of these poorly understood mammals, and the practical goal of developing the ability to predict sexual receptivity in rhinos based on their behaviour. Achievement of these goals will greatly enhance the future success of captive breeding programs for these two endangered species.

The purpose of this General Introduction is to provide background information on endocrine function in mammals, and to describe the basic life history of the five species of rhinoceros.

Mammalian Reproductive Endocrinology

Unless otherwise noted, the information in this section comes from Norris (1997).

Female Reproductive Hormones and the Estrous Cycle

The female reproductive cycle, called the estrous cycle in most mammals other than primates, is composed of two distinct phases – the follicular phase and the luteal phase. The follicular phase involves the maturation of oocytes in the ovaries, a process which is initiated by the production of gonadotropin-releasing hormone (GnRH) in the

hypothalamus. GnRH is passed through special blood vessels to the anterior lobe of the pituitary gland, which in turn releases follicle-stimulating hormone (FSH). FSH passes through the bloodstream to the ovaries, where it stimulates primary follicles to begin to enlarge and differentiate, and causes cells of the granulosa to produce estrogens. These estrogens are then released into the general circulation. FSH also causes the granulosa cells to produce inhibin, which feeds back on the pituitary to repress further FSH release.

Rising estrogens eventually reach a critical level, which causes the hypothalamus to release a large surge of GnRH. The GnRH passes to the anterior lobe of the pituitary, which in turn produces luteinizing hormone (LH) and releases it into the bloodstream. LH causes developing follicles to ovulate (release of oocytes into one of the two oviducts which extend from the ovary to the uterus) and, after ovulation, inhibits further estrogen production. In most mammals, ovulation occurs 24 to 60 hours after the estrogen peak, and 12 to 24 hours after the LH surge. Ovulation marks the onset of the luteal phase. The number of follicles ovulating is species specific, varying from a norm of one in human females to a dozen or more in female pigs. The cells in the ruptured follicle then undergo a transition and begin to secrete progesterone. At this stage the follicle is called a corpus luteum or CL. Progesterone prepares the body for fertilization by initiating the many physiological changes associated with pregnancy while inhibiting the development of other follicles.

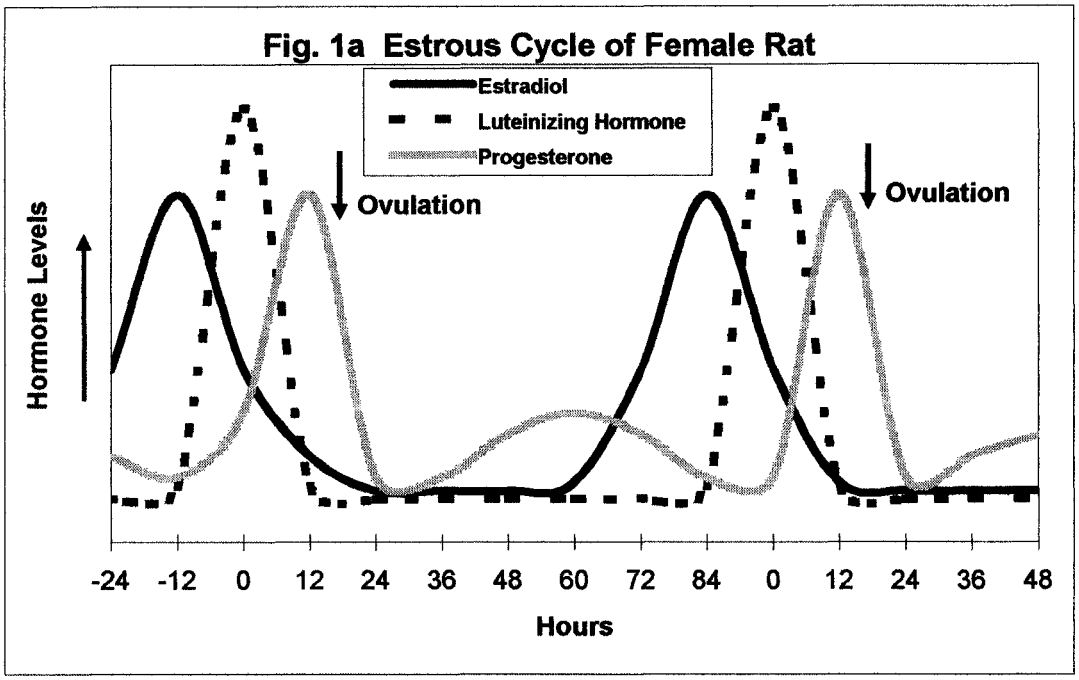
The CL continues its production of progesterone after fertilization has occurred, as progesterone plays a role in the maintenance and development of pregnancy. If,

however, fertilization does not occur, production of progesterone declines. In most species the CL secretes progesterone for only a short period (e.g. 5 to 8 days in human females, 17 days in buffalo), after which it begins to regress (Arthur et al., 1996; Raven and Johnson, 1989). The cessation of progesterone production by the CL marks the end of the luteal phase. In the relative absence of estrogen and progesterone the pituitary can again initiate the production of FSH, starting another estrous cycle. Fig. 1 illustrates the typical mammalian reproductive cycle, using the rat and cow as examples.

Male Reproductive Hormones

In many mammalian species post-pubertal males are capable of copulating at any time. Secretion of GnRH and hence FSH and LH is more or less continuous, although depending on the species multiple surges may occur, even within one day. To date anecdotal reports have assumed (but not tested) that males of all five species of rhinoceros fit this pattern.

The synthesis and release of androgens by the interstitial cells (steroidogenic cells located in the testes between the seminiferous tubules) is primarily controlled by LH. Testosterone is the major circulating androgen, although others such as androstenedione and DHT (5α-dihydrotestosterone) may circulate in significant amounts. Within the testes, testosterone seems to be the most important androgen influencing sperm production. Testosterone also stimulates the growth spurt which occurs at puberty and the development of sex organs and secondary sex characteristics.



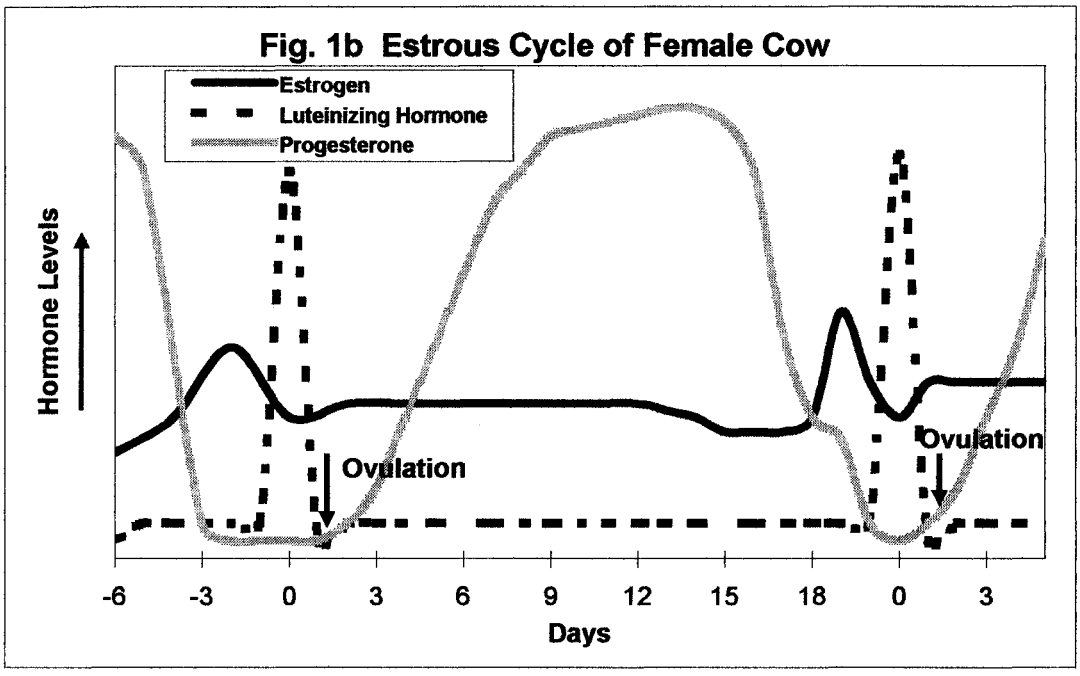


Fig. 1 Hormone levels during the reproductive cycle of the female rat & cow.
a) The 4-day cycle of the female rat. Adapted from Norris (1997), pg. 396.
b) The 21-day cycle of the female cow. Adapted from Pineda (1989), pg. 333.

Spermatogenesis is initiated by FSH, which stimulates the mitotic proliferation of spermatogonia (1st stage of spermatogenesis) and their development into primary spermatocytes (2nd stage). Testosterone may also play a role here by initiating meiotic divisions of the primary spermatocytes (stages 3 and 4), leading to the formation of spermatids.

Methods of Endocrine Analysis

Two types of immunoassay were used in this research: an enzyme immunoassay (EIA - also known as enzyme-linked immunoabsorbent assay or ELISA), and a radioimmunoassay (RIA). The purpose of both of these competitive binding assays is to accurately determine the amount of antigen (e.g. steroid hormone) in a given sample. The basic EIA protocol is as follows, although there are variations (Fig. 2). A "coating" antibody specific to the antigen (hormone) being measured is adsorbed onto a solid substrate (e.g. a microtitration plate). A measured amount of sample is added, along with a fixed amount of enzyme-labelled hormone. Hormone molecules from the sample compete with molecules of enzyme-labelled hormone for binding sites on the coating antibody (the greater the proportion of sample hormone which achieves binding, the greater the concentration of hormone in the sample). Finally, a substrate is added which undergoes a colour reaction with the enzyme-labelled hormone – the darkest possible colour result means that only the enzyme-labelled hormone has bound (100% binding), therefore no sample hormone is present. The colour change is measured photometrically

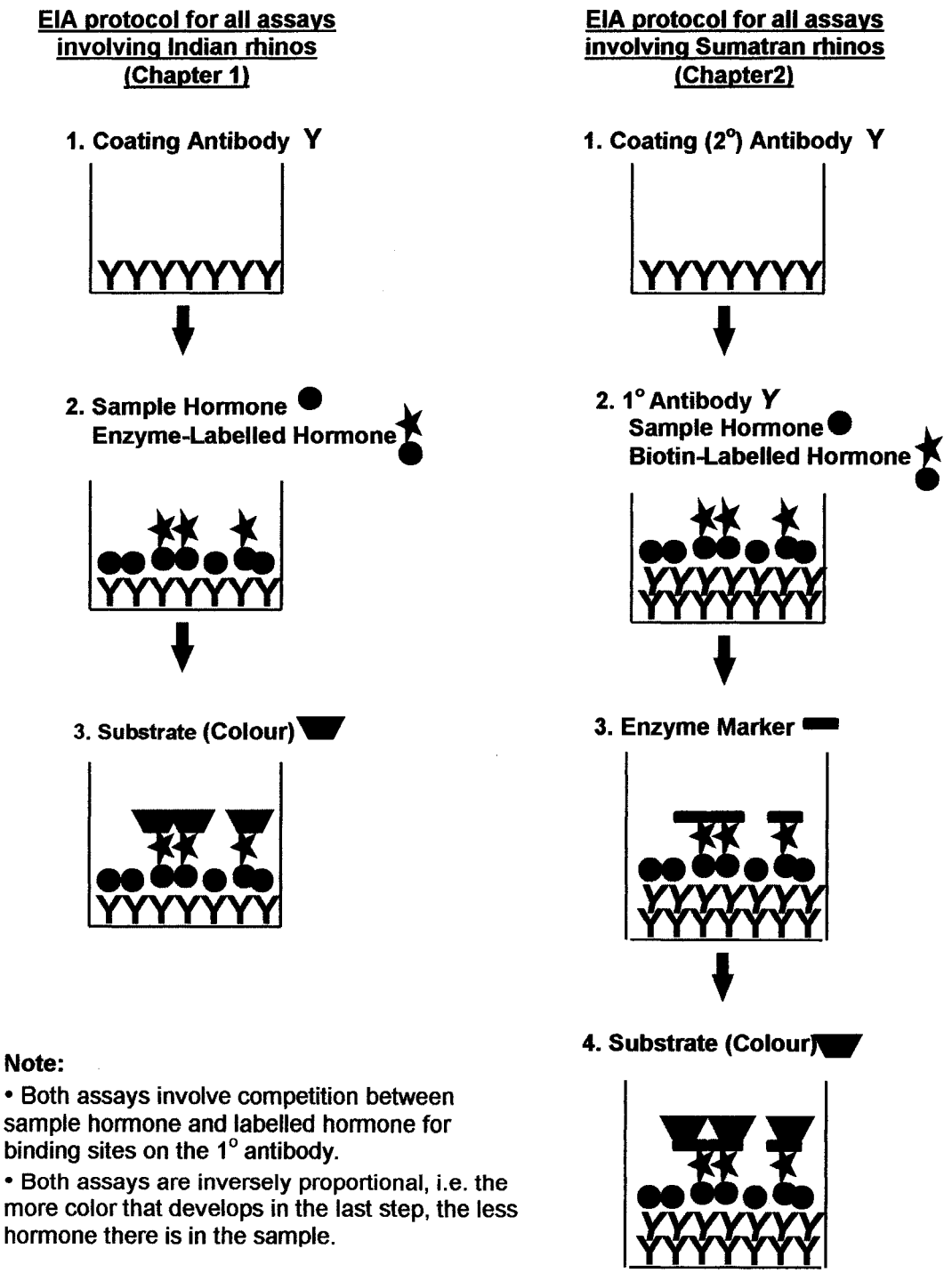


Fig. 2 Illustration of EIA protocols.

and compared against a standard curve to give the exact concentration of hormone in the sample.

The use of RIA relies on the assumption that an antibody cannot distinguish an unlabeled or "cold" antigen (e.g. steroid hormone) from a radioactively-labelled or "hot" antigen. Like EIA, RIA involves competition between unlabelled or "cold" hormone (from the sample being analyzed) and labelled or "hot" hormone for binding sites on the antibodies. Rather than binding the antibodies to a substrate as in EIA, the antibodies and hormone molecules are free-floating in solution. To establish the standard curve, a constant amount of antibody and hot hormone is added to a series of tubes, along with increasing amounts of cold hormone. The greater the amount of cold hormone added, the lower will be the amount of hot hormone which succeeds in binding to the antibody. By measuring the amount of radioactivity bound to the antibody and plotting this (y-axis) against the known concentrations of cold hormone (x-axis), a standard relationship can be established.

To estimate the amount of unknown, cold antigen in a given sample, a similar competition is set up using the same quantities of antibody and hot antigen as was used to determine the standard curve. Once the amount of bound hot antigen has been measured, the quantity of cold antigen in the sample can be determined by extrapolating to the y-axis.

Family *Rhinocerotidae*: Life History and Reproduction

The family *Rhinocerotidae* includes 4 genera and 5 species: the Indian or Greater One-Horned Asian rhinoceros (*Rhinoceros unicornis*), the Javan or Lesser One-Horned Asian rhinoceros (*Rhinoceros sondaicus*), the Sumatran or Asian Two-Horned rhinoceros (*Dicerorhinus sumatrensis*), the Black rhinoceros (*Diceros bicornis*) and the White rhinoceros (*Ceratotherium simum*).

Rhinos have poor vision but excellent hearing and smell (Nowak, 1999). These two senses seem to be very important for communication (Laurie et al., 1983; Nowak, 1999). All species of rhino are known to drop their dung in well-defined piles, often furrowing around the piles with their horns. These piles, as well as urination spots, rubbed branches and hoof/horn scrapes, are believed to act as territory markers (Nowak, 1999) and may also be used to identify other individuals in the area. All ages and both sexes of Indian rhino defecate in communal dung heaps, and this deposition of scent may help males in determining the availability of receptive females. Indian rhinos also have pedal scent glands (Laurie et al., 1983; Nowak, 1999) which presumably function when the animals scrape the ground with their feet after urination or defecation, further aiding in the identification of individuals.

Studies on Indian, Sumatran, Black and White rhinos (Berkeley et al., 1997; Brett et al., 1989; Heistermann et al., 1998; Hindle and Hodges, 1990; Hindle et al., 1992; Hodges and Green, 1989; Kasman et al., 1986; Kassam and Lasley, 1981; Patton et al., 1999; Radcliffe et al., 1997; Roth and Brown, 1999; Roth et al., 2001; Schaffer et al.,

1994; Schwarzenberger et al., 1993, 1996 & 1998) indicate that the reproductive biology of the family *Rhinocerotidae* tends to be species-specific. Single offspring are, however, the rule for this family (Nowak, 1999).

The Challenge of Breeding Rhinos in Captivity

All species of rhinoceros are on the Red List of Threatened Animals published by the World Conservation Union or IUCN (IUCN, 1996). According to the IUCN the status of the Indian rhinoceros in the wild is "Endangered", meaning that the population size and geographic distribution of this species are small and rapidly declining and the distribution is severely fragmented. The Sumatran rhinoceros is classed as "Critically Endangered", because it faces a very high and immediate risk of extinction in the wild. Indian and Sumatran rhinos are also listed in Appendix I of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), meaning that they are rare or endangered and international trade for primarily commercial purposes is prohibited (Environment Canada, 1995).

Captive breeding is an essential part of the conservation strategy for rhinos because of the difficulties and uncertainties involved in conserving these animals in the wild (Foose and van Strien, 1997; Kasman et al., 1986; Khan, 1989). Khan (1989) stated that, to preserve any threatened species in perpetuity, a captive population capable of long-term viability must be maintained to guard against any unforeseen extinction of the wild population. To meet this goal it was recommended (Khan, 1989) that the captive

populations of Indian and Sumatran rhinos be expanded to a minimum of 150 rhinos. This goal has yet to be met, as the Indian captive population is currently at 137 animals while the Sumatran captive population is comprised of only 14 animals (Foose, 2002; Wirz-Hlavacek, 2001).

All species of rhinoceros, but especially the Sumatran rhino, have proven to be very difficult to breed in captivity (Czekala and Callison, 1996; Dinerstein et al., 1988; Foose and van Strien, 1997; Fouraker and Wagener, 1996; Hindle et al., 1992). Until recently, only one Sumatran rhino had been bred and born in captivity – this birth occurred in a zoo in Calcutta, India in 1889 (Reynolds, 1961). In 2001, after several miscarriages, a female Sumatran at the Cincinnati Zoo in Ohio bore a healthy male calf. Three reasons for low reproduction rates in all rhino species, both in the wild and in captivity, are: 1) long gestation periods; 2) long inter-birth intervals; 3) late reproductive maturity and 4) pathologies of the reproductive tract (an extremely serious problem for Sumatran females in captivity). Three additional factors also limit reproduction rates in captivity. The first is the limited knowledge of the reproductive biology of any of the rhino species. In particular, almost nothing is known about the behaviour or physiology of the Sumatran rhino in the wild. This is most probably the reason that viable populations have not been established in captivity or under intensive management.

Particularly lacking is information regarding the proximate triggers of reproduction under natural conditions. *In situ* studies of Indian and Black rhinos indicate that the breeding process tends to be governed by the female. Females in estrus urinate

frequently, thereby attracting the attention of nearby males. Having located an estral female, the male will follow her intermittently for several days, repeatedly testing her receptivity until she acquiesces or is no longer in estrus. This courtship often involves lengthy chases and fighting (Goddard, 1966; Laurie, 1982; Laurie et al., 1983; Nowak, 1999). It is noteworthy that urine squirting by captive estral females does not always attract the sexual interest of nearby males.

The literature concerning the *in situ* proximate triggers of reproduction for Sumatran rhinos is contradictory, with some authors reporting that females have small permanent territories and males seek out estral females while others report that dominant males guard exclusive territories and wait for estral females to approach them. Groves and Kurt (1972) and van Strien (1985) found that males visit the territories of females and possibly fight over the latter after the young are weaned. Van Strien (1985) reported that males sometimes visited salt licks with the evident objective of meeting a female.

The second factor which limits reproduction rates in captivity is that Indian and Sumatran rhinos are mostly solitary in the wild, with adult males and females associating only for breeding (Flynn and Abdullah, 1984; Fouraker and Wagener, 1996; Groves and Kurt, 1972; IUCN, 1978; Laurie, 1982; Laurie et al., 1983; Nowak, 1999; van Strien, 1985). Placing a captive pair together when the female and/or the male are not sexually receptive can result in serious aggression, especially as neither animal can run away. Unfortunately, it is not always easy to recognize the state of sexual readiness in a rhino. Tong (1960) noted two instances in which pairs of Indian rhinos could not be reconciled

and had to be permanently separated. A pair of Indian rhinos at the Fort Worth Zoo (FW Zoo) in Texas, USA has been introduced many times and has often fought but never copulated (Wagener, pers. comm., 1998). Breeding programs for Sumatran rhinos have been underway for several years at sites in Sungai Dusun (Peninsular Malaysia) and Way Kambas (Sumatra, Indonesia) without achieving any pregnancies; in fact, male-female introductions have resulted in more fights and injuries than breeding (Khan et al., 1999).

The third difficulty arising from captive breeding is the fact that Indian – as well as Black and White - males and females often appear to come into breeding readiness at different times (Gowda, 1967; IUCN, 1966; Tong, 1960; Vahala et al., 1993). Jeanne Jacobsen of the FW Zoo reported (pers. comm., 1998) that their male Indian rhino displays "rutting behaviour" approximately every fifty-four days (roughly 13 days before the female begins to show signs of estrus). The male's "rut" lasts for one to two weeks and consists of vocalizing, urine spraying and charging the female rhinoceros and the keepers. Unfortunately, by the time the female is sexually receptive the male has usually lost interest. To date this pair has failed to breed. Staff at the Toronto Zoo (TO Zoo) in Ontario, Canada have reported (pers. comm., 1998) that their male Indian rhinoceros tends to begin courtship displays a day or two before the female does and then quiets down once the female begins her display. The Toronto rhinos, however, are proven breeders with three calves to date.

According to Tong (1960), after two years of breeding failure, successful mating of a pair of Indian rhinos at the Whipsnade Zoo in Great Britain required hormone

therapy for the male. During the two years before this therapy the male appeared indifferent to the female when she was in estrus, yet he seemed interested in breeding two days later. Hormone therapy was also required for the successful breeding of a male Black rhino housed at the Mysore Zoo in India (Gowda, 1967). In a study at Dvur Kralove Zoo in the Czech Republic, Vahala et al. (1993) noted that, in a population of Northern White rhinos, the males tended to show sexual interest in the females one to three days before the females were in estrus.

The phenomenon of asynchronous male/female breeding states has not been reported for Sumatran rhinos, possibly because no one has conducted a formal study of their behaviour in captivity. The fact that this phenomenon is unknown in wild populations of any rhino species suggests that the problem may be an effect of captivity, although the scarcity of comprehensive *in situ* studies makes it difficult to be certain.

Responding to the Challenge

The endocrine cycle of female reproduction has been documented for the Indian rhinoceros (Bellem and Goodrowe, unpublished data; Kasman et al., 1986; Kassam and Lasley, 1981), and some work has been done on Sumatran females (Heistermann et al., 1998; Roth et al., 2001). Analysis of female hormones has been used successfully at TO Zoo to assist in the breeding of its Indian rhinos, and this breeding resulted in a healthy calf. The use of hormone analysis alone is not always successful in timing introductions, however, as evidenced by the pair of Indian rhinos at the FW Zoo. The FW female has

been studied extensively and the pair has been introduced many times, yet has never copulated. There are also disadvantages to the use of hormone analysis. The collection and processing of blood, urine and/or fecal samples requires a substantial commitment of time and money, as well as proper facilities and a trained staff.

Previous studies on all species of rhinoceros have focused on either reproductive physiology (Berkeley et al., 1997; Brett et al., 1989; Heistermann et al., 1998; Hindle and Hodges, 1990; Hindle et al., 1992; Radcliffe et al., 1997; Roth et al., 2001; Schaffer et al., 1994; Schwarzenberger et al., 1993, 1996 & 1998) or behaviour (Dinerstein and Price, 1991; Dinerstein et al., 1988; Goddard, 1966; Laurie, 1982; Vahala et al., 1993; van Strien, 1985); there are no reports that combine these disciplines. Kasman et al. (1986) studied female Indian rhinos and mentioned that keepers were noting behaviours associated with estrus (whistling, increased urination), but no results were presented. Kassam and Lasley (1981), also studying female Indian rhinos, matched urinary hormone metabolites with behavioural data collected by the animal keepers, but no information was given regarding the methods of data collection or analysis, nor even which behaviours related to estrus. Patton et al. (1999) analyzed fecal pregnanes and mating behaviour in the Southern White rhino, but the only behaviours mentioned are mounting and copulation, and little information was provided regarding data collection or analysis. In addition, there are no published studies on male reproductive hormones of any rhino species.

The theoretical goal of this project was to develop a more comprehensive understanding of rhinoceros reproduction by determining whether relationships exist between the female estrous cycle, male testosterone levels and the behaviour of female and male Indian and Sumatran rhinos. By way of example, the results may help to explain how males and females who are solitary and widely dispersed (e.g. the density of Indian rhinos is as low as 0.4 rhinos per km^2 in grasslands as tall as 8 m – Laurie et al., 1983) manage to locate one another and to synchronize their physiological states. This project used an experimental protocol which integrated the disciplines of endocrine physiology and ethology.

The practical goal of the project was to create a behavioural chart, validated by hormone analysis, which would assist animal managers in predicting the times when rhinos are ready to breed. Such a chart could 1) replace, or at least reduce the need for, expensive, time-consuming and labour-intensive hormone analysis; 2) lessen the chance of injury to the animals and 3) improve reproductive rates.

Before research on Sumatran rhinos could proceed it was necessary to first develop technology that would allow for easy monitoring of the reproductive cycle of the female Sumatran rhinoceros. Although analysis of fecal progestogen metabolites by radioimmunoassay (RIA) had previously been found to be useful in monitoring the reproductive cycle of Black, White and two Sumatran female rhinos (Berkeley et al., 1997; Patton et al., 1999; Radcliffe et al., 1997; Roth et al., 2001), few of the facilities that house Sumatran rhinos are equipped to conduct assays involving radioactivity (i.e.

RIA). For facilities with limited research budgets and laboratory space enzyme immunoassay (EIA) offers an affordable alternative to RIA while still permitting accurate assessment of reproductive activity. Besides low cost, EIA has several other advantages over RIA, namely, ease and speed of performance, easy reading of end point and avoidance of the dangers and difficulties associated with the acquisition, use and disposal of radioactive isotopes (Bellem et al., 1995; Hodges, 1986; Hodges and Green, 1989; Kirkpatrick et al., 1990; Kirkpatrick et al., 1991). For these reasons it was deemed necessary to develop an EIA, preferably one comparable to the existing RIA, before beginning any studies of Sumatran rhinos.

CHAPTER 1 - THE INDIAN RHINOCEROS

(*Rhinoceros unicornis*)

INTRODUCTION

Life History of the Indian Rhinoceros

The Indian (or Greater One-Horned) rhino ranges in height (at the shoulder) from 148 to 193 cm (4'10" - 6'4") and in length (snout to root of tail) from 310 to 429 cm (10'2" - 14'1"). Weight ranges from 1,600 to 2,200 kg (3536 lbs. - 4862 lbs.) and the single nasal horn is normally 15 to 45 cm (6" – 18") in length. Both sexes have lower incisor tusks which reach up to 20 cm (8") in males. The most distinguishing feature of this species is the fact that it appears to be protected by large, studded armour plates – the skin is covered with large, hard, convex tubercles and folds around the neck, in front of, below and behind the shoulder and in front of, across and below the thigh. Males show enormous development of the neck folds. The grey brown skin is hairless except for fringes of stiff hairs around the ears and the tip of the tail, and the skin is quite pink within the folds (Laurie, 1982; Laurie et al., 1983; Nowak, 1999).

Formerly found across northern Pakistan, northern India and Assam, Nepal, Bhutan and northern Bangladesh, the Indian rhino is now restricted to the Brahmaputra Basin Valley in Assam (north eastern India), West Bengal (a small area in India, just south of Bhutan) and the Chitwan Valley region of the Nepal Terai (Foose and van Strien, 1997; IUCN, 1978; Khan, 1989; Nowak, 1999). Approximately 2400 animals survive in the wild (Wirz-Hlavacek et al., 1998), and 137 in captivity (Wirz-Hlavacek, 2001). The Indian rhino prefers early successional, open and marshy habitats such as

alluvial plain grasslands (where grass can reach a height of 8 m) and adjacent swamps and forests, although some animals live in drier forest areas, especially in Nepal. Due to human encroachment the range of Indian rhinos is now severely restricted, forcing them to inhabit cultivated areas, pastures and modified woodlands (Foose and van Strien, 1997; IUCN, 1978; Khan, 1989; Laurie et al., 1983; Nowak, 1999).

Indian rhinos are generally solitary. They may rest or feed in close proximity at wallows and open grazing grounds but then move independently, with the exception of cow-calf pairs and temporary associations of a few sub-adults, usually males (Laurie, 1982; Laurie et al., 1983; Nowak, 1999). Laurie (1982) reported that 5.2% of observations of Indian rhinos were of groups of more than two individuals and 0.2% of groups of more than three individuals. Corresponding values for the two African species (10.6% and 1.7% for Black rhinos, 17.2% and 3.4% for White rhinos) indicate that the Indian rhino is less social than its African relatives (Laurie, 1982; Owen-Smith, 1975).

Population density varies. Some areas have densities of 0.4 to 2.0 animals per km^2 (Laurie et al., 1983; Nowak, 1999), while Laurie (1982) reported densities of up to 4.85 per km^2 in the areas with the highest diversity of vegetation, as well as easy access to water and agricultural land. Dinerstein and Price (1991) found densities of 1.7 to 3.2 per km^2 in grasslands and a maximum of 13.3 per km^2 in riverine forest. Only the strongest of the males participate in breeding. These males have home ranges (2 to 8 km^2) which are somewhat exclusive but not truly territorial – the ranges of dominant

bulls overlap with each other and with the ranges of non-breeding males (Laurie, 1982; Laurie et al., 1983; Nowak, 1999).

Indian rhinos are grazers, with grass (as many as 50 species) making up 70 to 89% of their diet, depending on the season (Laurie, 1982). Grass is supplemented by aquatic plants, fruit, leaves and branches from trees and shrubs. The prehensile upper lip helps in grasping and biting off food. Animals living near human settlements have been known to raid agricultural crops for food, a problem which is steadily worsening as the destruction of their natural habitat forces rhinos ever closer to humans (IUCN, 1978; Laurie et al., 1983; Nowak, 1999). Daily access to water and regular visits to a natural salt lick are critical (Laurie et al., 1983; Nowak, 1999). Laurie (1982) found that significant seasonal variations in plant productivity led to movements of rhinos between vegetation types – home ranges were therefore smallest in the areas of greatest food diversity.

The Indian rhino is not divided into sub-species, but the IUCN does recognize two distinct populations (IUCN, 1996). The first population is located in Bhutan, India (Uttar Pradesh) and Nepal and the second in India (Assam and West Bengal). Currently, captive breeding programs do not recognize the division between populations (Foose and van Strien, 1997).

Endocrinology and Reproduction in the Indian Rhinoceros

Laurie (1982) described the courtship rituals of Indian rhinos *in situ*. Adult males followed the tracks of females, sniffing the ground and performing flehmen. Having located an estrus female the male accompanied her, at a distance, for up to several days, occasionally making attempts to approach more closely. The female usually repelled the male's advances by turning and snorting, but sometimes the male continued to advance, with his head down, until they were standing horn to horn. "Fights developed...with both rhinos trying to push each other back, the female in particular roaring and bleating loudly between lunges, horn clashes and tusk displays. Once a male turned a female over on her back and several times deep tusk wounds were inflicted. If the female turned and ran the male chased her, sometimes over several kilometres." (p. 328)

Female Indian rhinos are polyestrous year-round, with an average cycle length of 43 to 48 days (Kasman et al., 1986; Kassam and Lasley, 1981; Laurie, 1982; Tong, 1960). The gestation period for the Indian rhinoceros is approximately 15.5 to 16.5 months or 462 - 491 days (IUCN, 1978; Laurie et al., 1983; Nowak, 1999), while the inter-birth interval is 3 to 5 years (Dinerstein and Price, 1991; Laurie, 1982; Nowak, 1999). Calves usually remain with their mother until just before the next calf is born (Nowak, 1999). Females are full-grown at 6.5 years (Laurie et al., 1983; Nowak, 1999) and reach sexual maturity between the ages of 5 and 7.5 (Dinerstein and Price, 1991; Fouraker and Wagener, 1996; Nowak, 1999; Wirz-Hlavacek et al., 1998). Males are full-

grown at 10 years (Laurie et al., 1983; Nowak, 1999) and reach sexual maturity at the age 9 or 10 (Fouraker and Wagener, 1996; Wirz-Hlavacek et al., 1998).

Analysis of urinary steroid metabolites has been shown to be a practical and accurate method for monitoring female reproductive function in a variety of mammalian species (Kasman et al., 1986; Lasley, 1985). Major reproductive events in the mature female Indian rhino can be accurately assessed by the collection of regular urine samples (at least three times per week – Kasman et al., 1986) and the measurement of estrogen and/or progesterone urinary metabolites (Kassam and Lasley, 1981; Loskutoff et al., 1983). The combination of these two profiles allows a complete evaluation of ovarian steroid production. Although a regular pattern of estrogen fluctuations is a good indication that ovulation is occurring, estrogen profiles alone do not constitute proof. Confirmation of ovulation necessitates the measurement of progesterone production to reflect CL function (Kasman et al., 1986; Kassam and Lasley, 1981).

The major estrogen metabolite (> 95%) in the urine of the female Indian rhinoceros is conjugated estrone (EC), and 95-99% of this conjugated estrone is in the form of estrone sulfate. Estrone sulfate is consistently the major estrogen metabolite in all stages of the reproductive cycle and should reflect total estrogen excretion (Kasman et al., 1986; Kassam and Lasley, 1981). Loskutoff et al. (1983) presented preliminary data indicating that pregnanediol-3-glucuronide (PdG) is a major urinary progestogen metabolite in a number of species and is useful for monitoring corpus luteum function in the Indian rhinoceros.

Kassam and Lasley (1981) studied estrogen profiles in two Indian rhino females. They found that urinary estrogen increased progressively for a period of 13 to 18 days, with elevations beginning approximately 10 days before overt behavioural estrus and then returning to base-line values between 3 and 8 days after estrus. Peak estrogen values, at or just before the onset of behavioural estrus, ranged from 5.3 to 12.5 μg/mg Cr. The period of sexual receptivity (based on female behavioural patterns rather than male-female contact) averaged 3 days (n = 5 animals, range 2 to 5 days). Tong (1960), however, reported that sexual receptivity is limited to a 24-hour period, rather than the several days over which behavioural signs of estrus may be apparent.

According to a study by Kasman et al. (1986) of 6 female Indian rhinos, baseline estrone sulfate (ES) values in urine were 0.8 ± 0.3 μg/mg Cr. The ES values stayed baseline from day 25 to day 18 prior to D0, then began to increase (D0 was the day of the precipitous decline of ES – presumably, this decline was soon followed by ovulation). The highest value of ES just before D0 was 164.0 μg/mg Cr. After the sharp drop which occurred on D0, the ES values continued to decrease slowly back to baseline over a period of 1 to 7 days. The average length of the follicular phase was 14.8 days (range 13-19).

In the same study, baseline PdG values (days 23 to 6 prior to D0) were 20.4 ± 1.3 ng/mg Cr. PdG began to rise between days 5 and 1 prior to D0 and remained elevated until approximately day 18 after D0, at which time the level dropped sharply. The average PdG value over the time of elevation was 125 ± 13.4 ng/mg Cr (range 40.0 to

278.0 ng/mg Cr). The rise of PdG at the time of declining ES reflects the formation of an active CL and confirms ovulation. The luteal phase, defined as the interval from D0 to the day of the precipitous decline of PdG, was 19.0 days (range 17-21).

No studies have been published on male reproductive hormones in any rhino species – from anecdotal reports it is generally assumed that post-pubertal males are capable of breeding at any time.

Purpose of Study

This research was initially designed to test two hypotheses. The first was that female behaviour will relate to female reproductive hormones, in that certain behaviours will occur far more often (or only) when estrogen levels are high (indicating that the female is in estrus). Based on earlier studies of Indian, Black and White rhinos, the female behaviours expected to begin or become more frequent with rising estrogens included urination, defecation, foot scraping and whistling (Goddard, 1966; Kasman et al., 1986; Laurie, 1982; Laurie et al., 1983; Nowak, 1999). The second hypothesis was that male behaviour will relate to the reproductive hormones of nearby females, particularly increasing estrogen levels. Male behaviours expected to begin or become more frequent with rising estrogens, based on earlier studies of Indian, Black and White rhinos, included urination, defecation, foot scraping, loud vocalizations and increased aggression, particularly charging (Goddard, 1966; Jacobsen, pers. comm., 1998; Laurie, 1982; Laurie et al., 1983; Nowak, 1999). To test these hypotheses, a male-female pair of

Indian rhinos at the Toronto Zoo (TO Zoo) in Ontario, Canada was studied over the course of six of the female's estrus periods. During this time urine samples (for endocrine analysis) were collected from the female and the behaviour of both animals was observed.

After studying the first pair of Indian rhinos two other hypotheses were developed. The first was that some female behaviours (especially aggressive or fearful behaviours such as bellowing, blow-snorting and head bobbing) will be related to the testosterone (T) levels of nearby males, rather than to female estrogens, and that these behaviours will occur more often (or only) when T is high. The second new hypothesis was that some male behaviours (especially aggressive behaviours such as bellowing, blow-snorting and charging) will begin or become more frequent when male T levels are high. Further data collection - three estrus periods from a male-female pair at the Fort Worth Zoo (FW Zoo) in Texas, USA - thus also included the collection of fecal samples (for endocrine analysis) from the male.

Based on anecdotal information, the T profile was expected to follow one of four trends: 1) no discernible pattern, which is the case with Black and White rhino males (Roth, pers. comm., 2000); 2) T increases 24 to 60 hours after each of the female's EC peaks (i.e. at the time of ovulation); 3) increased T whenever the male was displaying overt sexual behaviour or 4) increased T whenever the male was introduced to the female.

MATERIALS and METHODS

Experimental Animals

Captive animals were chosen as study subjects because of time and financial restrictions and ease of data collection as compared to studying animals in the wild. Two male-female pairs of Indian rhinos were studied. The first pair, which had previously produced 2 calves, was housed at the TO Zoo. The female (Indira) was captive born in 1975 and shipped to Toronto in 1979. The male (Patrick) was captive born in 1974 and arrived at the TO Zoo in 1989.

The second pair was housed at the FW Zoo. The female (Arati) was born wild in Nepal (estimated birth date September, 1989) and arrived in Fort Worth in May 1990. The male (Arun) was also born wild in Nepal (estimated birth date November, 1989) and arrived in Fort Worth at the same time as the female. At the time the Fort Worth portion of the study began both animals were 9 years old, so they should have been sexually mature. Despite many introductions from 1997 to the present, these animals have never copulated, although an earlier study of the female (Bellem and Goodrowe, unpublished data) had revealed that she was cycling regularly.

Housing and Care of Animals

The Toronto pair was housed separately (except for the time of breeding introductions) but had continual auditory and olfactory contact and occasional visual

contact. They were kept indoors overnight in cement pens (average size 21' by 18'). During the day one of the rhinos was put into an indoor exhibit (roughly 36' by 24') while the other was either released into an outdoor exhibit (roughly 174' by 80') or left in the overnight pen, depending on the weather. Daily routine varied considerably in terms of which exhibit enclosure the rhinos were released into and what time they were brought in and out.

In the morning the rhinos were each fed half a bale of timothy hay if they were kept inside or a full bale each if they were put in the outside paddock (1 bale is usually 12 flakes - 6 lbs. or 2.3 kg per flake). Overnight they were each given a full bale of timothy hay, 1.8 to 2.8 kg (4-6 lbs.) of Toronto Zoo Herbivore Cubes (Land-o-Lakes, Toronto, Canada), 6 apples, 6 carrots and 1 head of iceberg or romaine lettuce. The Toronto Zoo Herbivore Cubes were made by Land-o-Lakes for the Zoo according to the specifications of the Zoo's nutritionist. Water was always available (E. Valdes, pers. comm., 2001).

The Fort Worth pair was kept apart until introductions began in the spring of 1997, although like the Toronto animals, the Fort Worth rhinos were always within auditory and olfactory distance of each other. Overnight the pair was housed in cement stalls (15' by 20'), and during the day they were released into adjacent outdoor exhibit enclosures (roughly 150' by 30'). They were fed Coastal Bermuda grass hay: 0 to 8 kg (0-18 lbs.) in the morning (depending on how much remained from the night before), 22 to 27 kg (48-60 lbs.) at night and roughly 11 kg (24 lbs.) during the day in the outside yards.

Grain was also fed, in the form of a _" low fibre (not more than 14% fibre) pellet designed by the Zoo's nutritionist, and manufactured by Mazuri® (St. Louis, MO, USA). The female was given 3.5 kg (8 lbs.) of grain each morning, the male 3 kg (6 lbs.) to 5.5 kg (12 lbs.). When the animals came into the barn at night the female was given 4.5 kg (10 lbs.) of grain and the male 3 to 5.5 kg (6 to 12 lbs.). Fresh water was always available (A. Ward, pers. comm., 1998).

Sample Collection

Urine samples were collected from the Toronto female during the period commencing August 9, 1996 and ending December 9, 1996 and again from May 16, 1997 to September 23, 1997. Urine was collected three to four times per week, as soon as the zookeepers started morning chores (about 8 am). As was done in previous studies (Kasman et al., 1986; Kassam and Lasley, 1981), urine was aspirated from the floor using a clean syringe and placed into a 12 x 75 mm polypropylene tube. The tube was then capped, labelled with the date and the animal's name and frozen at -20° C as soon as was feasible.

Work with the Fort Worth pair began July 7, 1998 and ended December 2, 1998. Urine was collected from the female, 5 to 6 days per week, in a cup before it contacted the floor (occasionally urine had to be aspirated off the floor using a clean syringe). Urine was then transferred into a 12 x 75 mm polypropylene tube, labelled with the date and the animal's name and stored immediately at –20° C. Fecal samples were collected

from the male, 5 to 6 days per week, as soon as possible after defecation. Samples were collected by taking several small pieces from different areas of each dung pile and mixing the pieces together in a zip-lock plastic freezer bag. The bags were labelled with the date and the animal's name and stored immediately at -20° C.

Urine and fecal samples were later boxed with dry ice and shipped by overnight courier to the TO Zoo, where they were again stored at –20° C until analyzed for hormone content (estrogen and progesterone metabolites in the females' urine, and testosterone metabolites in the male's feces).

Behaviour Observations

Behaviour data were collected from all of the study subjects. Behavioural observations were conducted, usually on the same days as sample collection, for 2 to 3 hours in the morning. (Observations were conducted in the morning because rhinos are known to be most active in the early morning and evening, when feeding generally takes place, as well as at night - Groves and Kurt, 1972; IUCN, 1978; Laurie et al., 1983; Nowak, 1999; van Strien, 1974). Most of the behavioural data were collected by the author, but three other people assisted. All of the assistants were personally trained by the author, and standardized forms were used for recording data. Average inter-observer reliability, calculated by the formula given in Monette et al. (1990), was 88.4% (range 78.5 to 97.4%). According to Monette et al. (1990), 75% is an acceptable level of

reliability and 85% can be expected from well-trained observers using a well-constructed coding scheme.

The design of the behaviour study was developed using preliminary data collected from the Toronto pair. Behaviours were classified as either continuous or discrete. Continuous behaviours (e.g. standing, lying down, walking and eating) were recorded at 5 minute intervals throughout the observation period. Each day's results were reported on the basis on the percentage of the observation period occupied by each behaviour. Discrete behaviours such as vocalizations, trotting/cantering and urine spraying were counted each time they occurred. Results were reported either as percentage of observation period or as number of occurrences per hour of observation. The rhino ethogram in Appendix I lists and defines all behaviours recorded in this study.

At the time the Fort Worth portion of the study began, keepers at this site were timing introductions of the male to the female based on the female's vaginal discharge, assuming that she ovulated around the time of maximum discharge. For this reason, observations of vaginal discharge were included in this part of the study. It was not possible to collect and measure all secretions so the observations were ranked subjectively (0 indicating no discharge and 3 the maximum amount observed).

Definition of Estrous Cycle

In this study an estrous cycle was defined as the interval between two pre-ovulatory EC peaks. To be labelled pre-ovulatory an EC peak had to rise two or more

standard deviations above the EC baseline and be followed within 15 days by a post-ovulatory PdG peak (to be labelled post-ovulatory a PdG peak had to rise two or more standard deviations above the PdG baseline). To calculate baseline concentration, the mean of all values was taken, any values more than two standard deviations on either side of the mean were discarded, and the new mean of the remaining values was designated the baseline. One-tailed *t*-tests (Evans, 1992) were used to confirm that the EC and PdG peaks were significantly greater than their respective baselines.

Endocrine Analyses

Hormonal analyses of Indian rhino samples were performed in the endocrine lab at the TO Zoo.

Urine Analyses

Urine samples were analyzed by EIA for estrogen metabolites (estrone conjugates, EC) and progestogen metabolites (pregnanediol-glucuronide, PdG). These EIAs were first described by Munro et al. (1991), then adapted for use at the TO Zoo in lowland gorillas (Bellem et al., 1995) and in Indian rhinos (Bellem and Goodrowe, unpublished data).

Creatinine (Cr) Assay

Measurement of creatinine (Cr) was used to compensate for differences in the concentration of urine samples (Bellem et al., 1995; Hodges, 1986; Kasman and Lasley, 1986 and Taussky, 1954), so EC and PdG values were presented as mass per milligram

(ng/mg) of Cr. Urine samples were analyzed for Cr using a microcolorimetric assay previously described by Taussky (1954). Samples with Cr values under 0.1 mg/ml were eliminated from endocrine analyses (Bellem et al., 1995).

Estrone Conjugates (EC) Enzyme Immunoassay

The anti-EC polyclonal antibody (Ab) R522 was provided by C. Munro of the University of California, Davis (refer to Appendix IV for details on all reagents), and the cross-reactivities (see Appendix II) were taken from Bellem et al. (1995) and Munro et al. (1991). This Ab cross-reacted primarily with estrone-3-glucuronide at 100% and estrone-3-sulfate at 66.6%. The primary Ab stock was prepared by a 1:10 dilution in Coating Buffer (Appendix III contains recipes for all solutions used) and stored in 100 µl aliquots at –20° C until needed. Twenty-four hours before the assay was to be conducted, the primary Ab stock was further diluted 1:500 in Coating Buffer to make a 1:50, 000 working Ab solution. NUNC MaxiSorp microtitration plates were coated with 50µl/well of the working Ab and tapped lightly to ensure that the wells were evenly coated. The first column on these plates (wells A1 to A12) was not used because of reportedly high variability in Ab binding (A.C. Bellem, pers. comm., 1998). Each plate was then covered with an acetate plate sealer to prevent evaporation and incubated overnight (minimum 12 hours) at 4° C in a styrofoam container.

After overnight incubation, the plates were washed 5 times in a Dynatech microplate washer to remove unbound Ab, then tipped upside-down and banged against a paper towel-covered surface to remove excess Wash Solution. Plates were immediately

coated with 50 µl/well (including wells A1 and A2, which are non-specific binding or NSB wells) of EIA Assay Buffer and allowed to incubate at room temperature for 2 to 5 hours. The NSB wells were used to check whether the substrate was binding directly to the plate or to any molecules in the Assay Buffer or HRP.

Immediately prior to conducting the assay, estrogen standards, samples and controls were prepared in 12 x 75 mm glass culture tubes. The primary standard stock was prepared by weighing 0.1 mg of estrone-ß-D-glucuronide into 100 ml of ethanol (EtOH) for a 1 µg/ml solution. The top standard (10 ng/ml) was prepared by a 1:100 dilution of primary stock in EIA Assay Buffer, then divided into 1 ml aliquots and stored at –20° C until needed. In preparation for plate loading the top standard was diluted 1:2 in EIA Assay Buffer to yield an EC standard curve with concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039 and 0 ng/ml ('0' wells contained no Ab or steroid antigen). The functional sensitivity of the assay, based on the lowest standard dilution, was 0.039 ng/ml of urine.

Urine samples were diluted in Assay Buffer according to the 50% binding concentrations determined by parallelism (refer to Assay Validation – Parallelism, below). The primary control stock had previously been prepared by A.C. Bellem using urine from pregnant lowland gorillas, and was stored at -20° C until needed. The internal controls were made-up by diluting the control stock in EIA Assay Buffer - 1:10 for the high control (C1- 30% binding) and a further 1:5 for the low control (C2 – 30% binding).

When the plates were ready for loading, 20 µl volumes of standards, controls and samples were pipetted according to the plate map in Fig. 3. Standards were pipetted in triplicate: one standard curve in duplicate at the beginning of the plate and a single curve at the end to account for the effects of time lag and technician fatigue in plate loading. Controls and samples were pipetted in duplicate for purposes of quality control. Once all standards, controls and samples had been loaded, 50 µl of enzyme-labelled hormone (estrone-glucuronide-horseradish peroxidase or E-HRP) were pipetted into each well. In this plate-loading step, the NSB wells were loaded with E-HRP only. The original E-HRP (provided by C. Munro) was stored at -20° C. The primary stock was prepared by diluting E-HRP 1:100 in EIA Assay Buffer, then stored at 4° C until needed. The working solution was prepared, just before plate loading, by a further 1:250 dilution in Assay Buffer (final working dilution 1:25,000).

The loading step described above (addition of standards, controls, samples and E-HRP) had to be completed within a maximum of 10 minutes to minimize the effects of time lag in antibody binding. After loading, plates were covered and incubated for 2 hours at room temperature. Just before the end of the incubation period, substrate solution was prepared by combining 125 µl of 40 mM ABTS (3-ethylbenzthiazoline-6-sulfonic acid), 40 µl of 0.5 M H_2O_2 and 12.5 ml of Substrate Buffer. As soon as the substrate solution was prepared the plates were washed and dried and 100 µl of substrate solution were pipetted into each well. The plates were covered and slowly shaken on a flat rotator at room temperature until the substrate had reached sufficient colour intensity

	1	2	3	4	5	6	7	8	9	10	11	12
A	NSB	0	S4	S8	T1	T5	T9	T13	T17	T21	T25	S4
B	NSB	0	S4	S8	T1	T5	T9	T13	T17	T21	T25	S5
C		S1	S5	S9	T2	T6	T10	T14	T18	T22	T26	S6
D		S1	S5	S9	T2	T6	T10	T14	T18	T22	T26	S7
E		S2	S6	C1	T3	T7	T11	T15	T19	T23	0	S8
F		S2	S6	C1	T3	T7	T11	T15	T19	T23	S1	S9
G		S3	S7	C2	T4	T8	T12	T16	T20	T24	S2	0
H		S3	S7	C2	T4	T8	T12	T16	T20	T24	S3	0

Fig. 3 Microtitre plate map.
NSB = Non-Specific Binding; **0** = '0' standard (no hormone); **S1-S9** = standards, S1 is low and S9 is high;
C1 = high control (~ 30% binding); **C2** = low control (~ 70% binding); **T1-T26** = samples.

to indicate that the plates were developed ('0' wells between 0.9 to 1.1 optical density units). Colour development ranged from 15 to 60 minutes. The optical density of each well was read using a Dynex Technologies MRX plate reader (test filter 405 nm, reference filter 630 nm) interfaced with a Dell OptiPlex GX1 Pentium II Computer. The software used was Revelation Version 4.21 by Dynex Technologies, Copyright © 1999.

Pregnanediol-Glucuronide (PdG) Enzyme Immunoassay

The anti-PdG polyclonal Ab (P70) was provided by C. Munro, and the cross-reactivities (Appendix II) were taken from Bellem et al. (1995) and Munro et al. (1991). This Ab cross-reacted primarily with pregnanediol-3-glucuronide (100%) and 20α-hydroxyprogesterone (60.7%). The primary Ab stock was prepared by a 1:50 dilution in Coating Buffer and stored at -20° C until needed. Twenty-four hours before the assay was to be conducted, the primary Ab stock was further diluted 1:200 in Coating Buffer to make a 1:10,000 working Ab solution.

The primary standard stock was prepared by weighing 0.5 mg of 4-pregnen-20α-ol-3-one into 10 ml of EtOH for a 50 μg/ml solution. The top standard (500 ng/ml) was prepared by a 1:100 dilution of primary stock in EIA Assay Buffer, then divided into 1 ml aliquots and stored at –20° C until needed. In preparation for plate loading the top standard was diluted 1:2 in EIA Assay Buffer to yield a PdG standard curve with concentrations of 500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953 and 0 ng/ml ('0' wells contained only Assay Buffer). The functional sensitivity of the assay, based on the lowest standard dilution, was 1.953 ng/ml of urine.

Urine samples were diluted in Assay Buffer according to the 50% binding concentrations determined by parallelism (refer to Assay Validation – Parallelism, below). The primary control stock had previously been prepared by A.C. Bellem using urine from pregnant lowland gorillas, and was stored at -20° C until needed. The internal controls were made-up by diluting the control stock in EIA Assay Buffer - 1:10 for C1 and a further 1:5 for the C2.

The enzyme-labelled hormone used was pregnanediol-glucuronide-horseradish peroxidase or P-HRP, provided by C. Munro. The original P-HRP was stored at -20° C. The primary stock was prepared by diluting P-HRP 1:100 in EIA Assay Buffer, then stored at 4° C until needed. The working solution was prepared (immediately before beginning plate loading) by a further 1:250 dilution in Assay Buffer (final working dilution 1:25,000).

Except for these different reagents, the PdG assay protocol was exactly the same as that described for EC above.

Fecal Analysis

The decision to study male hormone levels was not made until data collection in Toronto was almost finished, so only a single male (Arun from Fort Worth) was included in this study. All of Arun's fecal samples were analyzed for androgen metabolites using a testosterone (T) EIA previously described by Walker (1999) for male red wolves.

Fecal Steroid Extraction – Androgen Metabolites

Thawed fecal samples were thoroughly mixed within their plastic bags. From each sample, 0.50 g of wet feces were weighed out and placed into labelled 16 x 125 mm glass extraction tubes. For each sample a labelled 12 x 75 mm polypropylene tube was filled with feces, capped and stored at -20° C in case of future need. Next 1.0 g of aluminum oxide (Al_2O_3) powder, 0.5 ml of dH_2O and 4.0 ml methanol (MeOH) were added to each glass extraction tube. The tubes were capped, vortexed for 30 seconds and mixed for one hour at room temperature on a platelet mixer. After mixing, the tubes were centrifuged for 10 minutes at 3500 rpm (centrifuge rotor radius 17.4 cm). The supernatants (methanol phase) were transferred into 12 x 75 mm polypropylene tubes and the labelling tapes were transferred from the glass extraction tubes to the polypropylene storage tubes. Extracts were stored at -20° C until required for analysis.

Testosterone (T) Enzyme Immunoassay

The anti-T polyclonal Ab (R156/7) was provided by C. Munro, and the cross-reactivities (Appendix II) were taken from Walker (1999). This Ab cross-reacted primarily with T (100%) and 5α-dihydrotestosterone or 5-DHT (57.37%). The primary Ab stock was prepared by a 1:50 dilution in Coating Buffer and stored at -20° C until needed. Twenty-four hours before the assay was to be run, the primary Ab stock was further diluted 1:200 in Coating Buffer to make a 1:10,000 working Ab solution.

The primary standard stock was prepared by weighing 0.1 mg of 17ß-hydroxy-4-androsten-3-one into 1 ml of EtOH for a 0.1 mg/ml solution, and the secondary stock was

prepared by diluting the primary stock 1:100 in EtOH for a 1000 ng/ml solution. Both stocks were stored at -20° C. The top standard (25 ng/ml) was prepared by diluting the secondary stock 1:40 in EIA Assay Buffer. This standard was then diluted 1:2 in Assay Buffer to yield a T standard curve with concentrations of 25, 12.5, 6.25, 3.13, 1.56, 0.781, 0.390, 0.195, 0.096 and 0 ng/ml ('0' wells contained only Assay Buffer). The functional sensitivity of the assay, based on the lowest standard dilution, was 0.096 ng/ml of fecal extract.

Fecal extracts were diluted in Assay Buffer according to the 50% binding concentrations determined by parallelism (refer to Assay Validation – Parallelism, below). The primary control stock had previously been prepared by S. Walker, using Fecal Steroid Extraction Method 1 (Walker, 1999) and the feces of male red wolves (collected during their breeding season). The primary stock was stored at -20° C until needed, and then the internal controls were made-up by diluting the control stock in EIA Assay Buffer - 1:65 for C1 and a further 1:10 for C2.

The enzyme-labelled hormone used was testosterone-horseradish peroxidase (T-HRP), provided by C. Munro. The original T-HRP was stored at -20° C. The primary stock was prepared by diluting T-HRP 1:100 in EIA Assay Buffer, then stored at 4° C until needed. The working solution was prepared, just before beginning plate loading, by a further 1:150 dilution in Assay Buffer (final working dilution 1:15,000).

The T EIA protocol was identical to that of the EC EIA described above, with the exception of the following. After the initial wash to remove unbound antibody, 50 µl of

EIA Assay Buffer were pipetted into each well and the plates were covered and incubated at room temperature for 30 minutes (instead of 2-5 hours) before plate loading. In the plate loading step, 50 µl of standards, controls and samples were added to each well (instead of 20 µl).

Assay Validation – Parallelism (EC, PdG and T)

Parallelisms for female Indian rhino urine on the TO Zoo EC and PdG EIAs had previously been established (Bellem, pers. comm., 1996). In accordance with these earlier parallelisms, the urine samples from Arati were run at 1:200 on the EC EIA and 1:10 on the PdG EIA (Bellem and Goodrowe, unpublished data). The suitability of these dilutions was confirmed by evaluating the percent binding of each sample (samples falling within the range of 25% to 75% binding were accepted).

Urine from Indira, however, had very high percent binding when analyzed at these dilutions, so two more parallelisms were run to determine the correct sample concentrations for this animal. To evaluate EC parallelism, a portion of each sample was pooled into a single tube and mixed well. The pooled sample was then serially diluted 2-fold in EIA Assay Buffer to yield a range of dilutions from neat (undiluted) to 1:8192. The parallelism plate was run as described above for the EC EIA, with the standard curve in triplicate, internal controls in duplicate and two dilution curves.

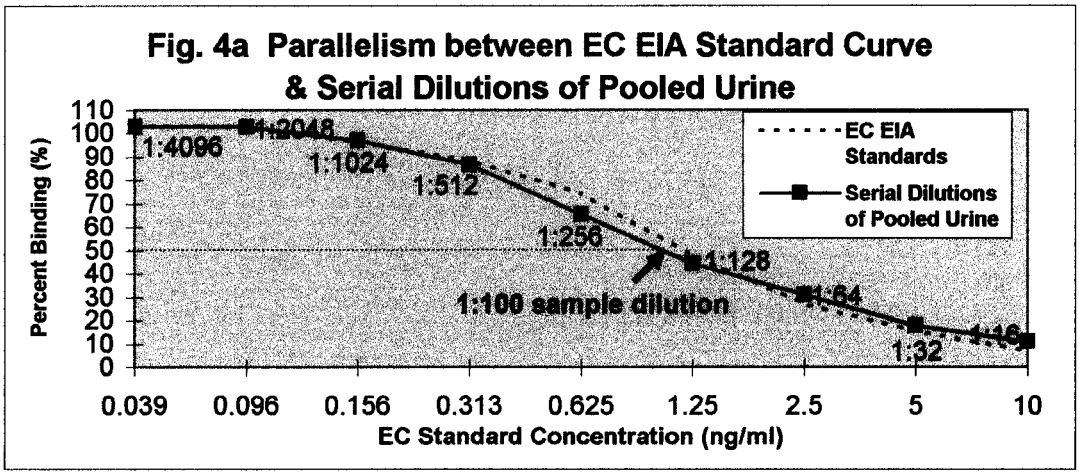
A parallel response of the sample dilutions and the EC standard curve indicated that immunoreactive estrogens were present in the urine and detected by the assay (Fig. 4a). A dilution of 1:100 was chosen for Indira's urine samples, based on approximately

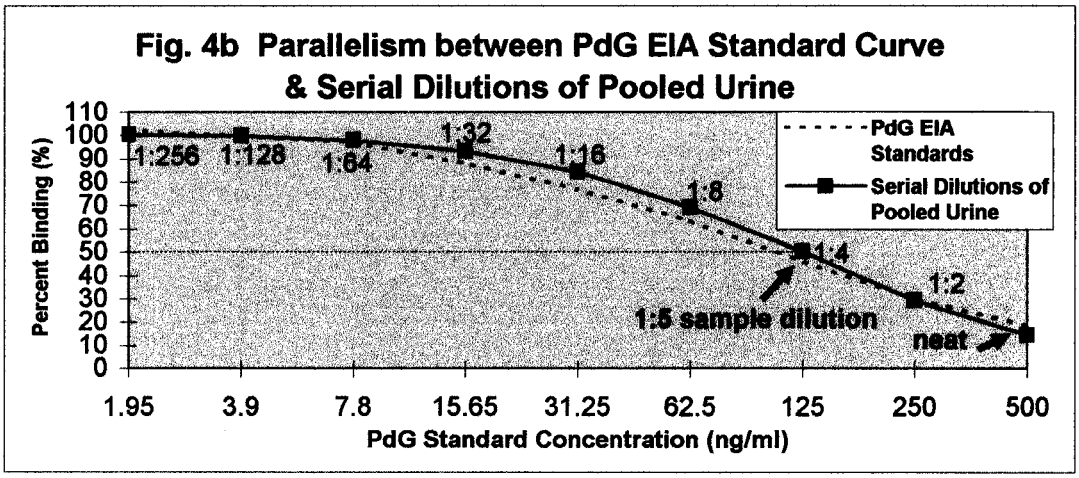
50% binding from the parallelism results. In Fig. 4 the mean percentage bindings measured for the standards were plotted (vertical axis) against their logarithmically transformed concentrations (horizontal axis). The sample dilutions were then plotted on the same graph.

The process described above was repeated to evaluate PdG parallelism for Indira's urine (Fig. 4b) and T parallelism for the fecal extracts from Arun (Fig. 4c). The only difference between these parallelisms and the one described above for EC is that the plates were run as described earlier for PdG EIA and T EIA, respectively. In both cases the sample dilutions and standard curves showed parallel responses. A sample dilution of 1:5 was chosen for Indira on PdG and 1:50 for Arun on T.

Assay Validation – Recovery (EC and PdG)

This validation had already been conducted by A.C. Bellem in the lab at the TO Zoo, using urine from Indian rhino females (Bellem and Goodrowe, unpublished data). The purpose was to examine the ability of the two assays to measure the required hormones in urine from Indian rhino females. To test the EC EIA, urine was spiked with increasing levels of exogenous EC (range 6.25 to 100 pg/well) then analyzed using the protocol described earlier for the EC EIA to obtain the observed pg/well values. The recovery, calculated as (observed value/expected value) x 100, was 93.8 ± 1.6%. To test the PdG EIA, urine was spiked with increasing amounts of exogenous PdG (range 0.312 to 5 ng/well) then analyzed using the protocol described earlier for the PdG EIA. The recovery was 96.2 ± 1.9%.





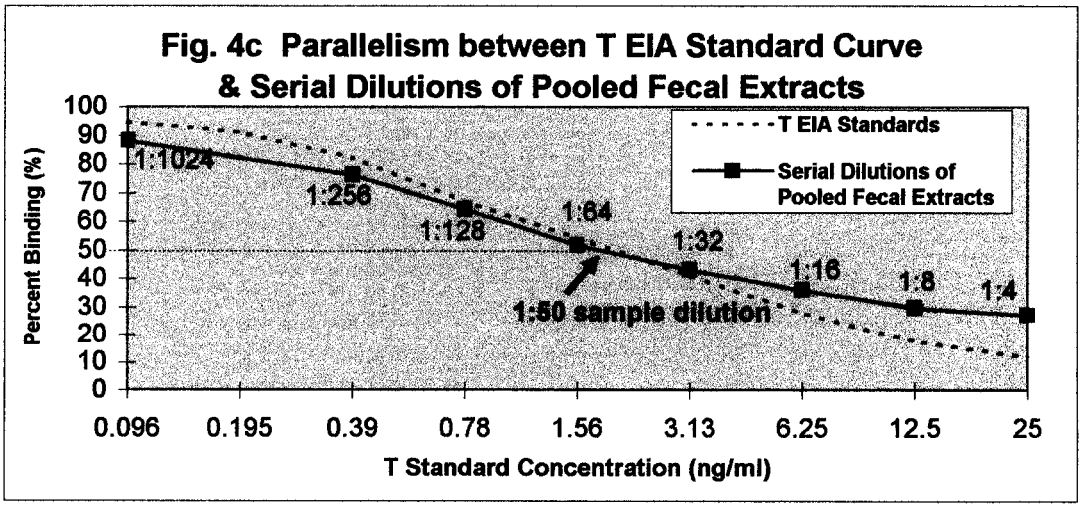


Fig. 4 Parallelisms for EC, PdG and T EIAs.

Assay Validation – Extraction Efficiency (T)

To assess the method of fecal steroid extraction described earlier, as well as the ability of the T EIA to measure T in fecal extracts from Indian rhino males, increasing concentrations of exogenous T were added to 0.5 g aliquots of feces (the aliquots were taken from one of Arun's fecal samples which had previously been found to be low in T). Unfortunately only a small quantity of T was available, so only two aliquots could be spiked. A third aliquot of feces was left unspiked to determine the quantity of endogenous hormone in the spiked aliquots.

The concentrations chosen for spiking, based on the standard curve from the T parallelism plate, were 6.25 and 0.781 ng/ml. These concentrations bound at 27.9% and 66.9%, respectively. The volumes of T needed for spiking were relatively small (281.3 and 35.2 µl) so no attempt was made to correct for them. Once the T had been added the three aliquots were vortexed for 30 seconds, allowed to sit for one hour, then vortexed again. Extraction proceeded as described earlier, and then the extracts were analyzed by the T EIA.

The percent extraction efficiency was calculated as the average, for each spiked aliquot, of (observed value/expected value) x 100. The observed value equalled the amount of hormone actually measured by the T EIA minus the endogenous hormone, as determined from the unspiked aliquot. Extraction efficiency was determined to be 123.13%. This high value was most likely due to cross-reaction of the T antibody with metabolites other than testosterone (refer to Appendix II for a list of cross-reactivities).

Based on anecdotal evidence, a range of 80 to 110% had previously been deemed acceptable, but the validation could not be repeated due to the shortage of T.

Quality Controls (EC, PdG and T)

For each assay (EC, PdG or T), the inter-assay coefficients of variation (CVs) were determined for each of the two quality controls (C1 and C2), using the results of all plates run on the assay. The CV was calculated as: (standard deviation ÷ mean) x 100. The EC inter-assay CVs were 7.6% at 33.2% binding and 14.2% at 75.5% binding. The PdG inter-assay CVs were 11.8% and 19.0% at 48.2% and 84.2% binding, respectively. T inter-assay CVs were 3.9% at 20.9% binding and 5.4% at 70.4% binding.

The intra-assay CVs were determined by running one full plate of C1 and one full plate of C2. The EC intra-assay CVs were 11.0% at 48.2% binding and 21.8% at 83.6% binding. The PdG CVs were 8.8% and 7.1% at 44.5% binding and 79.1% binding, respectively. The T CVs were 5.26% at 18.0% binding and 3.9% at 46.0% binding.

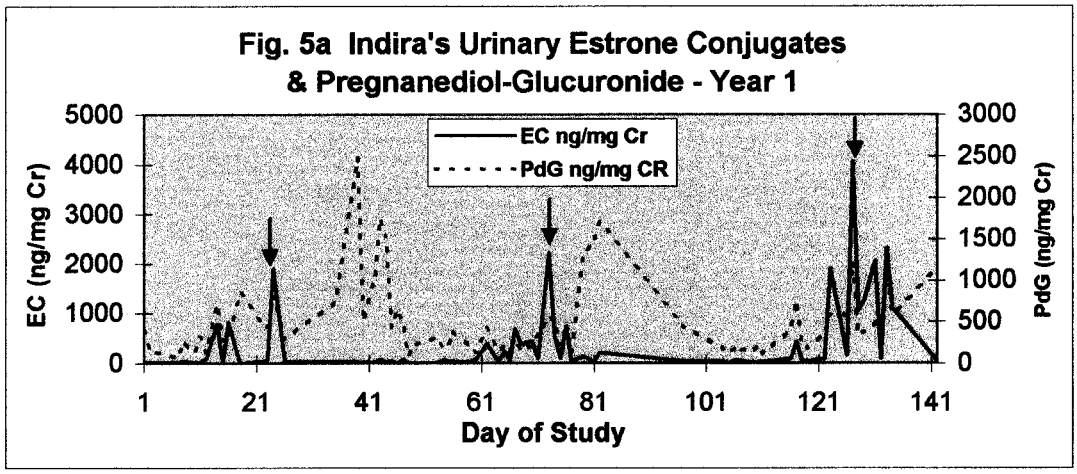
RESULTS and DISCUSSION

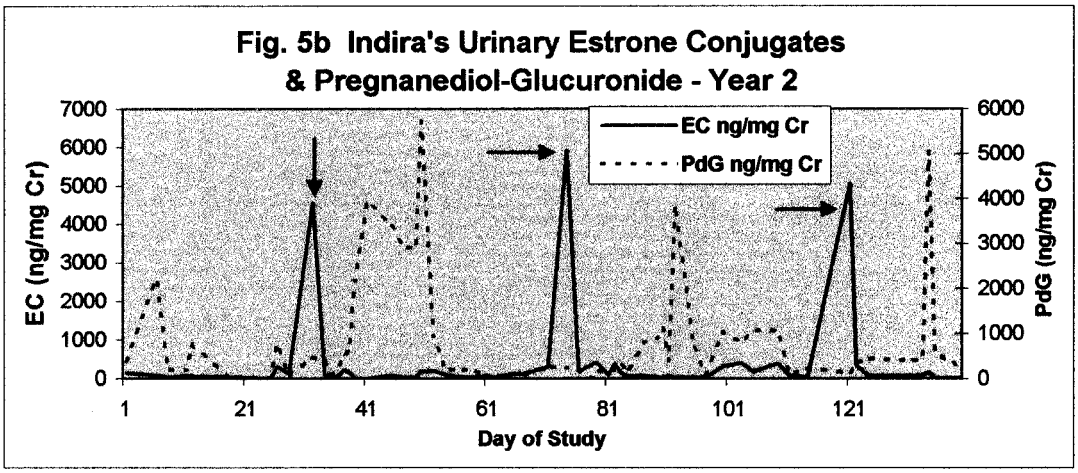
Endocrine Results

Figs. 5a and 5b show a total of six estrous cycles from Indira, the Indian rhino female at the TO Zoo, while Fig. 5c shows three estrous cycles from Arati, the female at the FW Zoo. One-tailed *t*-tests (Evans, 1992) revealed that all of Indira's EC and PdG peaks were significantly greater than baseline ($p < 0.05$ for the first three cycles, $p < 0.0005$ for the last three). Arati's EC peaks also were significant ($p < 0.025$), while her PdG peaks were marginally significant ($p = 0.055$). Significant EC peaks (indicative of estrus) are marked by arrows in Fig. 5. The average cycle length was 48 days for Indira ($n = 4$, range 41-54) and 46.5 days for Arati ($n = 2$, range 44-49). These values compare favourably with lengths of 43 ± 2 days reported by Kassam and Lasley (1981) and 48 days (range 39-64) reported by Kasman et al. (1986).

The third EC peak in Fig. 5a (Indira Day 127) was not followed by a post-ovulatory PdG peak, but this EC peak was nevertheless deemed to be pre-ovulatory. Sample collection was stopped on Day 142, at which time PdG was on an upward trend. Based on the first two cycles, if sample collection had continued a post-ovulatory PdG peak would probably have occurred within a few days.

Arati's estrous cycles (Fig. 5c) and Indira's last three cycles (Fig. 5b) were more clearly defined than Indira's first three cycles (Fig. 5a) in that the hormone profile was smoother, with fewer erratic increases and decreases. This is likely because the samples





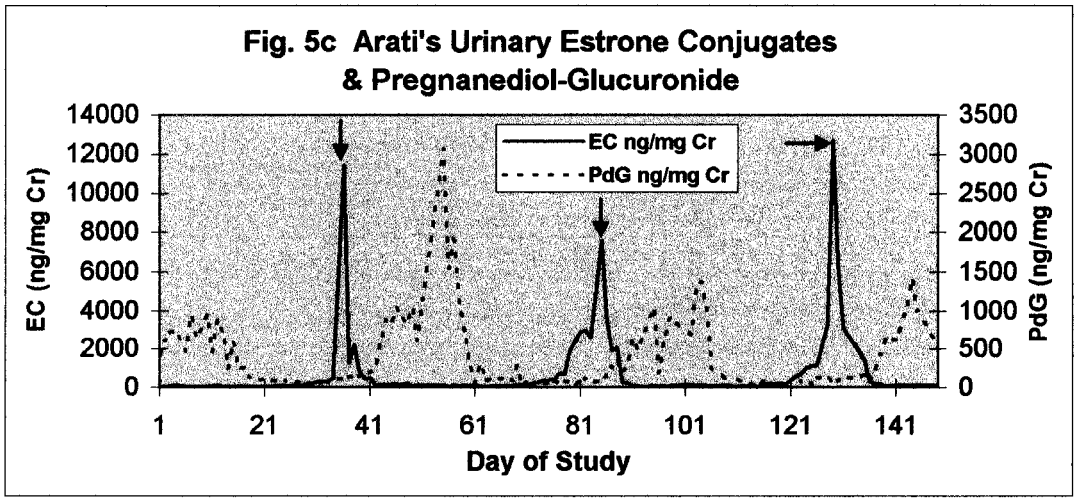


Fig. 5 Indian rhino estrous cycles.
a) 3 cycles from Indira - Year 1; **b)** 3 cycles from Indira - Year 2; **c)** 3 cycles from Arati.

Arati's estrous cycles (Fig. 5c) and Indira's last three cycles (Fig. 5b) were more clearly defined than Indira's first three cycles (Fig. 5a) in that the hormone profile was smoother, with fewer erratic increases and decreases. This is likely because the samples from Indira's first three cycles were processed when the author was just learning to conduct EIAs, therefore there was greater variation in the results. Indira's remaining samples and those from Arati were processed much later, when the author was more experienced.

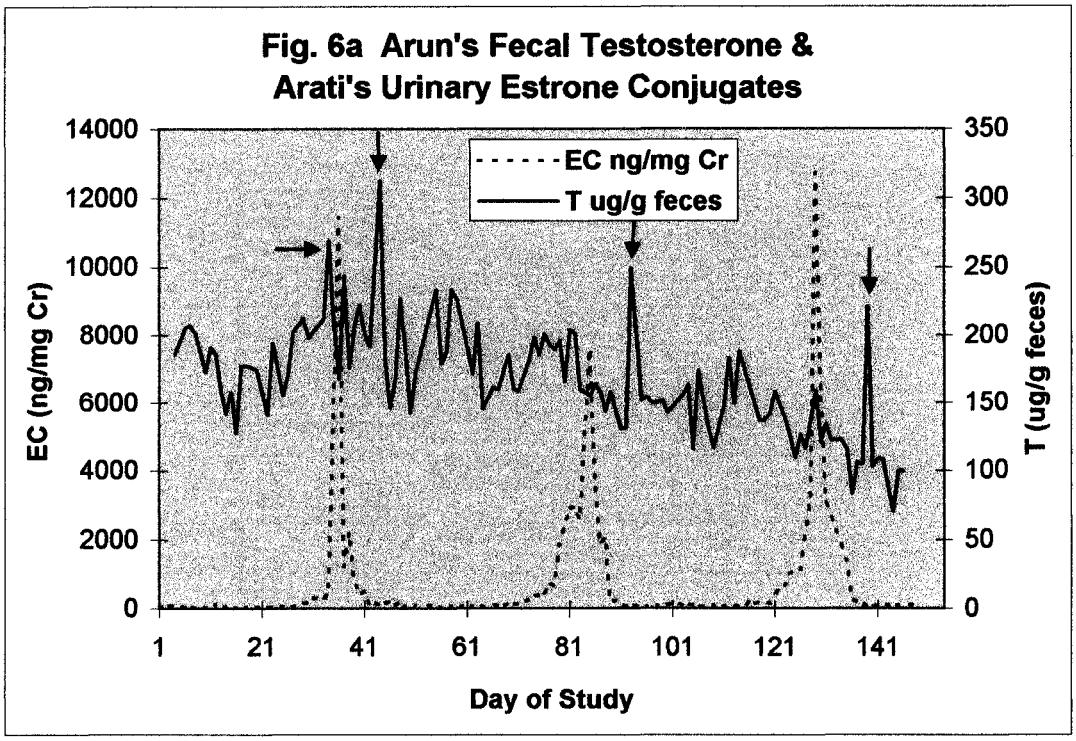
According to a two-tailed *t*-test (Evans, 1992) Arati's EC levels (mean 762.02 ng/mg Cr, range 18.19 to 12,715.09) were significantly greater ($p < 0.05$) than Indira's (mean 365.76, ng/mg Cr, range 0 to 5937.06). Their PdG profiles were not significantly different. This variation in EC is not necessarily meaningful as the literature reports marked differences among individual females. Kasman et al. (1986) studied 5 Indian females and reported that peak estrone sulfate ranged from 3500 to 164,000 ng/mg Cr, while peak PdG ranged from 40 to 278 ng/mg Cr. Bellem and Goodrowe (unpublished data) studied 6 Indian females whose peak EC concentrations ranged from 282 to 15,000 ng/mg Cr, while peak PdG ranged from 299 to 7996 ng/mg Cr. Reproductive ability and magnitude of steroid excretion are not necessarily related – in Bellem and Goodrowe's study (unpublished data) a 15-year-old female had the lowest EC levels among the 6 females studied, yet she bore two calves.

Most likely the difference in EC levels between Indira and Arati reflects a variation in physiology or the kind of random difference found in any small,

period, so their urine may have become mixed before collection. Such contamination could have diluted Indira's urine, thereby lowering her EC and PdG concentrations.

To determine whether the calf's urine added significantly to Indira's EC and PdG levels, urine samples were collected from the calf only, after he had been weaned and separated from his mother, for a period of 53 days. These samples were then analyzed for EC and PdG in the same manner as were the samples from the adult females. The calf's EC levels (mean 106.95 ng/mg Cr, range 13.71 to 289.23) and PdG levels (mean 373.69 ng/mg Cr, range 136.77 to 987.15) were quite low, in particular his urine did not show peaks significantly higher than baseline. In light of these results it was deemed reasonable to conclude that the calf's urine did not add significantly to Indira's EC or PdG levels.

In Fig. 6a Arati's EC is graphed with T from Arun, the male Indian rhino at the FW Zoo. Arun appeared to experience four T "spikes" over the course of the study (these spikes are marked by arrows in Fig. 6a). The first spike (Day 34) occurred two days before Arati's first pre-ovulatory EC peak, while the other three (Days 44, 93 and 139) occurred 8, 8 and 10 days, respectively, after each of Arati's three pre-ovulatory EC peaks. According to one-tailed *t*-tests (Evans, 1992) the first three spikes rose more than two standard deviations above baseline, while the fourth spike rose 1.3 standard deviations above baseline. (As was done for EC and PdG, to determine baseline T the mean of all values was calculated, any values more than two standard deviations on either



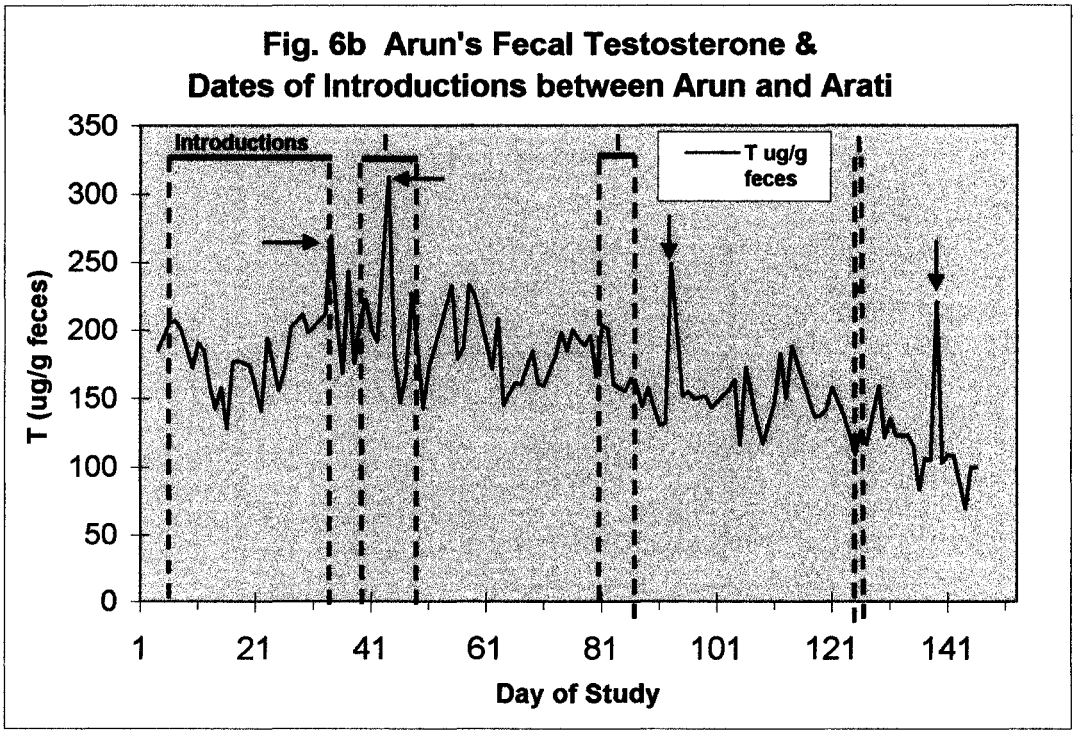
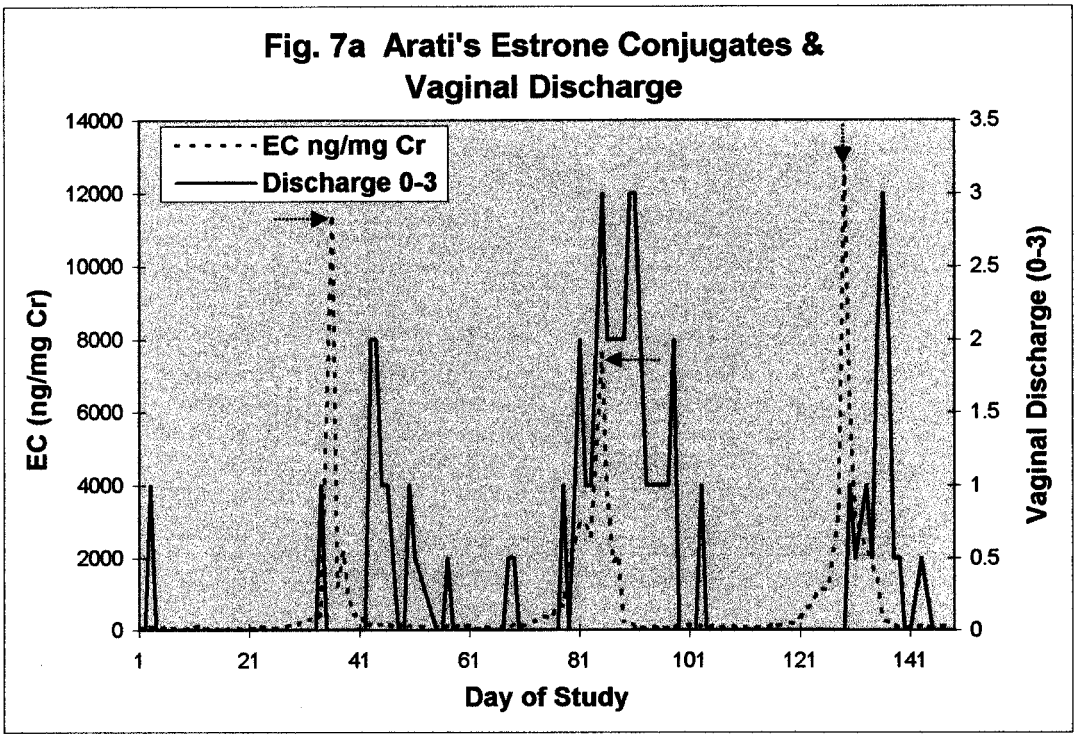


Fig. 6 Indian rhino T results.
a) Arun's T and Arati's EC; **b)** Arun's T and Dates of Introductions between Arun & Arati.

deviations above baseline. (As was done for EC and PdG, to determine baseline T the mean of all values was calculated, any values more than two standard deviations on either side of the mean were discarded, and the new mean of the remaining values was designated the baseline).

As was stated earlier, Arun's T profile was expected to follow one of four trends: 1) no discernible pattern, which is the case with some Black and White rhino males (Roth, pers. comm., 2000); 2) T increases 24 to 60 hours after each of the female's EC peaks (i.e. at the time of ovulation); 3) increased T whenever the male was displaying overt sexual behaviour or 4) increased T whenever the male was introduced to the female. There does indeed appear to be some association between Arun's T spikes and the dates that Arati and Arun were introduced to one another (i.e. placed together in the same pen). Introductions between Arati and Arun were conducted in four sets (Fig. 6b), the first set beginning six days before the study began. At this time the pair was not well-acquainted because they had previously been introduced on only five occasions between May 3, 1997 and March 23, 1998. The first T spike (Day 34) occurred just after the end of the first set of introductions, on the second day that the rhinos had been kept separate. It is possible that Arun was disturbed by the change in routine or the absence of the female. The next three T spikes each occurred during one of the three remaining sets of introductions.

As was discussed earlier, observations of Arati's vaginal discharge were included in the FW Zoo study. Since it was not possible to collect and measure all secretions the



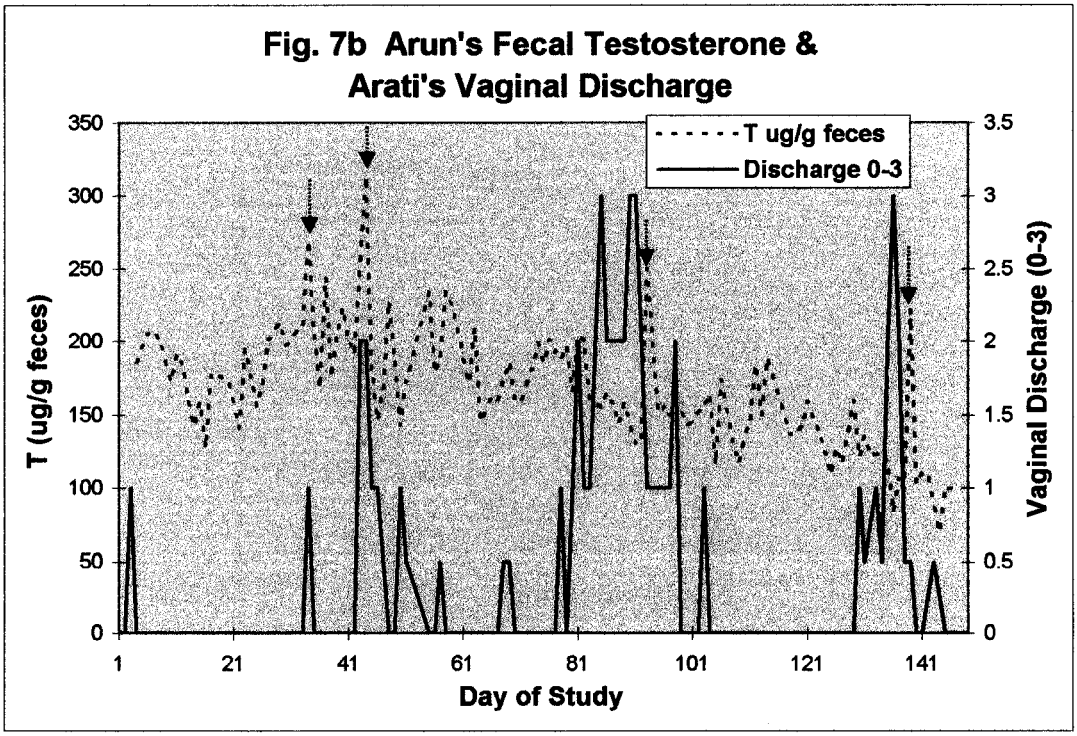


Fig. 7 Indian rhino vaginal discharge.
a) Arati's vaginal discharge and EC; **b)** Arati's vaginal discharge and Arun's T.

observations were ranked subjectively (0 indicating no discharge and 3 the maximum amount seen). In Fig. 7 vaginal discharge appears to be more strongly related to T than to EC (although, according to Pearson correlations, neither relationship was statistically significant). Discharge reached the maximum level several days after each of the three EC peaks (Fig. 7a), but on the day of, or immediately before, each of the last three T spikes (Fig. 7b).

Since the last three T spikes occurred 8, 8 and 10 days, respectively, after each of Arati's three pre-ovulatory EC peaks it seems possible that there was some association between EC and T. It would be difficult to explain the delay between the EC peaks and T spikes if, as has always been assumed, the temporal relation of estrogen and ovulation in rhinos follows the general mammalian pattern in which ovulation occurs 24 to 60 hours after estrogen peaks. Roth, however, has been monitoring the reproductive cycle of an 8-year-old female Indian rhinoceros using rectal ultrasound and urinalysis and has found (pers. comm., 2000) that ovulation occurs on average 13 days after estrogen rises (from a baseline of 12.5 ng/mg Cr to 48 ng/mg Cr). This pattern makes ecological sense given that these animals are solitary and widely dispersed in the wild; if females ovulated at the start of estrus they would have little time in which to attract mates (eggs would be viable for 72 hours, at the very most – Raven and Johnson, 1989). If Roth's results hold true for all Indian rhinos then Arun may be experiencing a T surge at the time of the female's ovulation. The time gap between EC peak and ovulation would also explain why Arati's vaginal discharge seems to be more strongly related to T than to EC.

Interestingly, Arun's T levels, including the four spikes, decreased gradually over time (Fig. 6a). One possible explanation for this decrease comes from the old adage "familiarity breeds contempt". By the end of the research period Arun may have been less surprised or excited by Arati's presence, which conceivably could lead to lower T levels. Arati's EC levels showed no such decrease - in fact the EC peak from her last cycle was the highest of all her EC values (Fig. 5c). Another explanation might be the fact that the study was conducted over the course of a summer and into the fall, so it may be that decreasing daylength affected T levels. Obviously, further study of T excretion by Arun and other Indian rhino males should be conducted in order to determine whether there is a consistent pattern and, if so, the nature of the pattern.

Behaviour Results

Pearson correlations (Evans, 1992) were performed on hormone and behaviour data in order to determine which behaviours were significantly correlated with hormone levels. Table 1 displays the results for the seven behaviours which were identified as being significantly correlated with EC (for definitions of all behaviours refer to the ethogram in Appendix I). These behaviours were: Weaving by Arun - Outdoors; Pacing by Patrick - Years 1 & 2 and also by Arun - Outdoors; Sniff/Taste Urine/Feces by Arun – Outdoors; Urine Spraying by Arun - Barn and Outdoors; Defecating by Arun – Outdoors; Blowing by Arun – Outdoors and Banging by Patrick - Year 2 and also by Arun

–Outdoors. Contrary to predictions of the first hypothesis of this study, no female behaviours were significantly correlated with female hormone levels.

Two behaviours, Head Bobbing by Arati – Outdoors and Blow-Snorting by Arun - Barn were significantly correlated with T (Table 2). Notably the only female behaviour of statistical significance, Head Bobbing, was correlated with male T rather than female EC.

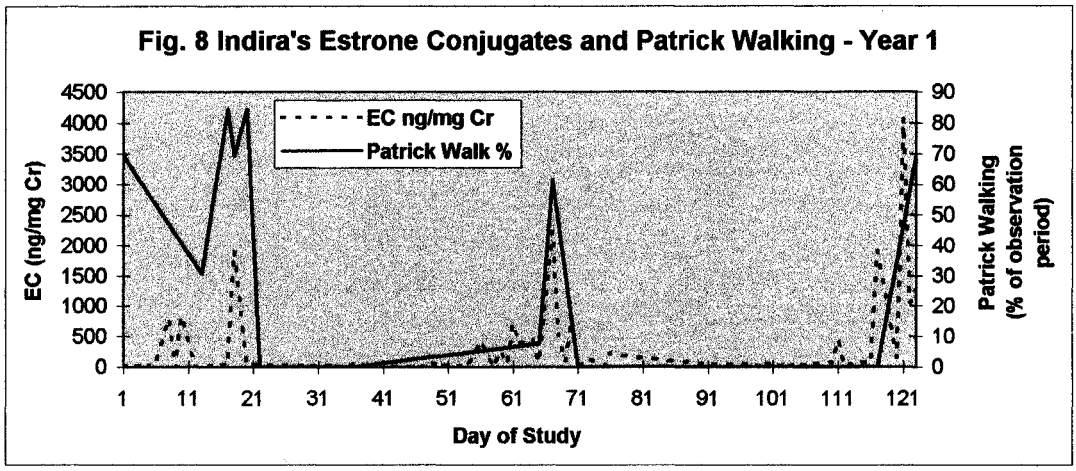
An additional twenty-two behaviours were plotted against hormone levels (Figs. 8 to 29). As is evident from the graphs, these behaviours appeared to relate to hormone levels (in that they occur only or more frequently around the time of EC peaks or T spikes), although the relationships were not statistically significant. Behaviours related to EC were: Walking - Fig. 8; Trot/Canter – Fig. 9; Weaving – Fig. 10; Pacing – Fig. 11; Charging – Fig. 12; Side-to-Side – Fig. 13; Sniff/Taste Urine/Feces – Fig. 14; Flehmen - Fig. 15; Urine Spraying – Fig. 16; Penis Extended – Fig. 17; Mooing – Fig. 18; Blowing – Fig. 19; Whistling – Fig. 20; Whistle-Blowing – Fig. 21; Whistle-Barking – Fig. 22; Teeth Grinding – Fig. 23; Banging – Fig. 24; Bleating – Fig. 25; Snorting – Fig. 26; Bellowing – Fig. 27 and Barking – Fig. 28. As was the case for the correlations, Blow-Snorting (Fig. 29) seemed to relate to T rather than EC. Possible reasons for the lack of statistical significance with regard to these behaviours include the fact that often these behaviours occurred just before or just after EC or T peaks, or else they occurred during only one or two of the three estrus periods. Failure to show statistical significance,

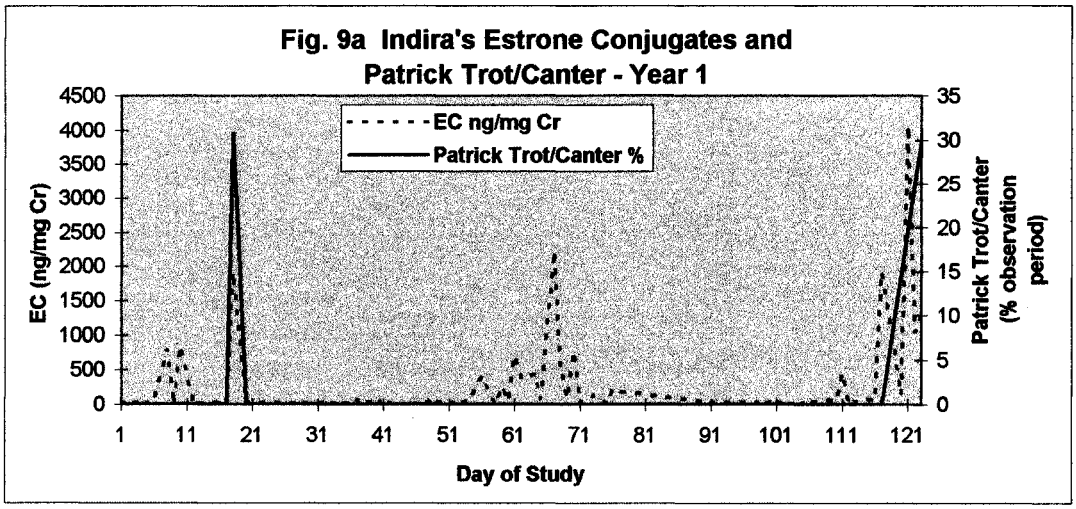
Table 1. Behaviours correlated with EC.

Behaviour	Subject	Pearson Correlation (Behaviour & EC)	Probability	Sample Size
Weaving	EC & Arun Weaving – Outdoors	$r = 0.60$	$p < 0.001$	$n = 69$
Pacing #1	EC & Patrick Pacing – Year 1	$r = 0.87$	$p = 0.001$	$n = 30$
Pacing #2	EC & Patrick Pacing – Year 2	$r = 0.44$	$p = 0.003$	$n = 43$
Pacing #3	EC & Arun Pacing – Outdoors	$r = 0.35$	$p = 0.003$	$n = 69$
Sniff/Taste Urine/Feces	EC & Arun Sniff/Taste – Outdoors	$r = 0.26$	$p = 0.030$	$n = 69$
Urine Spraying #1	EC & Arun Urine Spraying – Barn	$r = 0.43$	$p < 0.001$	$n = 104$
Urine Spraying #2	EC & Arun Urine Spraying – Outdoors	$r = 0.51$	$p < 0.001$	$n = 69$
Defecating	EC & Arun Defecate – Outdoors	$r = 0.25$	$p = 0.035$	$n = 69$
Blowing	EC & Arun Blowing – Outdoors	$r = 0.36$	$p = 0.002$	$n = 69$
Banging #1	EC & Patrick Banging – Year 2	$r = 0.34$	$p = 0.032$	$n = 40$
Banging #2	EC & Arun Banging – Outdoors	$r = 0.36$	$p = 0.002$	$n = 69$

Table 2. Behaviours correlated with T.

Behaviour	Subject	Pearson Correlation (Behaviour & T)	Probability	Sample Size
Head Bobbing	T & Arati Head Bobbing – Outdoors	$r = 0.33$	$p = 0.008$	$n = 63$
Blow-Snorting	T & Arun Blow-Snorting – Barn	$r = 0.40$	$p < 0.001$	$n = 94$





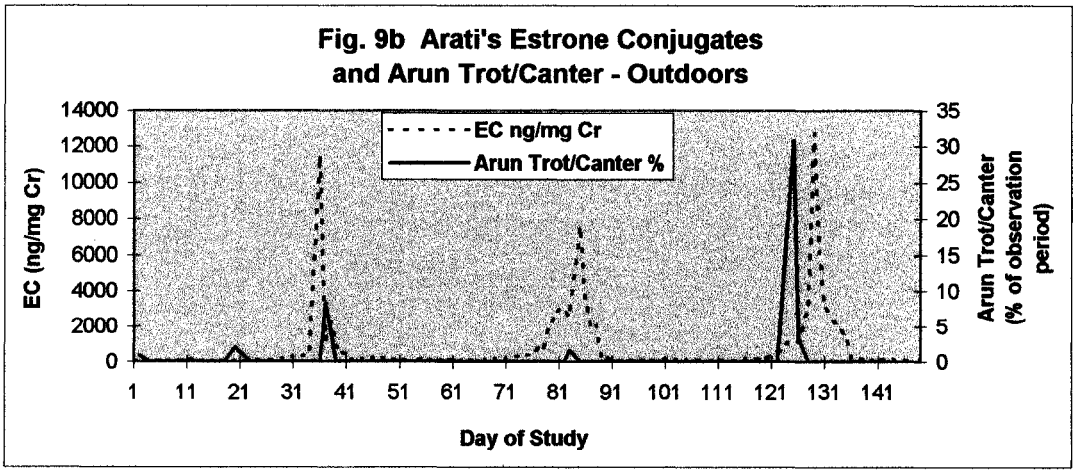
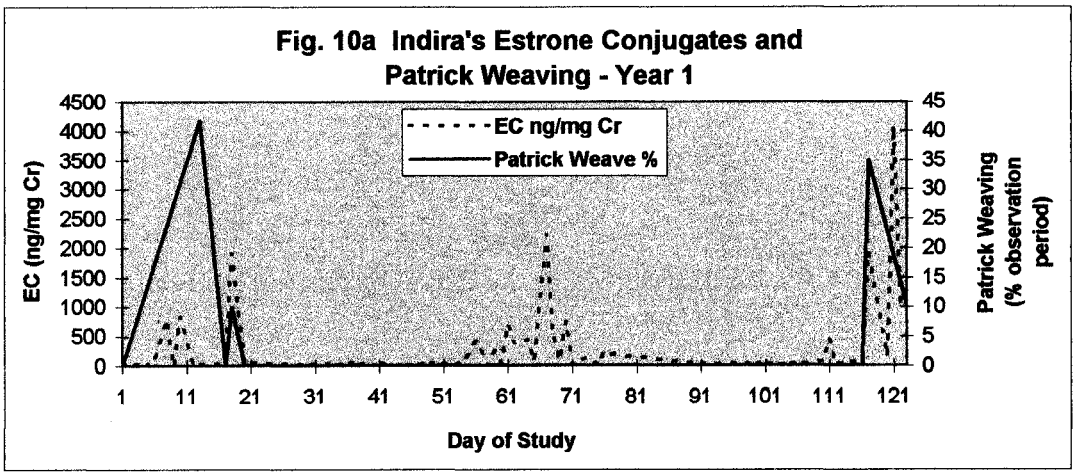
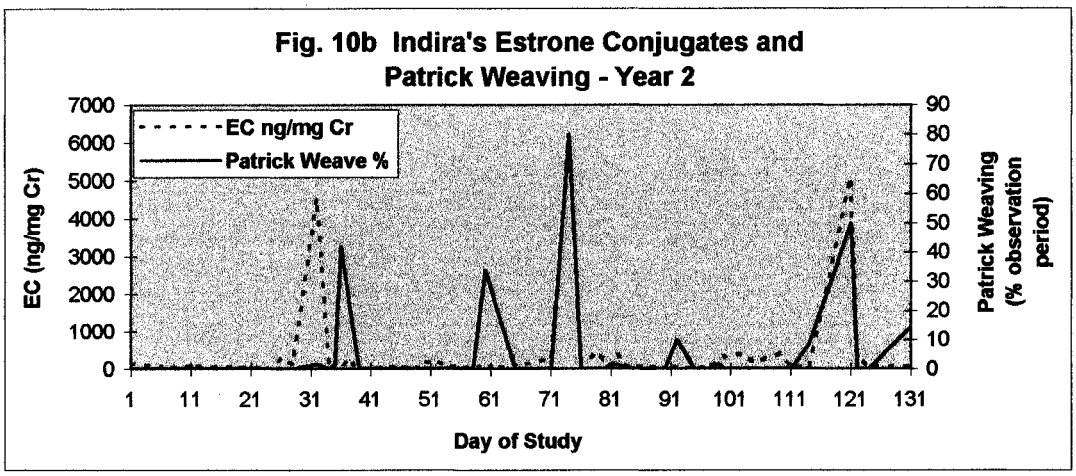


Fig. 8 Walking and EC.
Fig. 9 Trot/Canter and EC.





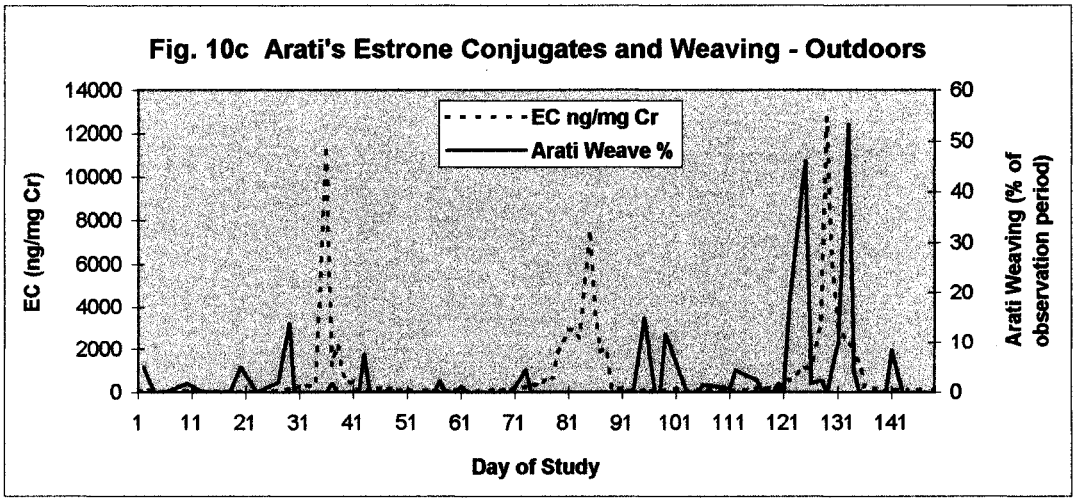
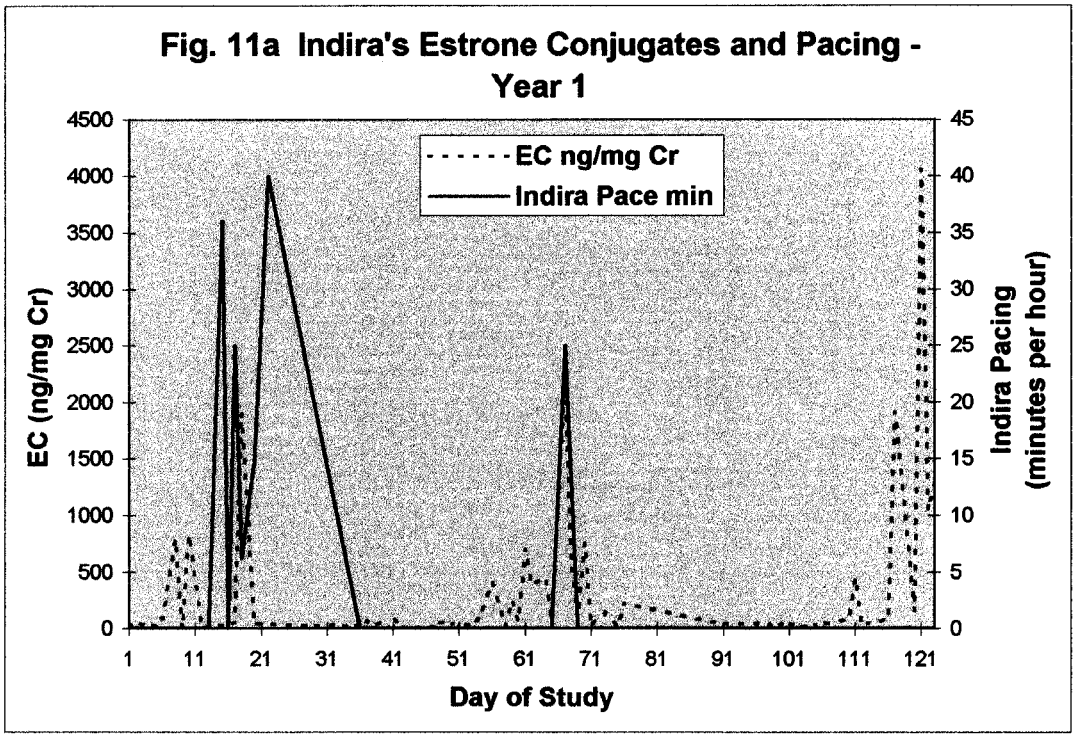


Fig. 10 Weaving and EC.



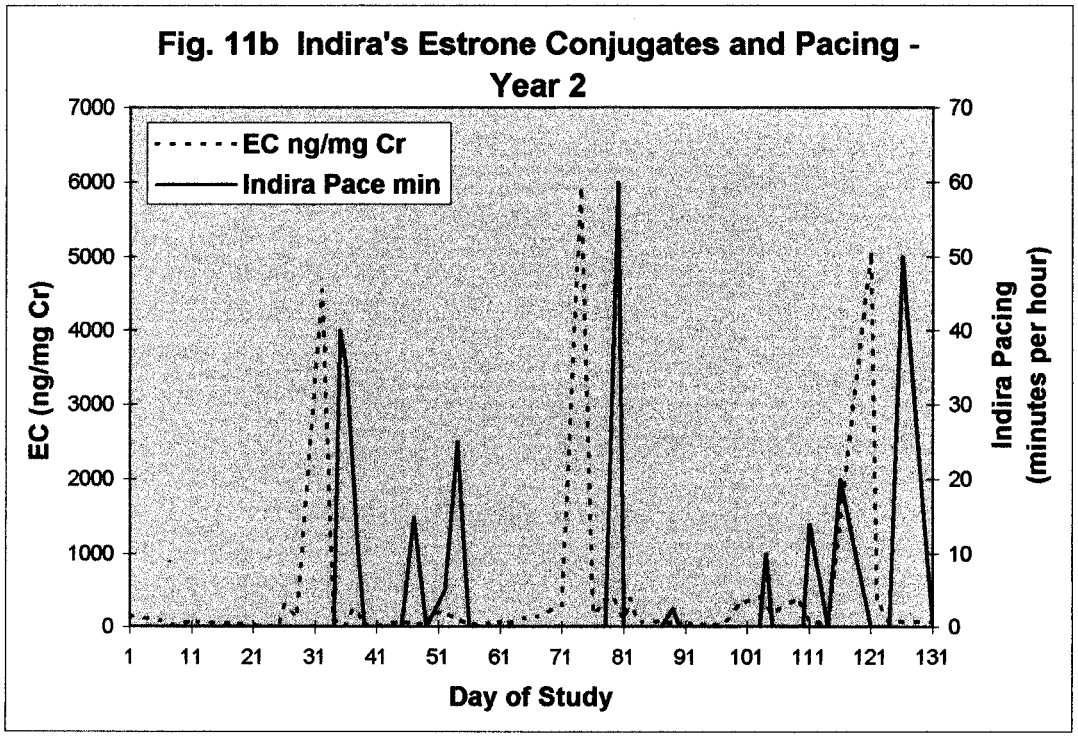
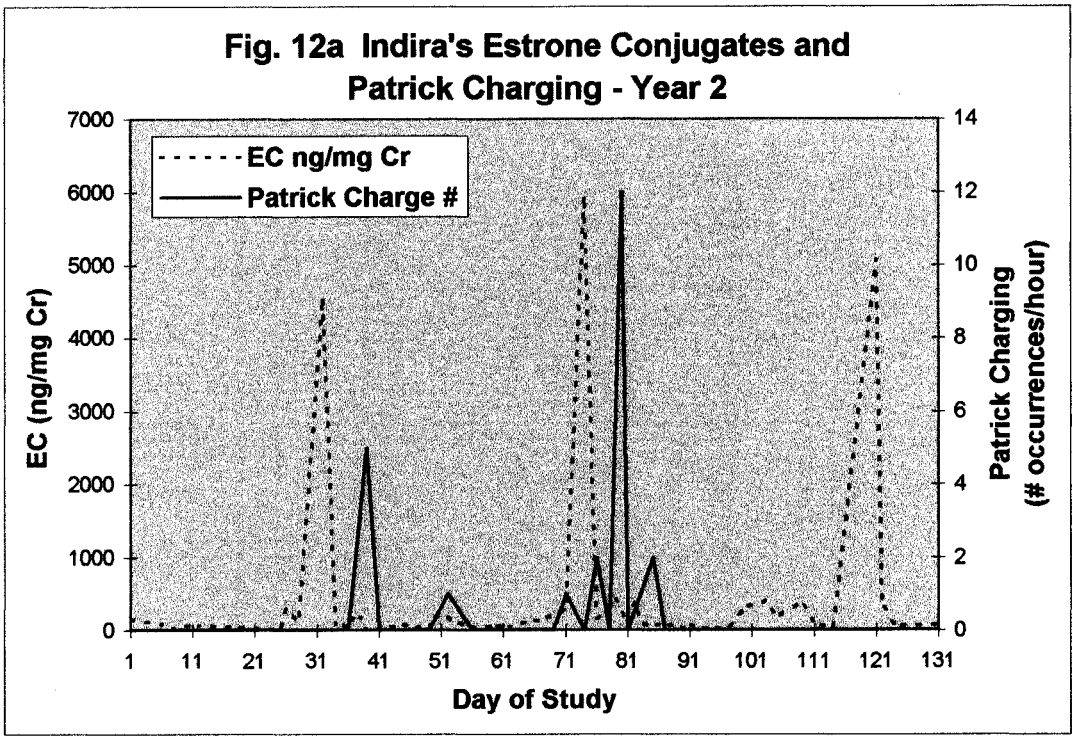


Fig. 11 Pacing and EC.



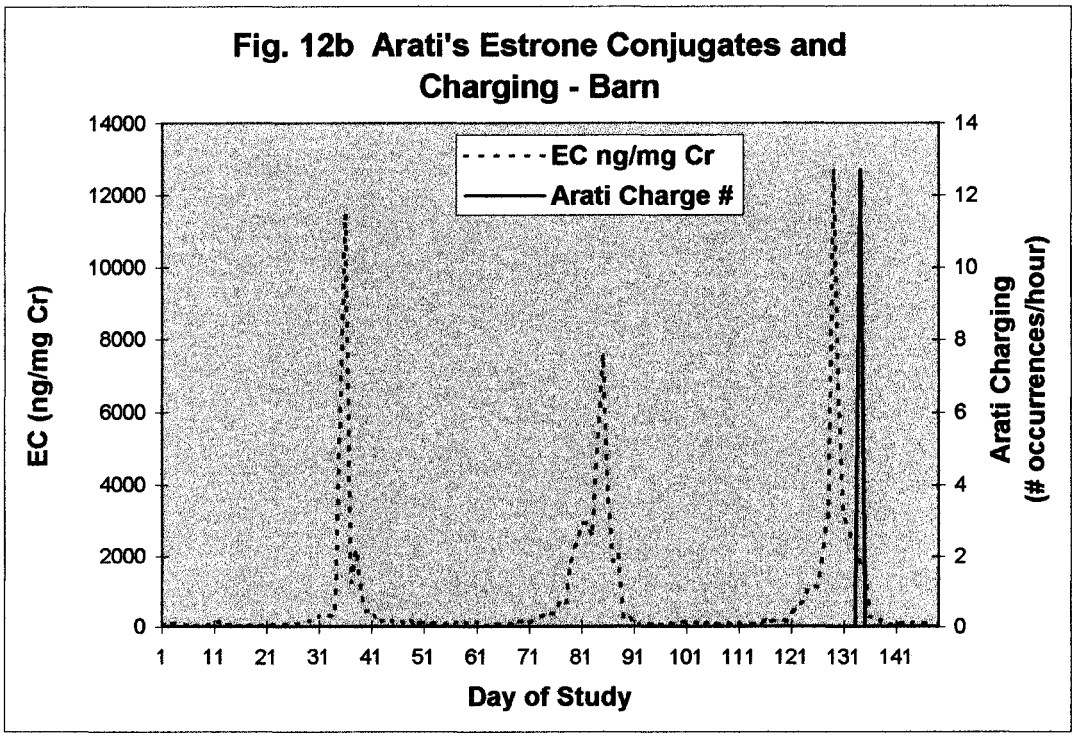
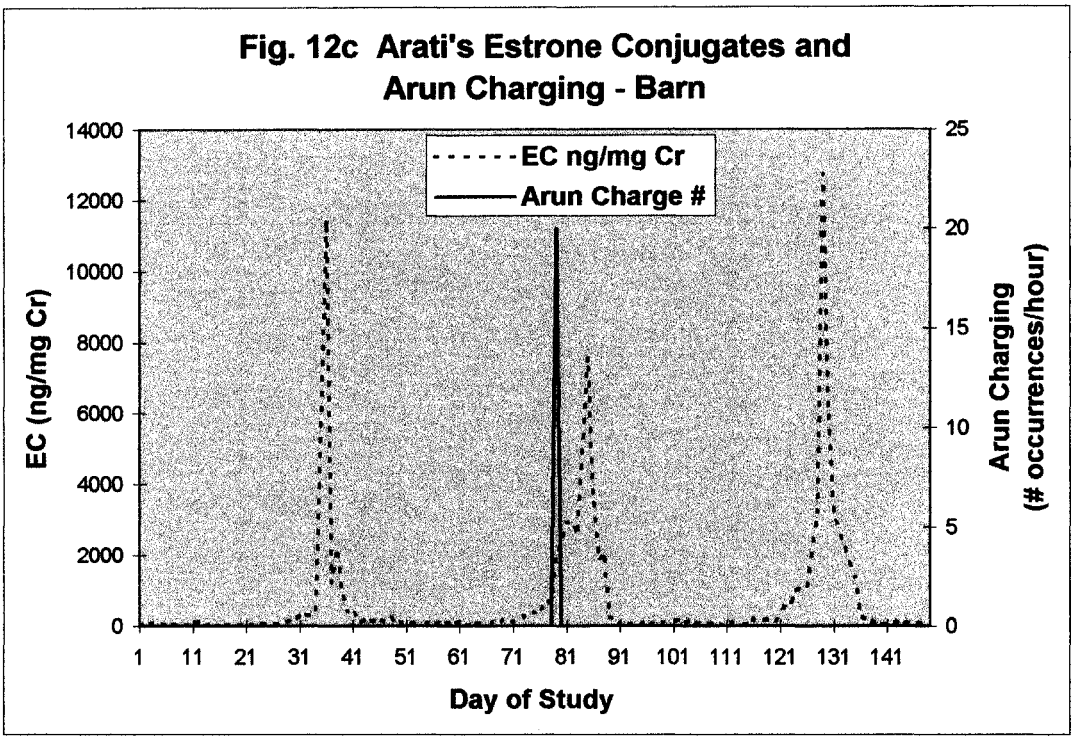


Fig. 12a,b Charging and EC.



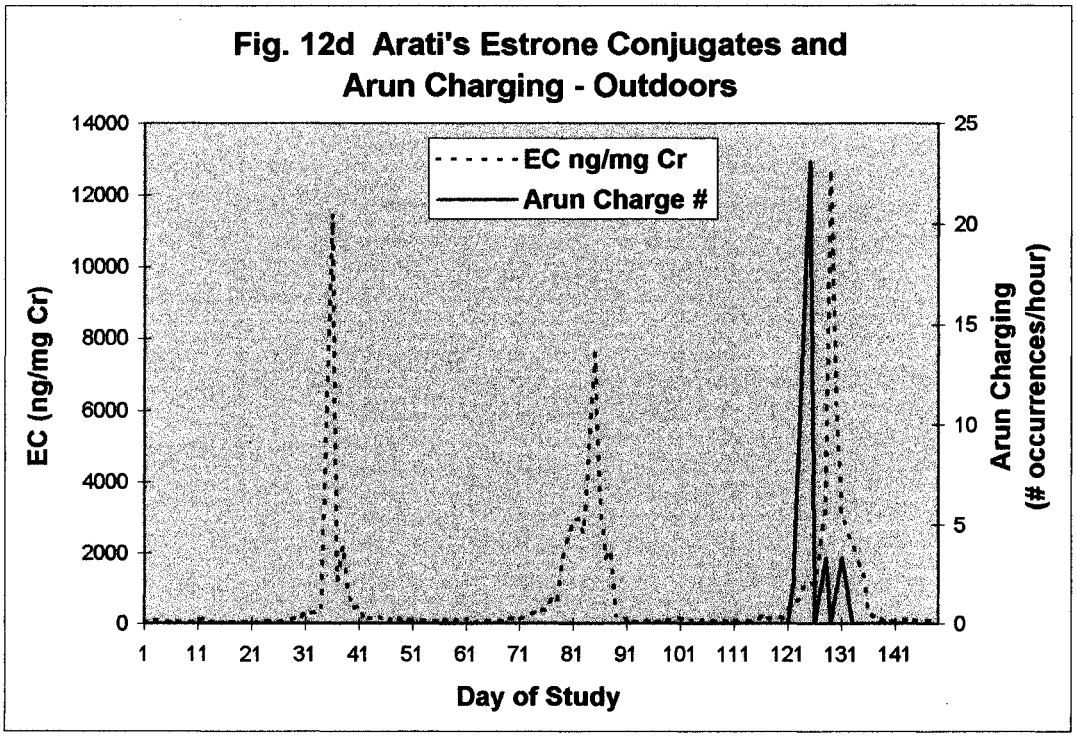
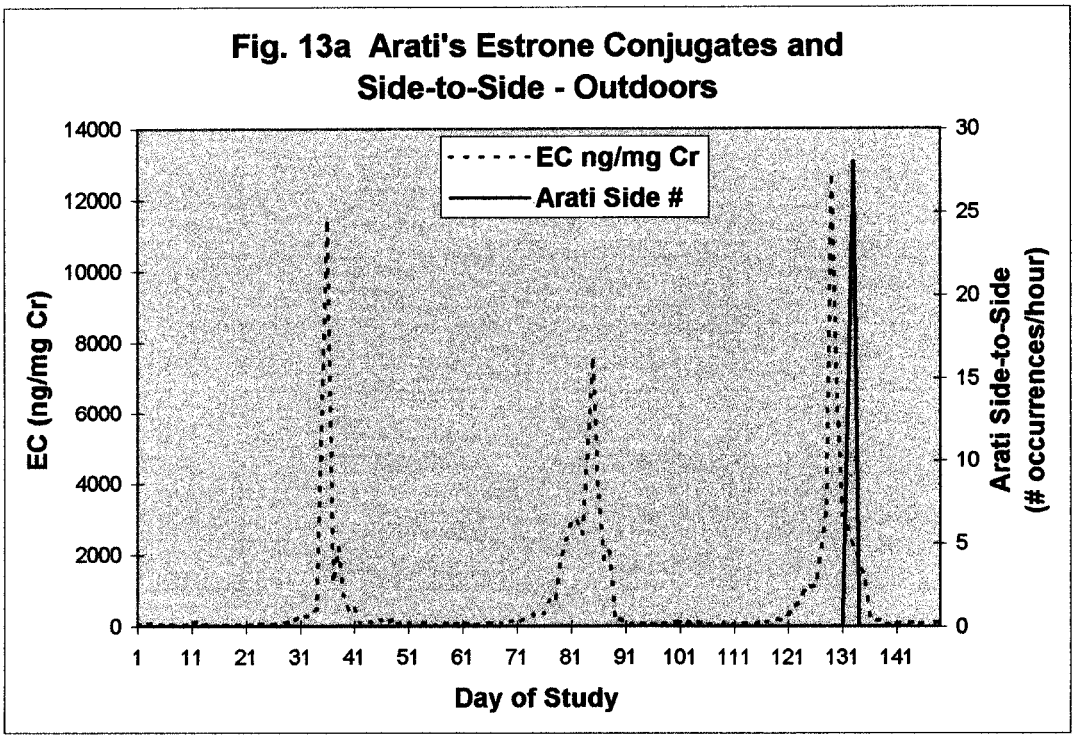


Fig. 12c,d Charging and EC.



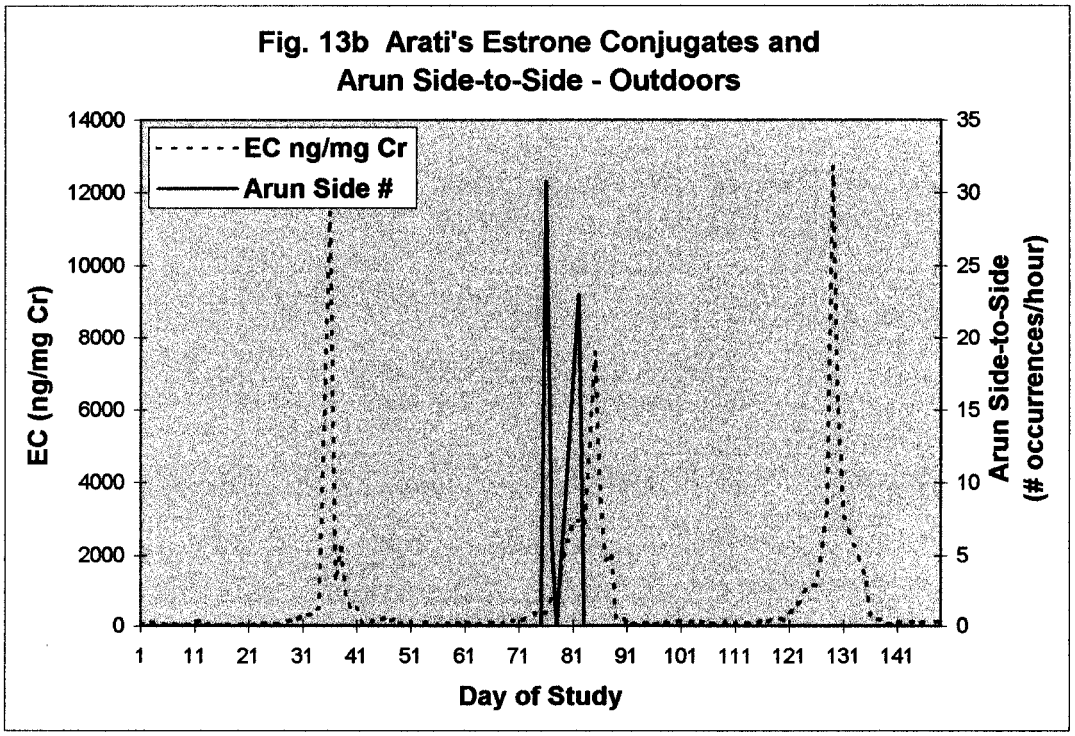
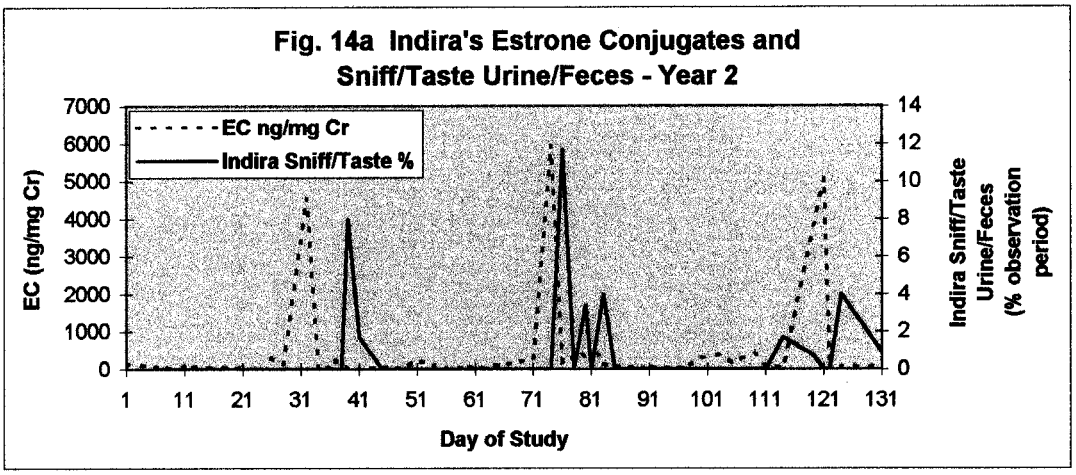
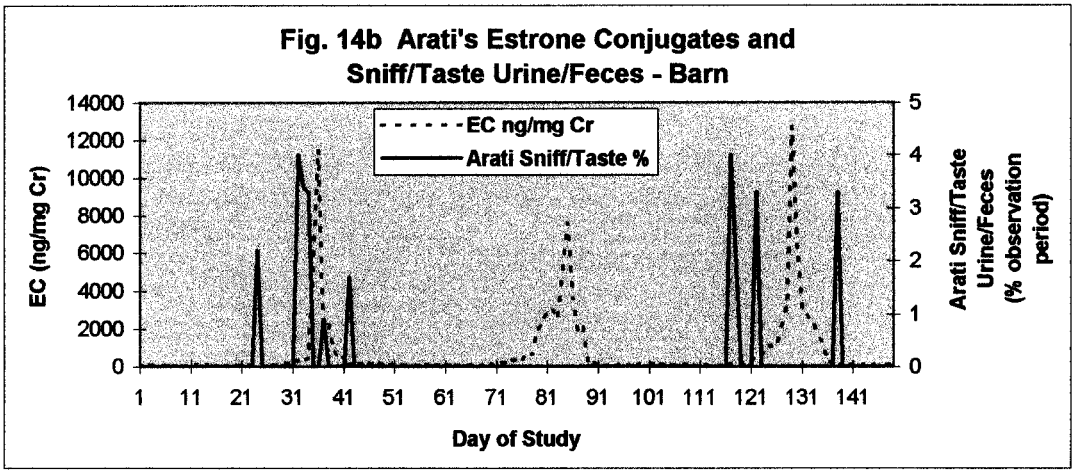


Fig. 13 Side-to-Side and EC.





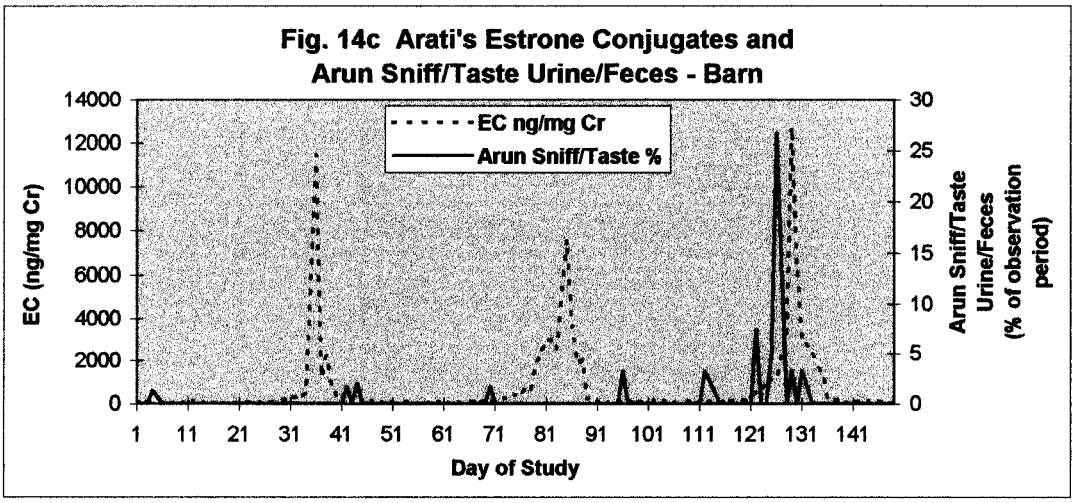
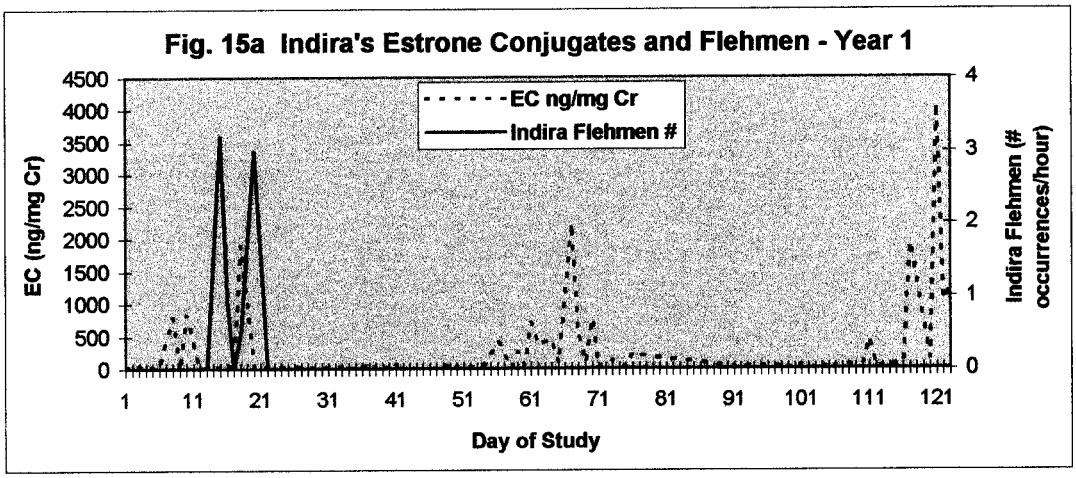
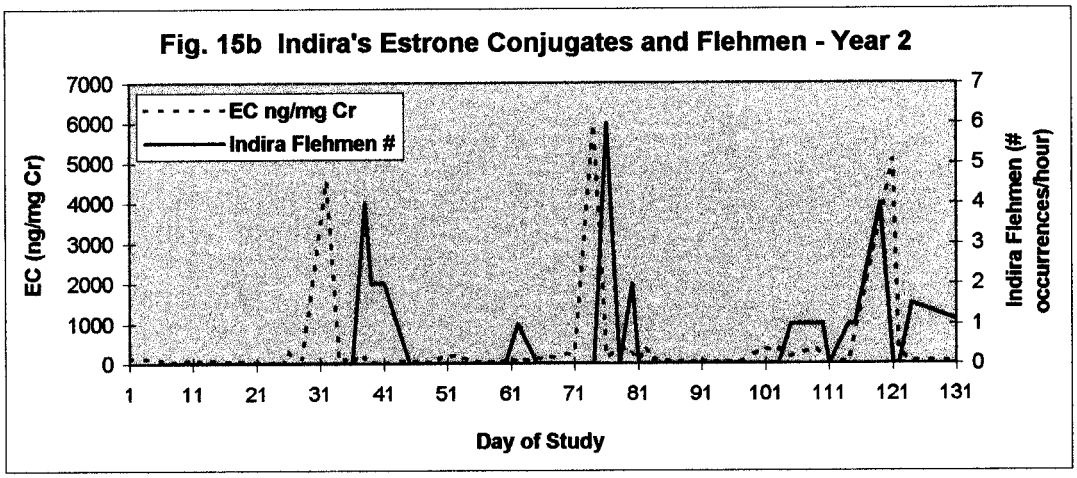


Fig. 14 Sniff/Taste Urine/Feces and EC.





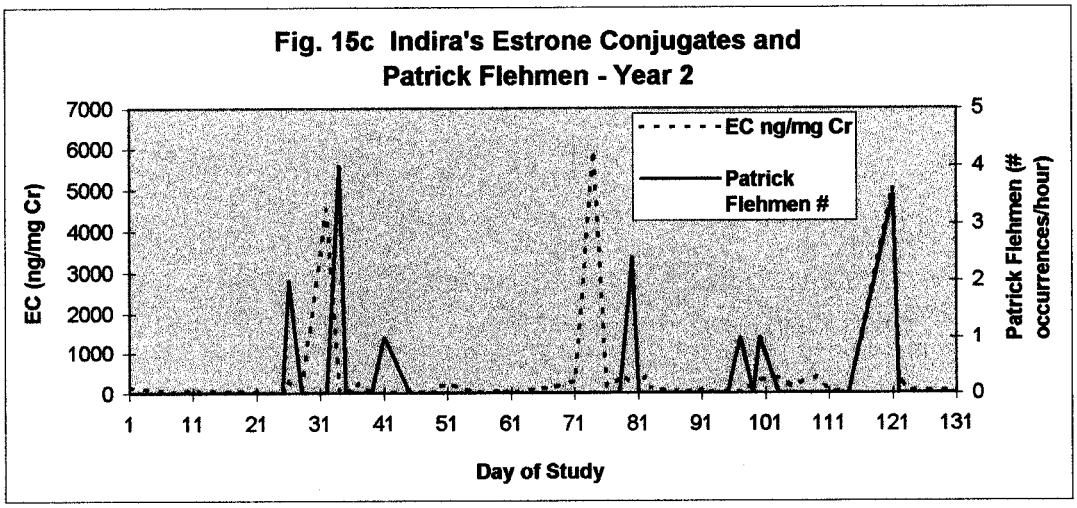
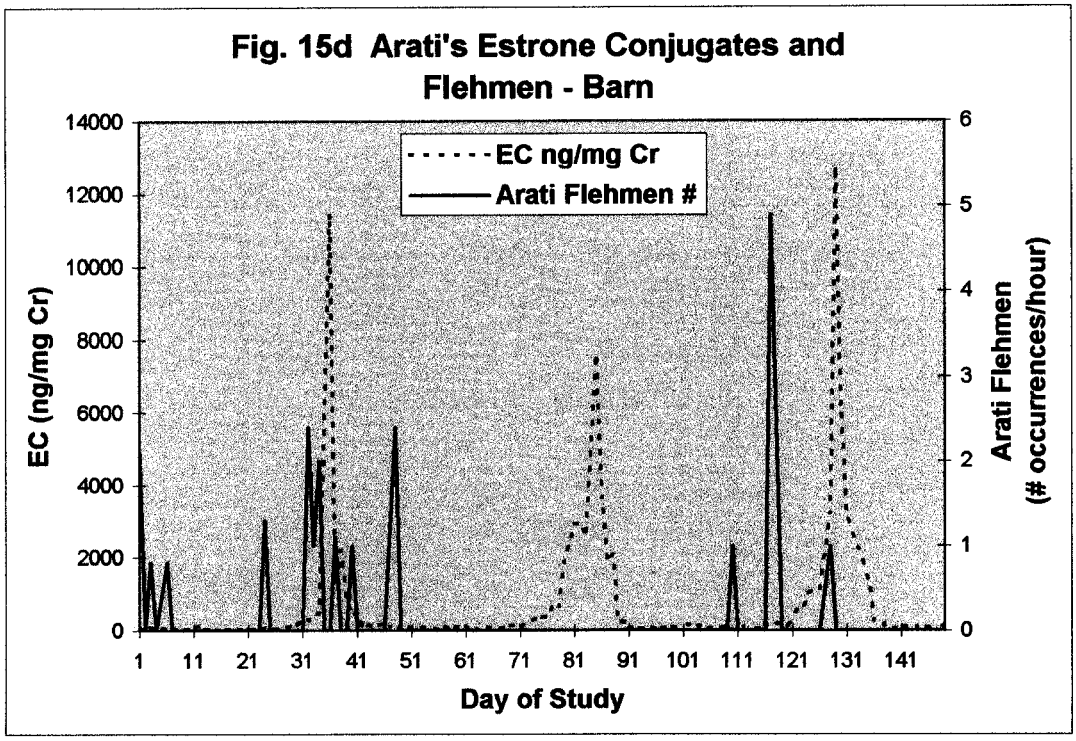


Fig. 15a,b,c Flehmen and EC.



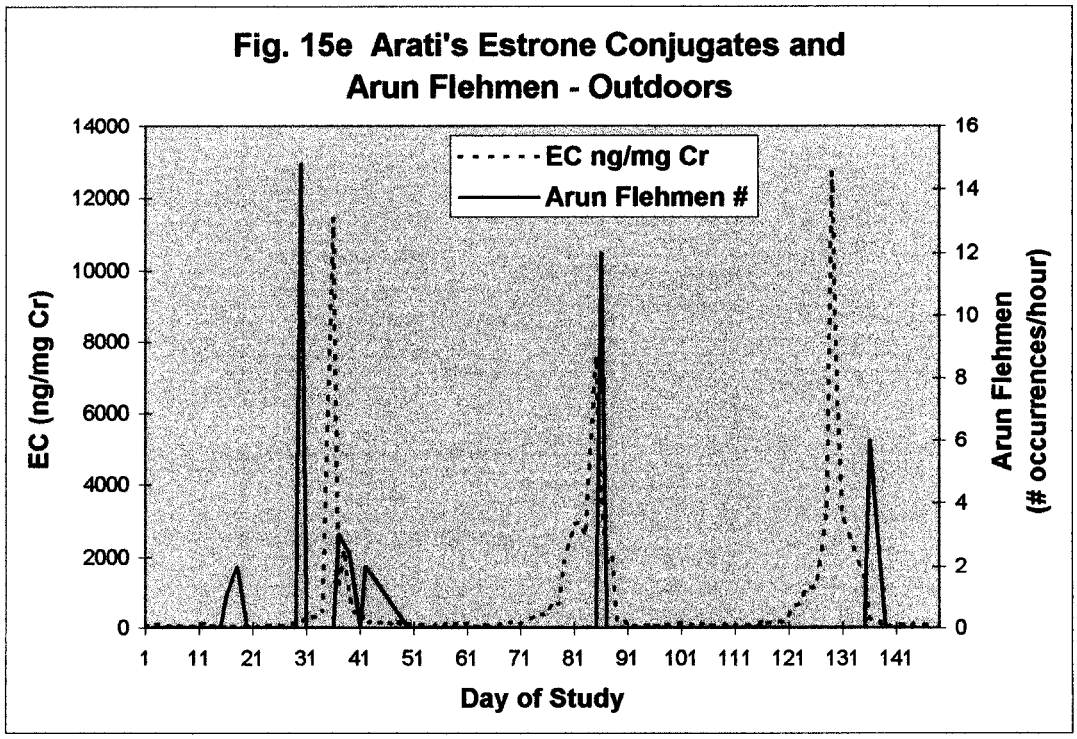
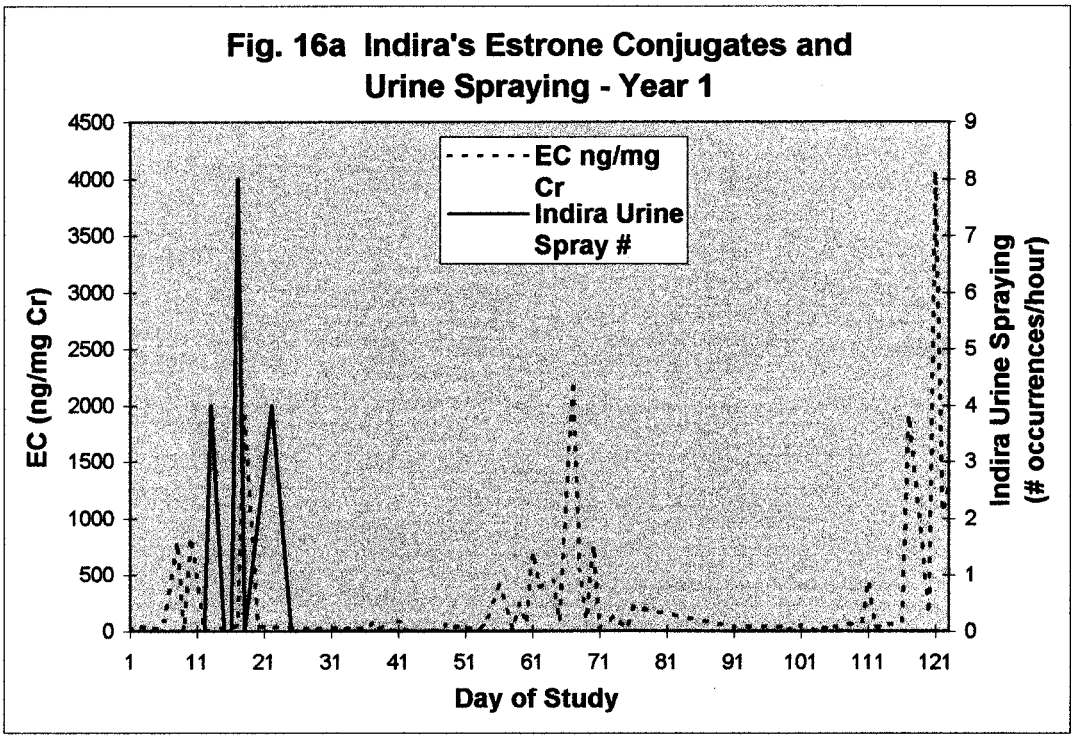


Fig. 15d,e Flehmen and EC.



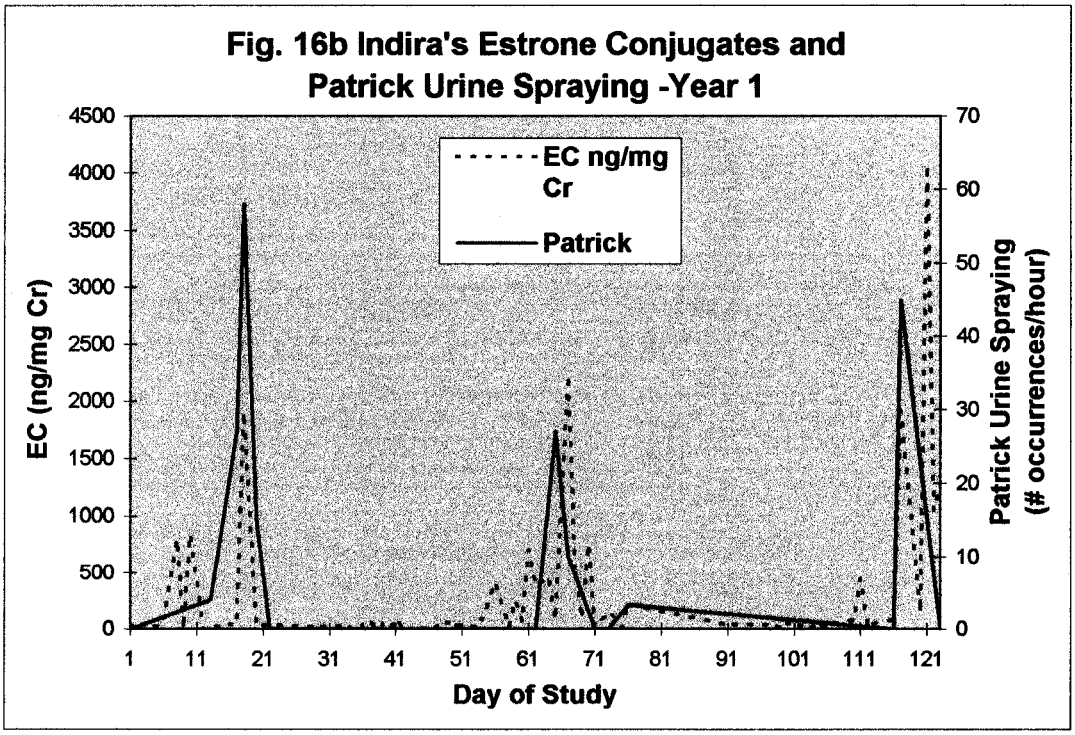
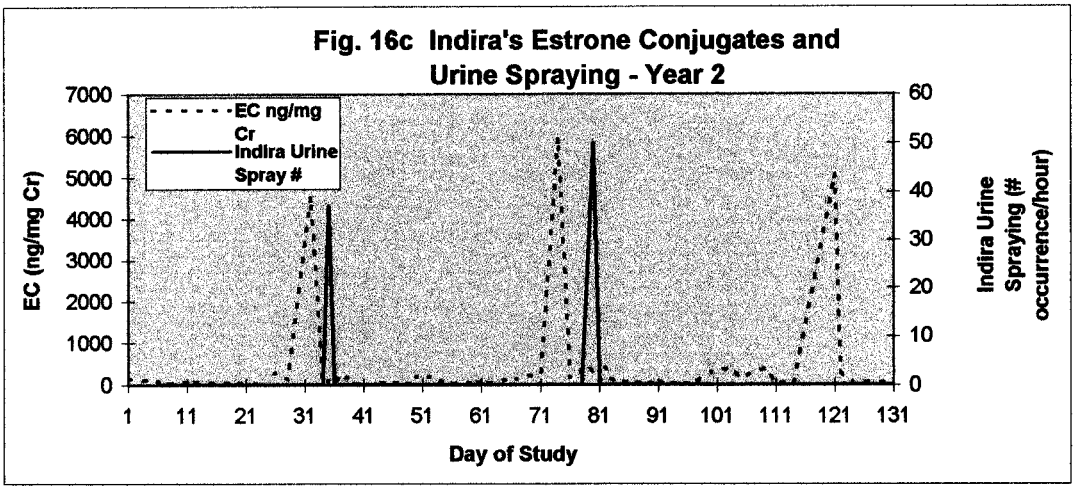
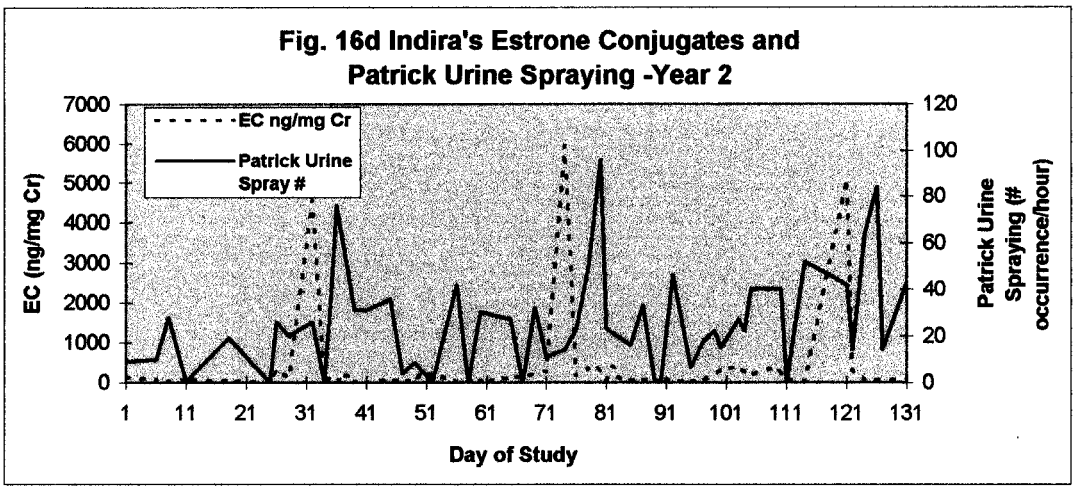


Fig. 16a,b Urine Spraying and EC.





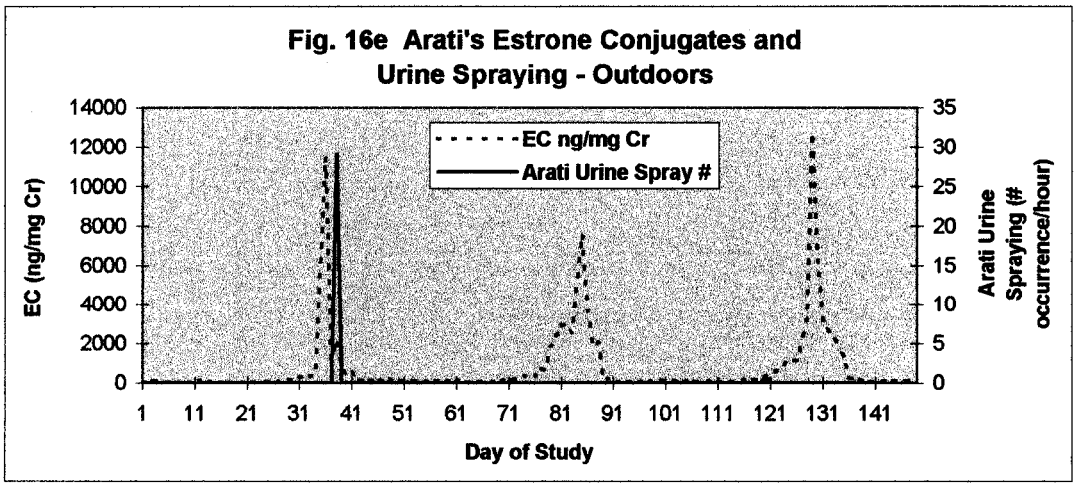
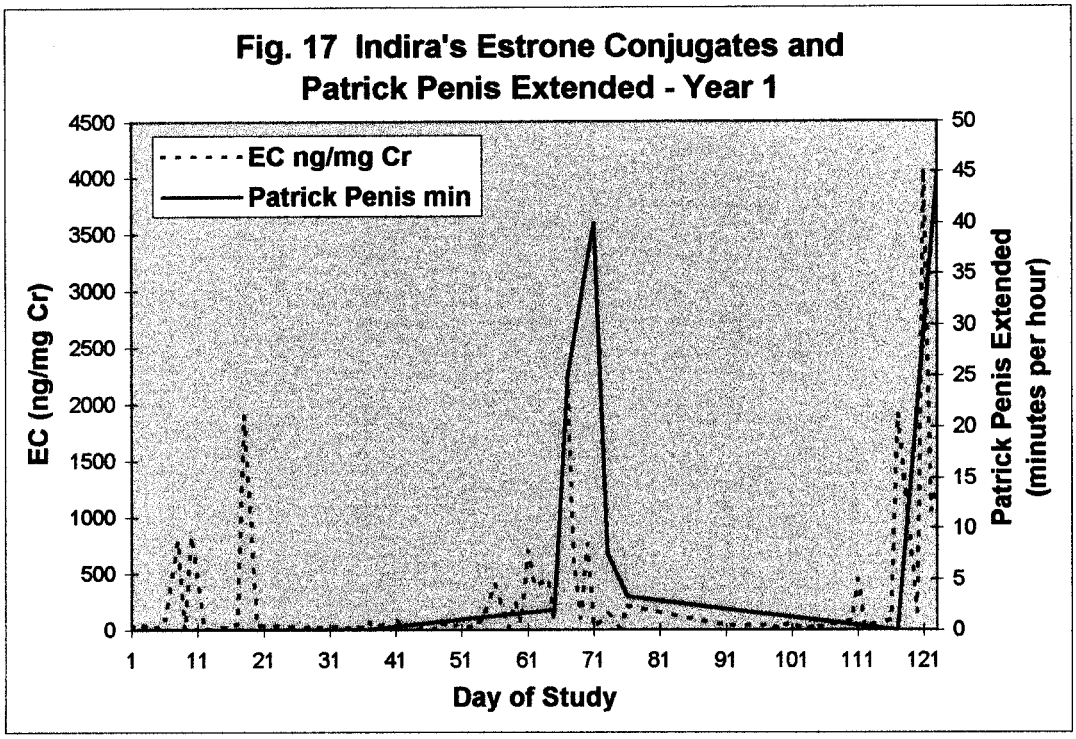


Fig. 16c,d,e Urine Spraying and EC.



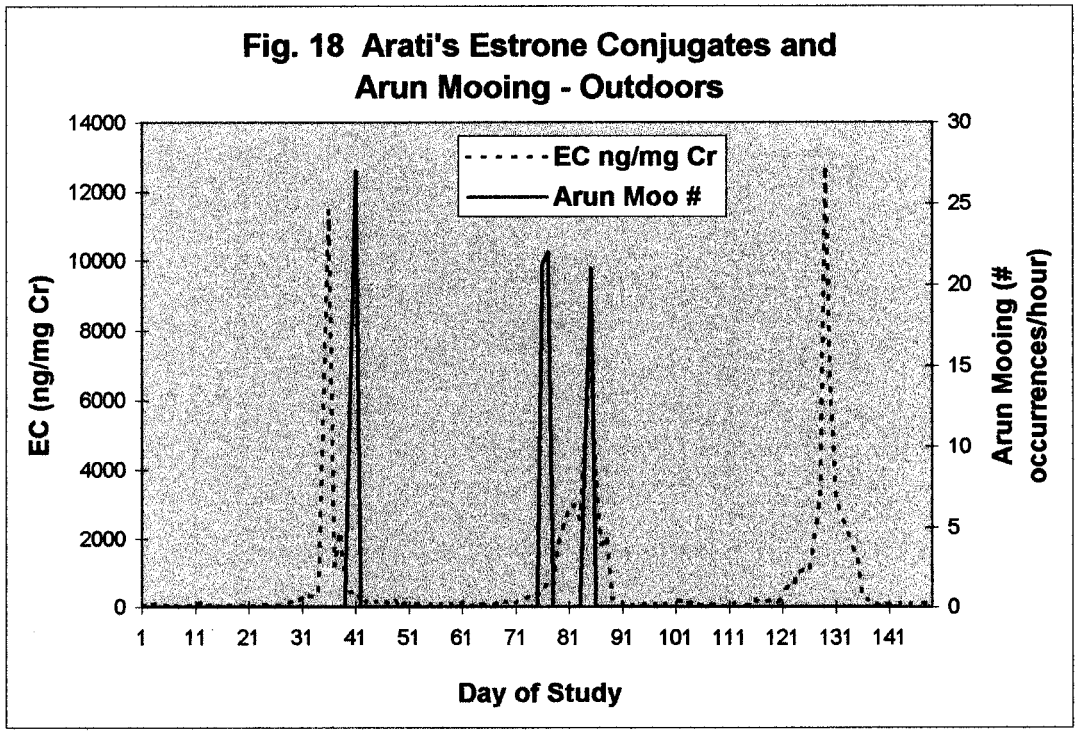
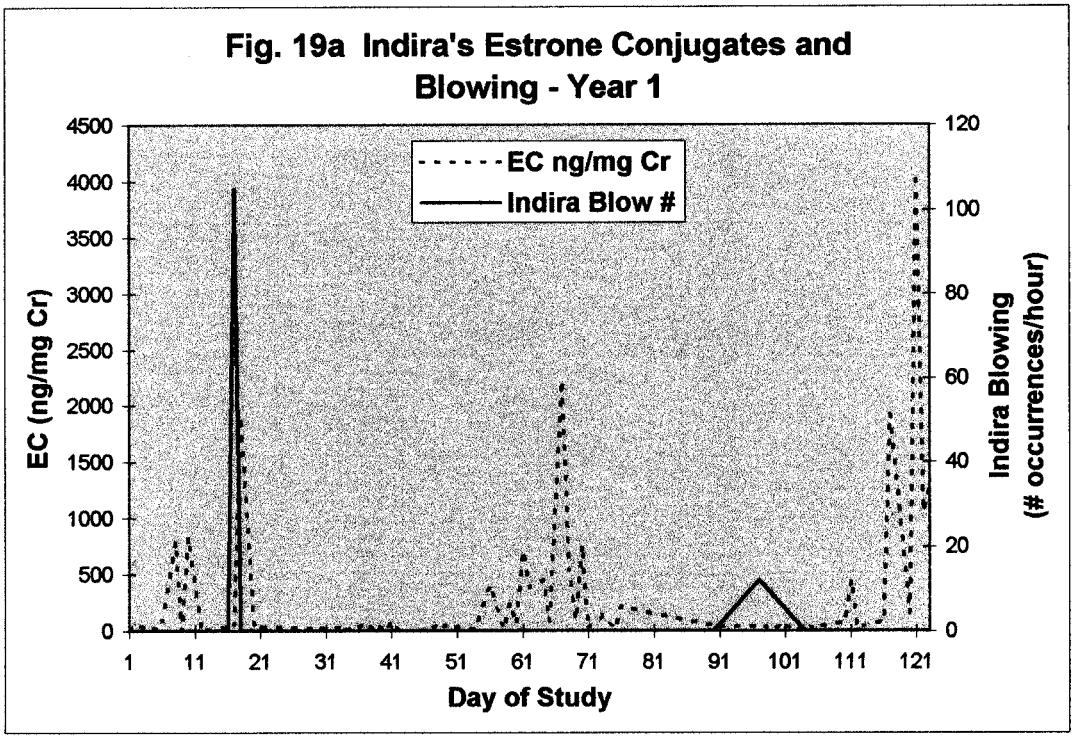


Fig. 17 Penis Extended and EC.
Fig. 18 Mooing and EC.



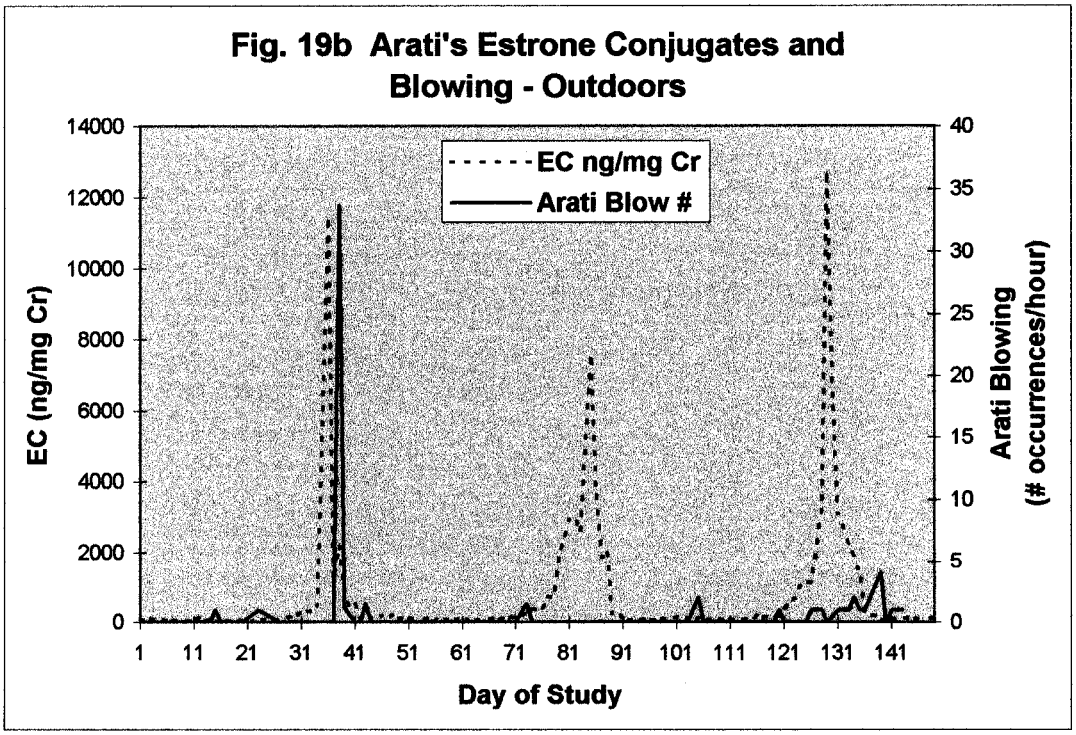
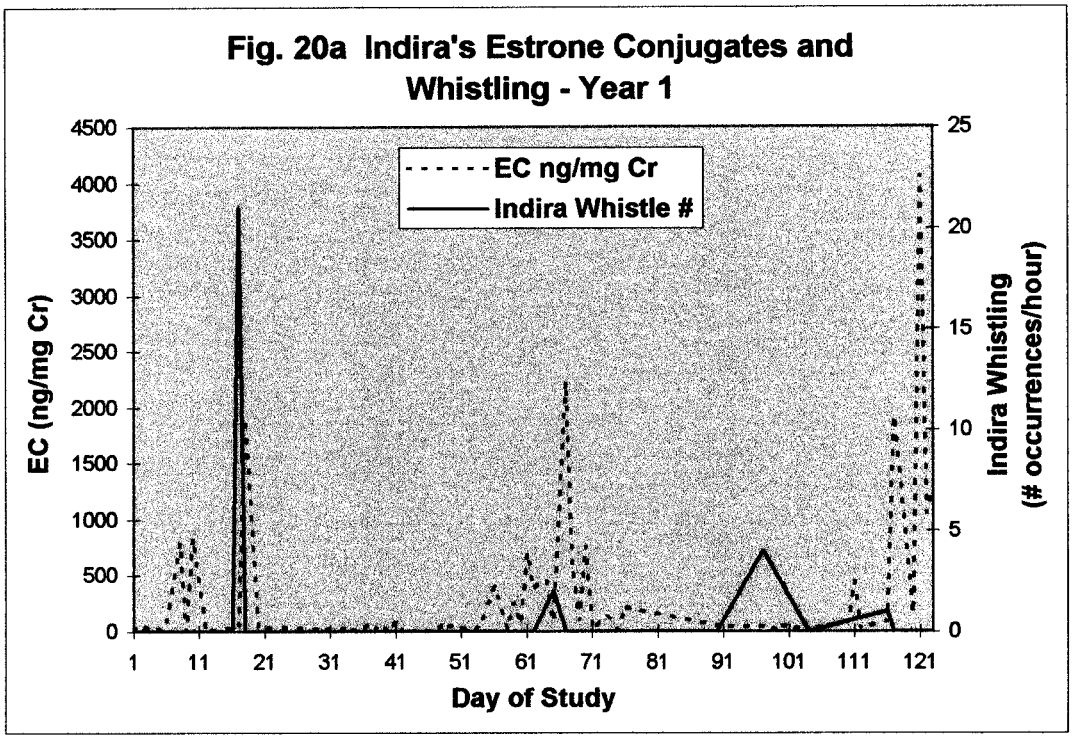


Fig. 19 Blowing and EC.



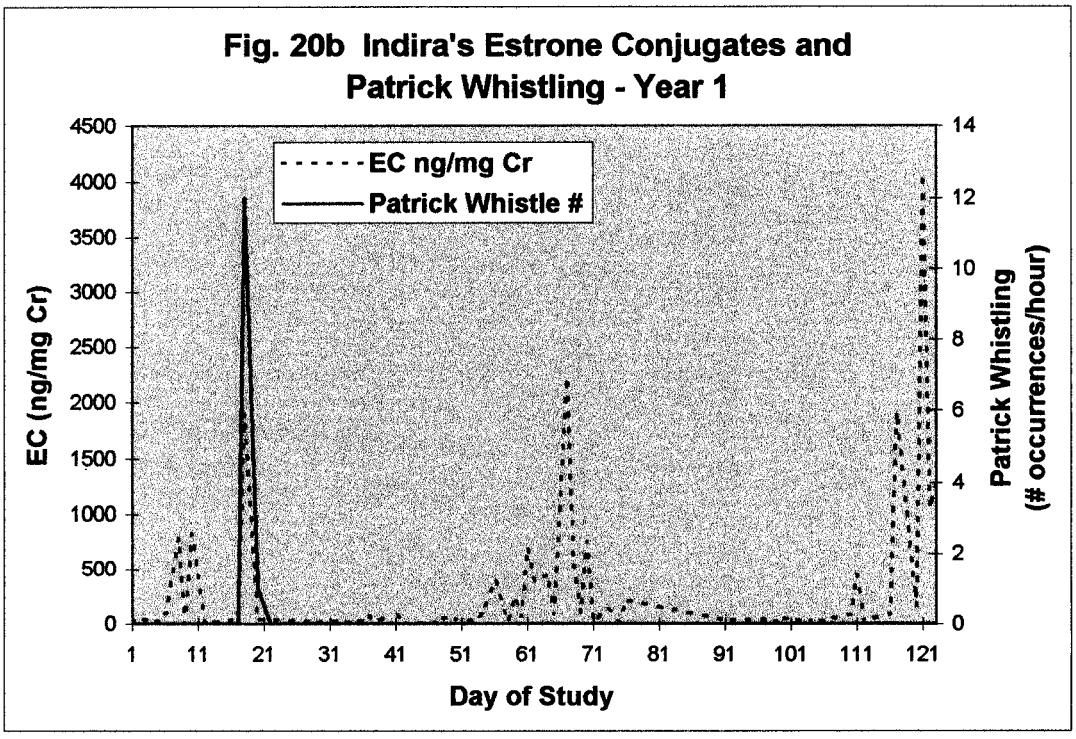
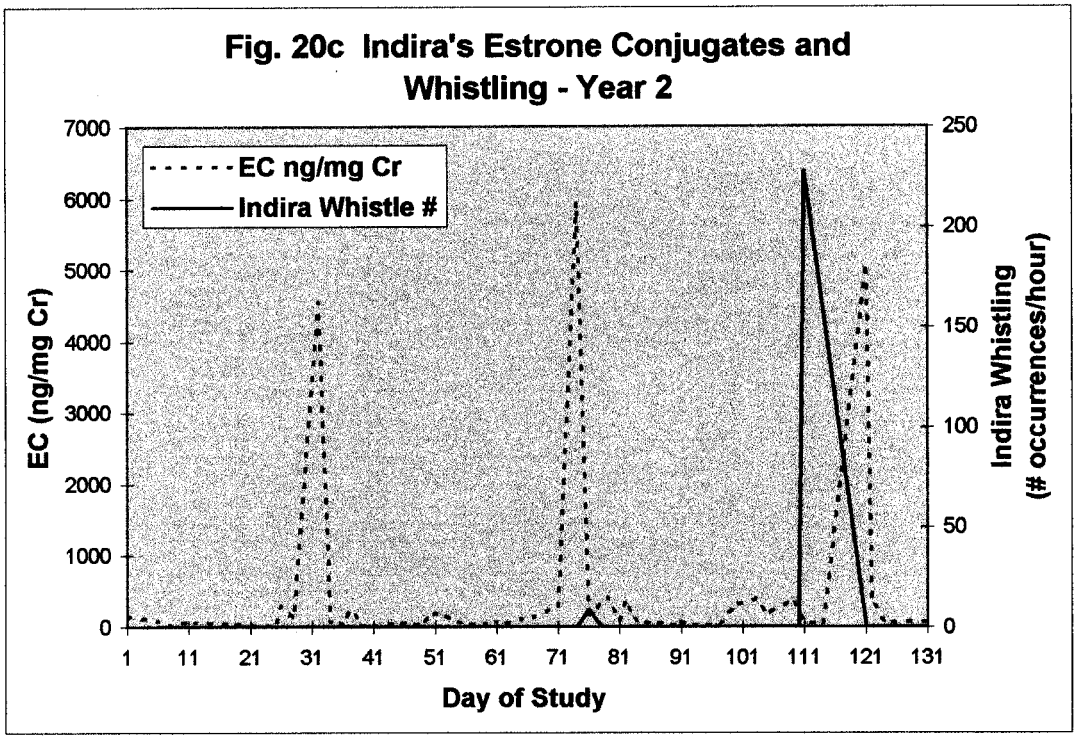


Fig. 20a,b Whistling and EC.



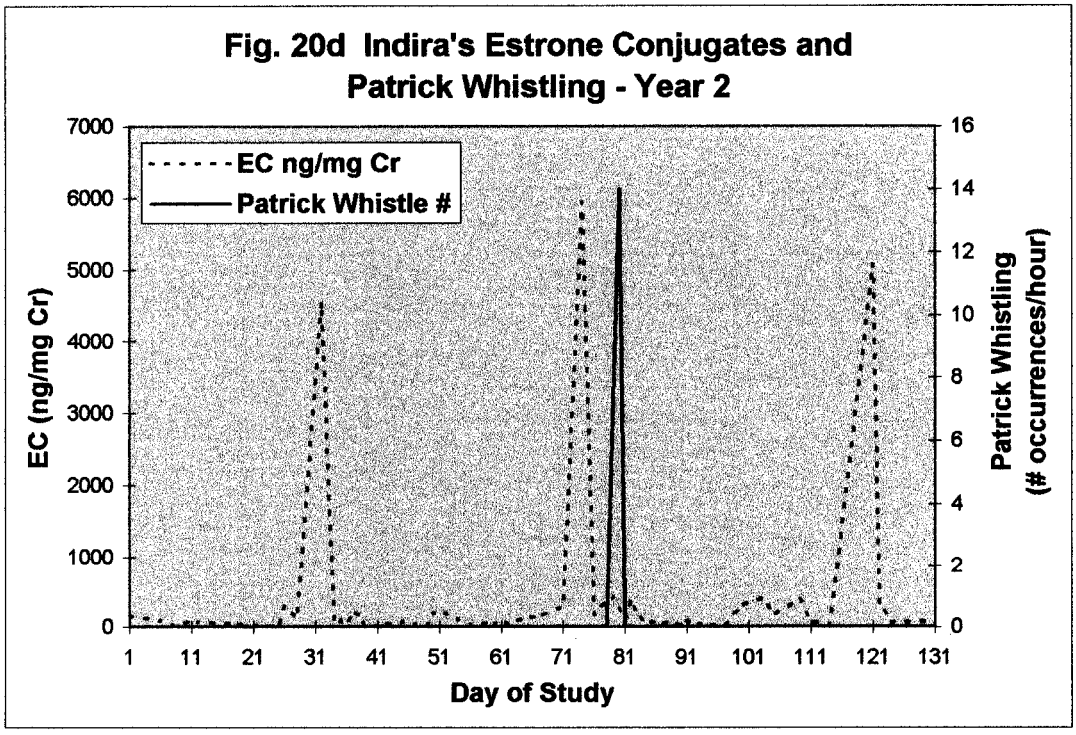
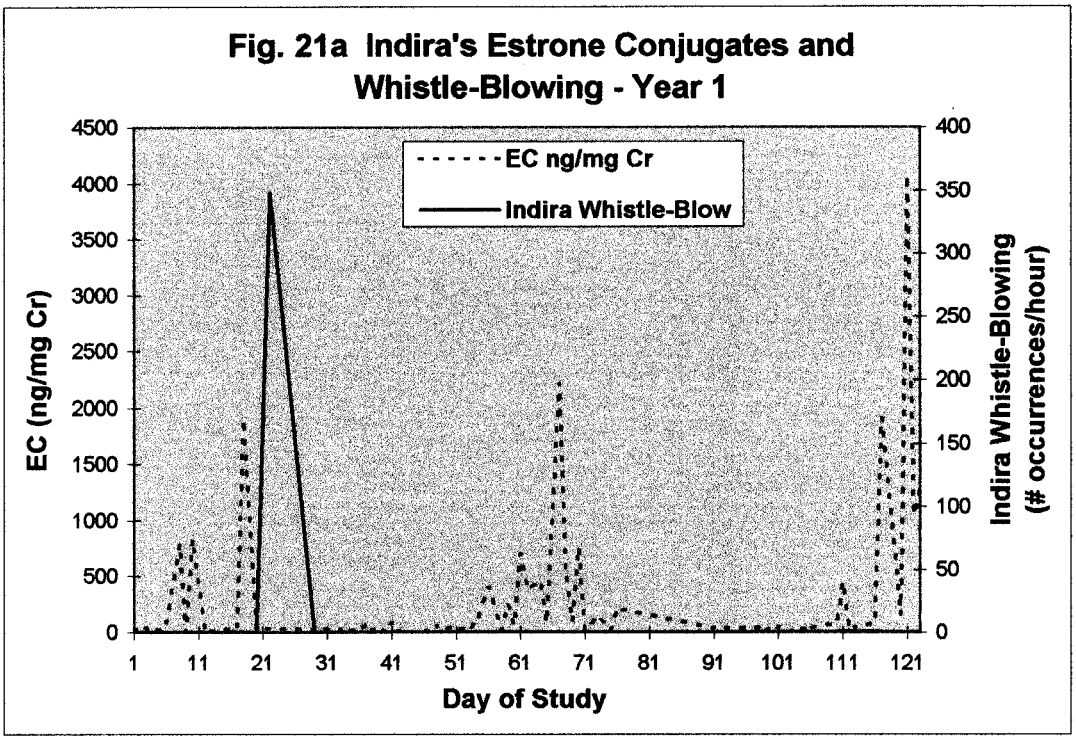


Fig. 20c,d Whistling and EC.



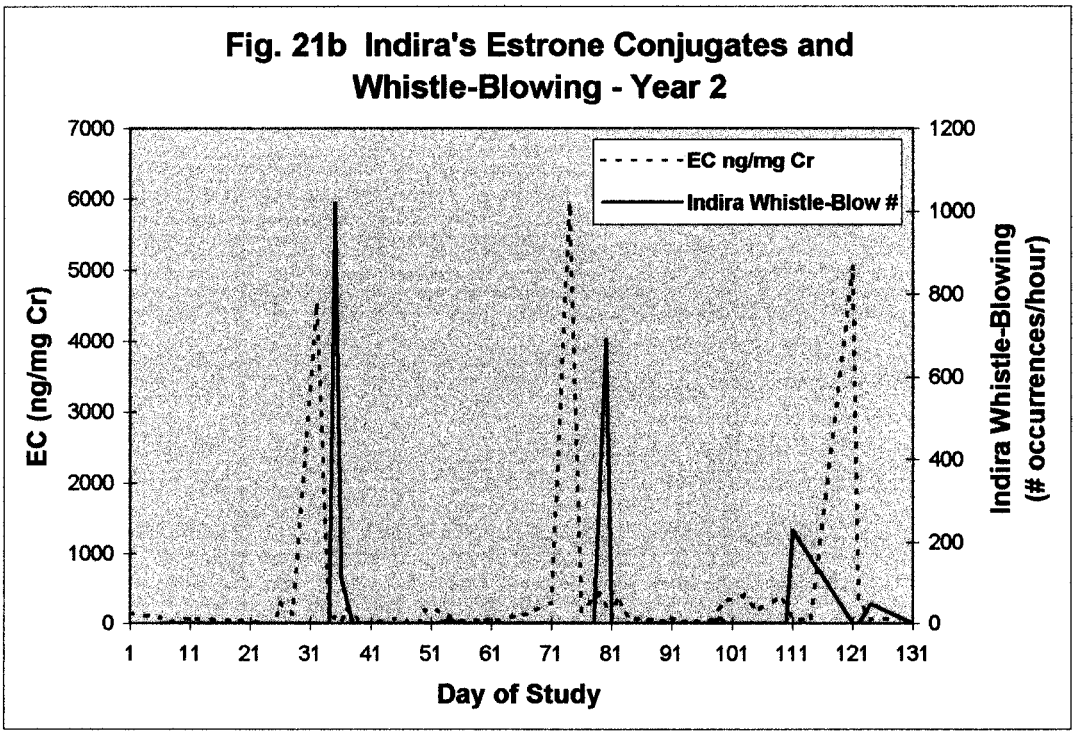
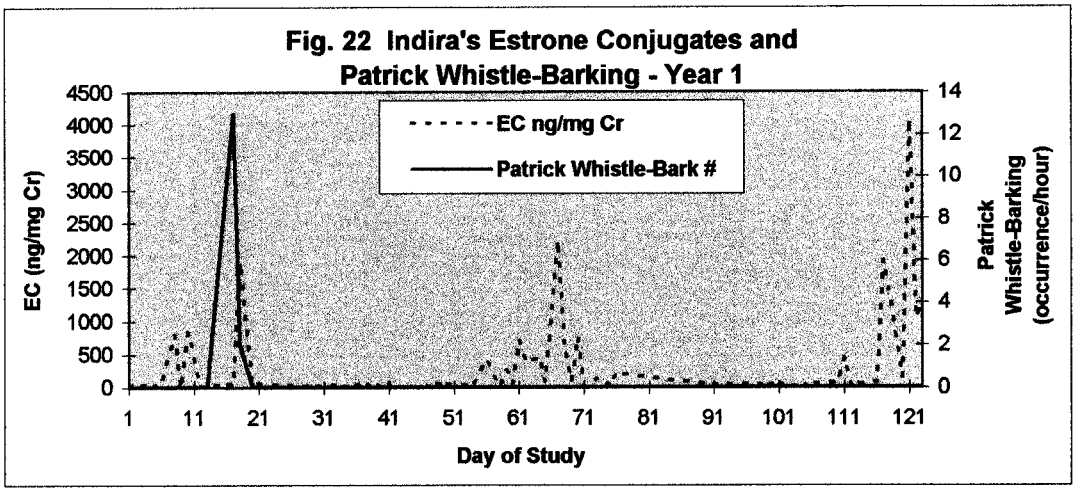
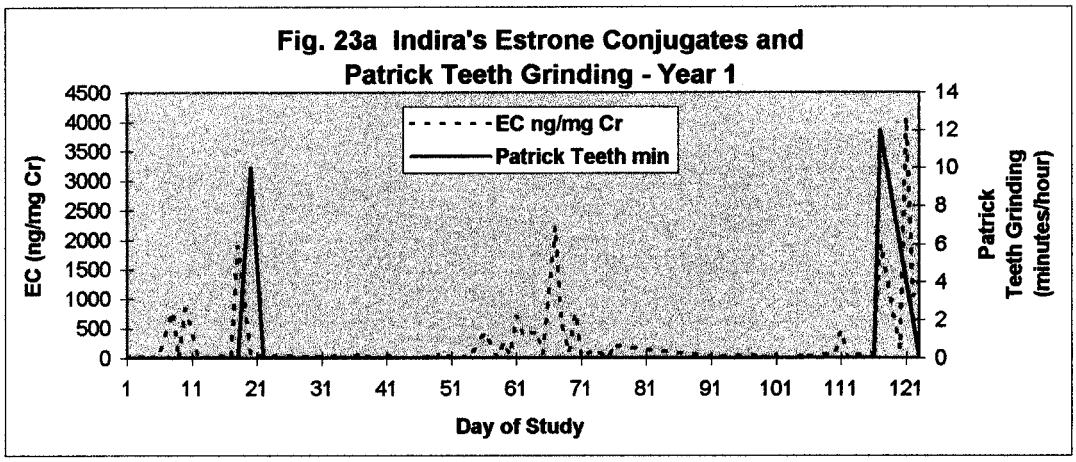


Fig. 21 Whistle-Blowing and EC.





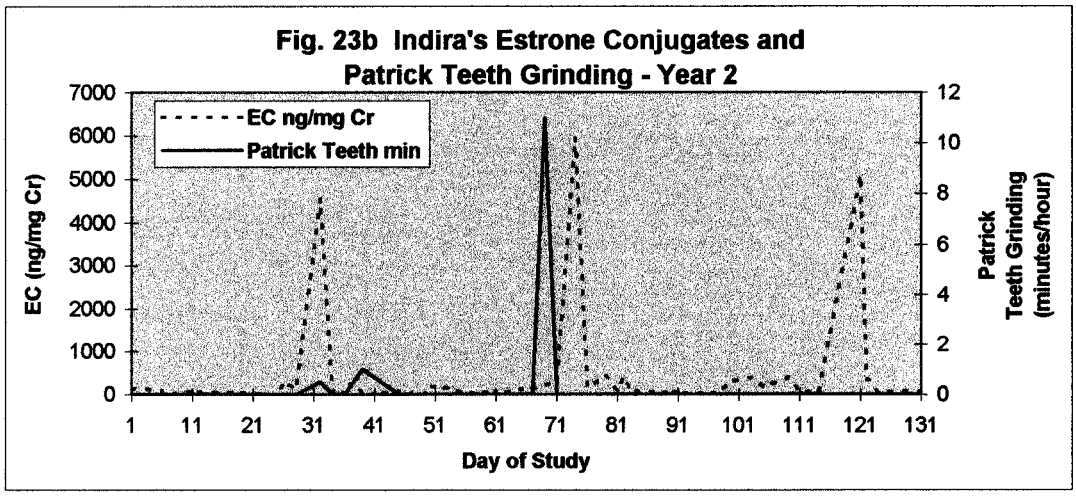
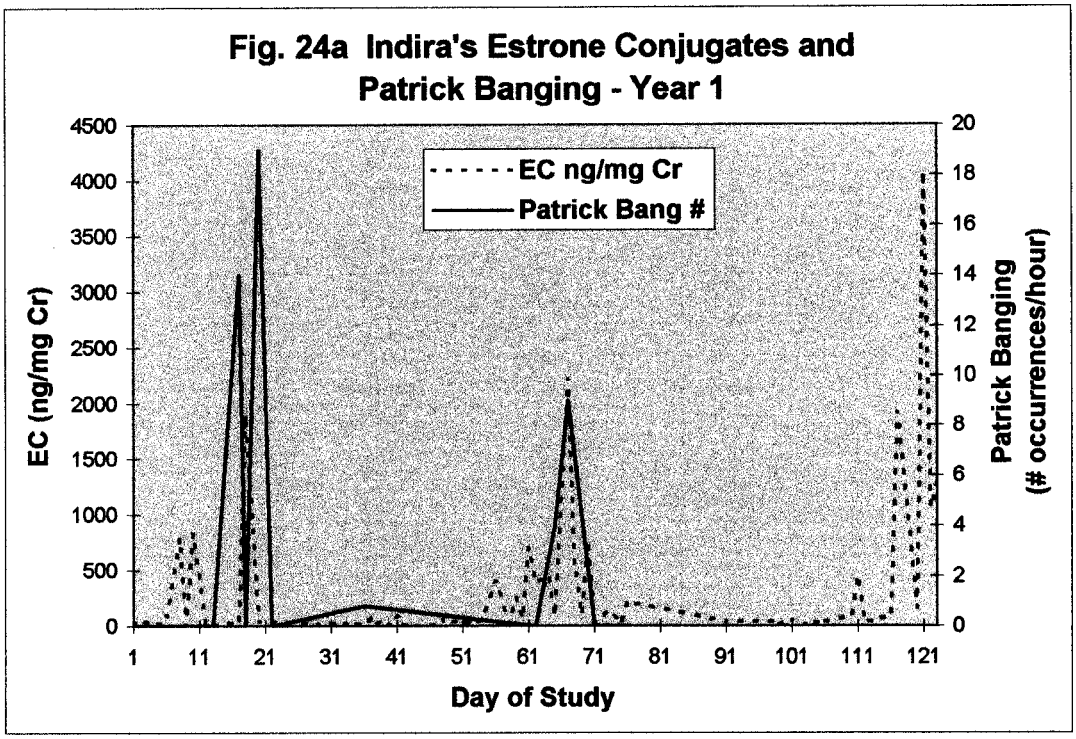


Fig. 22 Whistle-Barking and EC.
Fig. 23 Teeth Grinding and EC.



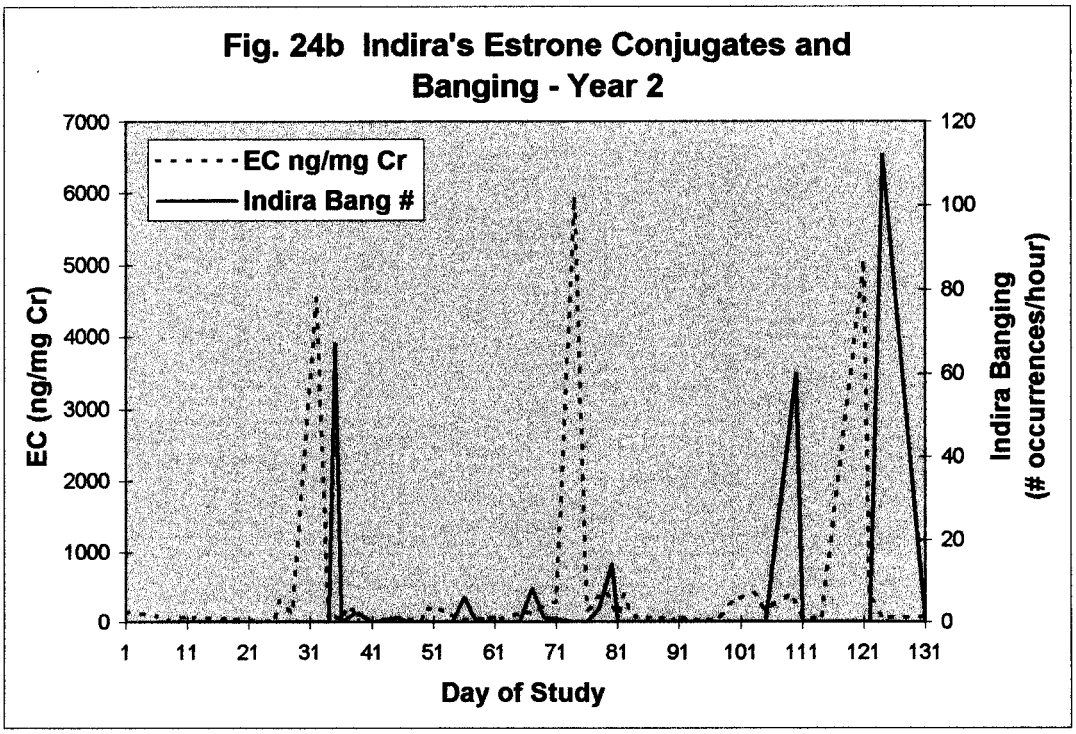
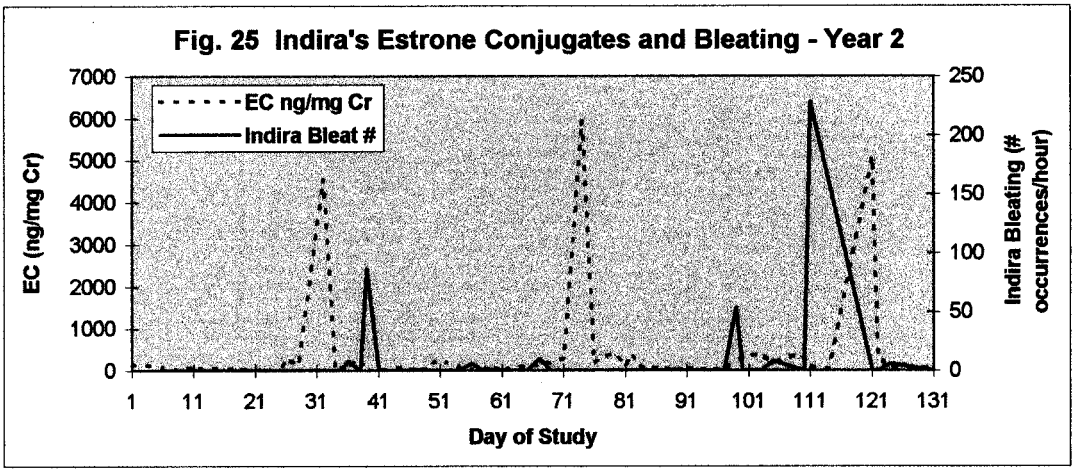
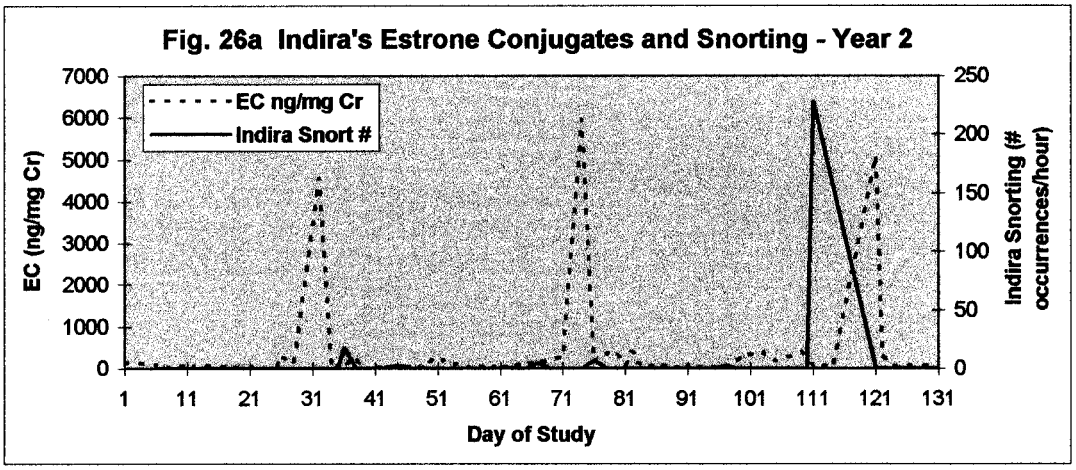


Fig. 24 Banging and EC.





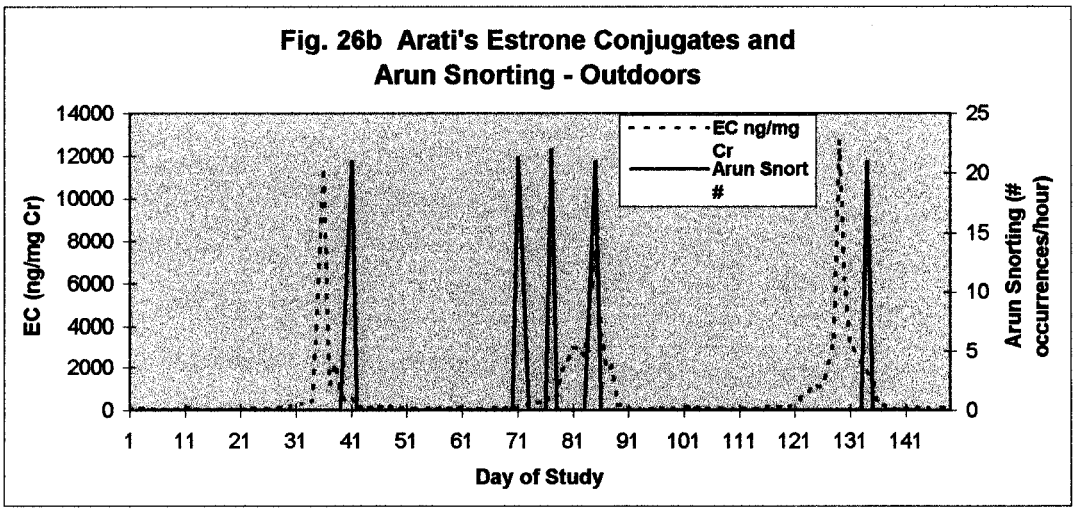
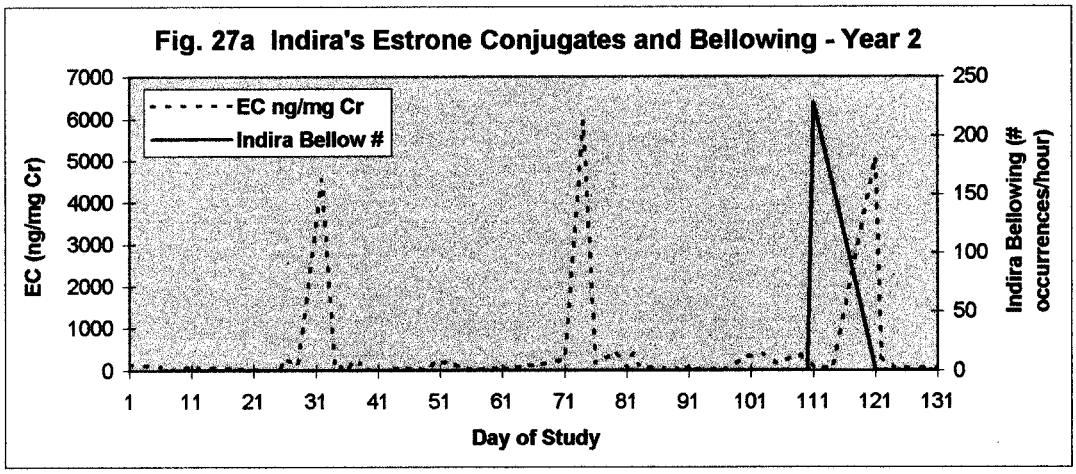
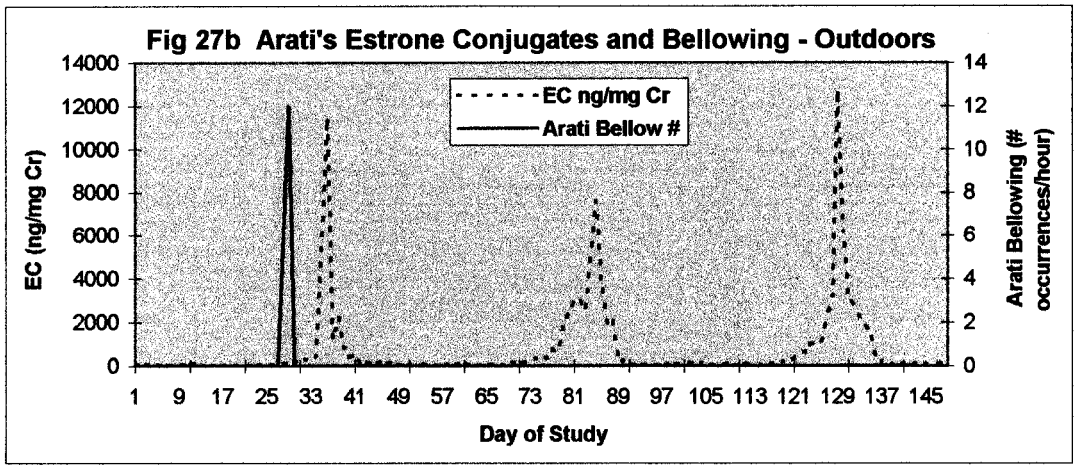


Fig. 25 Bleating and EC.
Fig. 26 Snorting and EC.





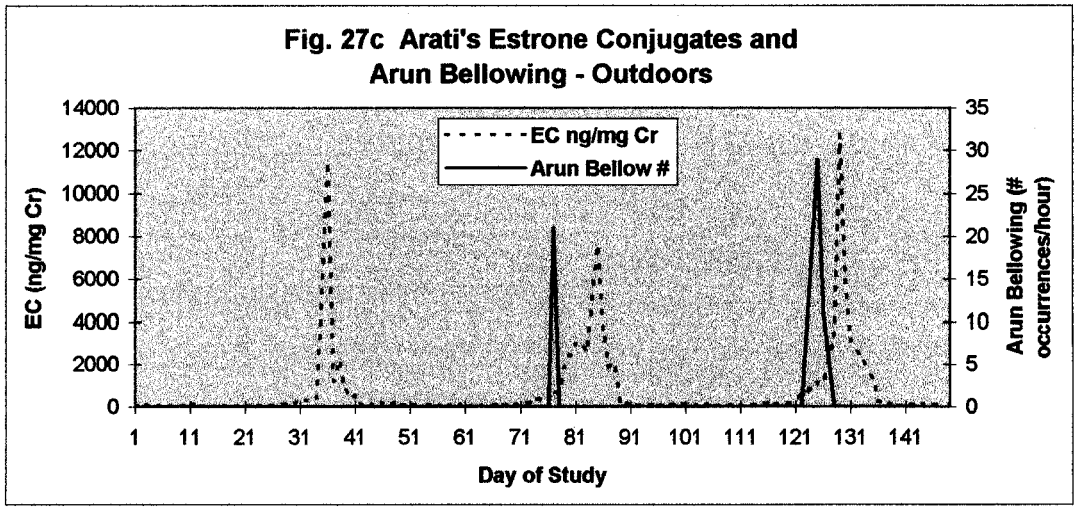
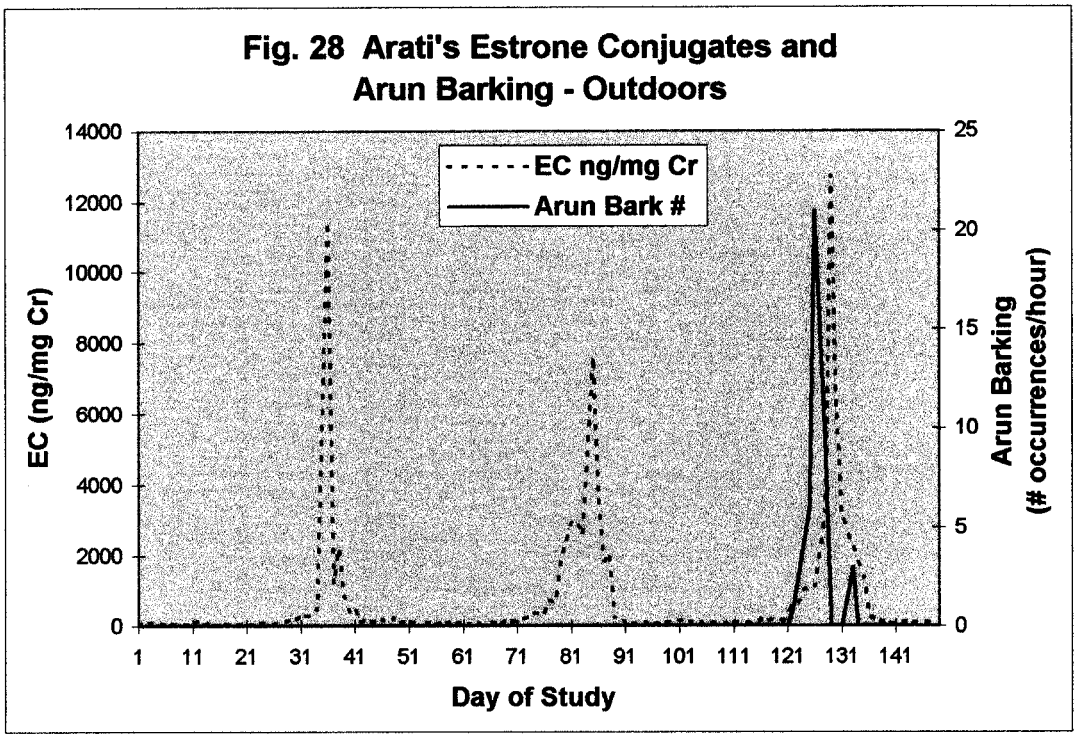


Fig. 27 Bellowing and EC.



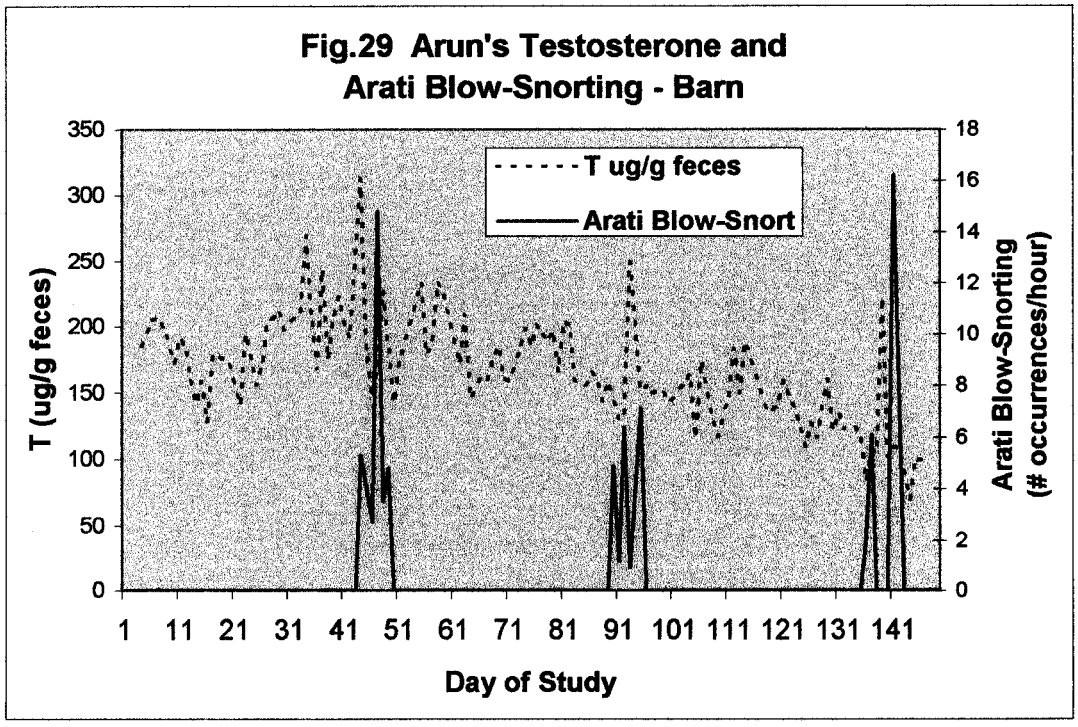


Fig. 28 Barking and EC.
Fig. 29 Blow-Snorting and EC.

however, does not preclude these behaviours from being useful indicators of estrus from an animal management perspective.

Overall, Urine Spraying appears to be the most useful behaviour for predicting estrus. In Arun's case, both in the Barn and Outdoors, Urine Spraying was significantly correlated with EC (Table 1). In addition, the graphs reveal that Urine Spraying was related to EC for both Indira and Patrick, Years 1 and 2, and also for Arati Outdoors (Fig. 16). This contrasts with Laurie (1982), who studied Indian rhinos *in situ* and reported that squirt urination was performed by breeding males only.

Pacing and Whistling were important predictors of estrus for the Toronto pair. According to Fig. 11, Indira's EC and Pacing were related for both Year 1 and Year 2, while EC and Patrick Pacing were significantly correlated for both years (Table 1). Figure 20 shows that Whistling was related to EC for both animals during both years. For Indira specifically, Flehmen (Fig. 15) and Whistle-Blowing (Fig. 21) were important behaviours, as both showed a relationship with EC during both years. Patrick exhibited three behaviours which were related to EC for both years – Weaving (Fig. 10), Teeth Grinding (Fig. 23) and Banging (Fig. 24).

Weaving and Side-to-Side were valuable predictors of estrus for the Fort Worth pair while they were outdoors. Arati Weaving Outdoors showed an association with EC when they were graphed together (Fig. 10), while Arun Weaving Outdoors was significantly correlated with EC (Table 1). In the case of both animals Side-to-Side Outdoors was related to EC (Fig. 13). For Arun specifically, Charging (Fig. 12) and

Sniff/Taste Urine/Feces (Fig. 14 and Table 1) were key behaviours, as both were related to EC in the Barn and Outdoors.

Blow-Snorting by the FW pair while in the barn was related to Arun's T levels. Fig. 29 shows that Arati Blow-Snorting Barn was associated with T, while Arun Blow-Snorting Barn was significantly correlated with T (Table 2). From observations of the pair while they were outside in the same enclosure, Blow-Snorting was a defensive/aggressive behaviour. Arati usually Blow-Snorted whenever Arun approached her, often at the same time as she backed away from him and Head Bobbed (T and Arati Head Bobbing Outside were significantly correlated – see Table 2). Arun usually Blow-Snorted when he first entered the enclosure that Arati was already in. Both animals uttered this vocalization whenever Arun was chasing Arati. In the barn, Blow-Snorting by Arun typically started a few days after Arati had begun to exhibit behaviours indicative of estrus, and Arati usually Blow-Snorted in response to Arun.

CONCLUSIONS

The results of the Indian rhino study support the initial hypotheses. The female behaviours originally expected to occur more often with high EC levels were urination, defecation, foot scraping and whistling. Of these, urination (in the form of Urine Spraying) and Whistling were found to be potential predictors of estrus, along with several other behaviours. The male behaviours expected to occur more often with high EC levels were urination, defecation, foot scraping, loud vocalizations and charging. Of these, urination (Urine Spraying) and Charging were found to be potential predictors of estrus, along with several others.

Three female behaviours, namely bellowing, blow-snorting and head bobbing, were expected to occur more often when the T levels of nearby males were high. Of these, Blow-Snorting and Head Bobbing were found to be related to high T. Three male behaviours, bellowing, blow-snorting and charging, were expected to occur more often when T levels were high, and it was found that Blow-Snorting was indeed related to high T.

This study was successful in identifying a "repertoire" of behaviours which were indicative of estrus (and of male T levels). Only Urine Spraying, however, was related to EC for all animals during all observation periods, and most behaviours occurred during only one or two estrus periods in a set of three. For these reasons it is important for animal managers to observe their individual charges in order to determine which

behaviours are most relevant. Observation of male as well as of female behaviour is important in determining sexual receptivity.

Both the practical and the theoretical goals of this study were achieved. The identification of estrus behaviours will assist animal managers in predicting the times during which rhinos are ready to breed, thereby eliminating or reducing the need for hormone analysis, lessening the chance of injury to the animals during introductions and ultimately improving reproductive rates. The data regarding the relationship of individual behaviours to particular hormonal events provide new insights into the reproductive biology of the species, and more specifically, the mechanisms by which these solitary, widely dispersed animals communicate information about their reproductive status and willingness to mate.

CHAPTER 2 - THE SUMATRAN RHINOCEROS

(*Dicerorhinus sumatrensis*)

INTRODUCTION

Life History of the Sumatran Rhinoceros

The Sumatran rhino is the smallest and most primitive of the five extant rhino species. Also known as the hairy rhinoceros, the Sumatran rhino is closely related to the woolly rhino *Coelodonta* and the large, unicorn-like *Elasmotherium* which inhabited Eurasia during the Pleistocene (Foose and van Strien, 1997; Nowak, 1999; van Strien, 1974). Adults range in height (Nowak, 1999) from 112 to 145 cm (3'8" – 4'9") at the shoulder and in length from 236 to 318 cm (7'8" - 10'4"). The weights of the 17 adults currently held in captivity range from 520 to 816 kg (International Workshop on the Sumatran Rhinoceros, Bogor, Indonesia, March 6-7, 2000). They have two horns; the anterior horn may reach up to 31 cm (1') in length while the posterior horn is only 5 to 10 cm (2" to 4") long. Unlike the Indian rhino, the Sumatran rhino's skin is dark grey-brown and relatively smooth, with a conspicuous fold just behind the shoulder and another in front of the hindquarters. The skin of young animals is usually covered with long, reddish brown hair that becomes darker and sparser with age (Groves and Kurt, 1972; Nowak, 1999; van Strien, 1974).

This species formerly ranged over much of Southeast Asia - throughout eastern Malaysia, Borneo and Sumatra and from eastern Bangladesh and the foothills of the Himalayas in Bhutan and eastern India through Burma, Thailand, Peninsular Malaysia and possibly even Vietnam (Foose, 2002; Foose and van Strien, 1997; Groves and Kurt,

1972; IUCN, 1978; Khan, 1989; Talbot, 1960; van Strien, 1974). Today fewer than 300 Sumatran rhinos survive in the wild, with Malaysia (Peninsular Malaysia, Sabah and Sarawak) and Indonesia (Sumatra) being the only significant range states. Fourteen (5.9) animals are maintained in captivity in 4 institutions (Foose, 2002).

Sumatran rhinos are primarily rainforest animals, although they can live in a variety of habitats, from swamps at sea level to drier areas high in the mountains. They prefer rugged landscape at higher elevations with easy access to water, in primary, tropical rainforest or, failing that, mountain moss forest, although they are sometimes attracted to forest clearings and fringes and regeneration areas (Foose and van Strien, 1997; Groves and Kurt, 1972; IUCN, 1978; Khan, 1989; Nowak, 1999; van Strien, 1974).

Van Strien (1985) observed that the need for salt had a considerable influence on behaviour. Each individual rhino maintained a permanent, well-defined home range which included a natural salt lick. Male territories averaged 30 km^2 and overlapped extensively with each other, although each seemed to contain an exclusive core area. Female territories were smaller but did not tend to overlap, except in the vicinity of salt licks. Van Strien (1985) concluded that females were much more territorial than males and were strongly driven to avoid one another. Adults of both sexes were observed to mark their ranges with feces, sprayed urine, bent or twisted saplings and hoof/horn scrapes. The population density in his study area (Gunung Leuser National Park in northern Sumatra) was 13-14 animals per km^2, though he noted that this figure was probably higher than normal because of the high number of salt licks in the area.

Sumatran rhinos are browsers and need large amounts of fibre-rich food. The diet of Sumatran rhinos includes fruit, leaves, twigs and bark. They often knock down small trees to browse on their twigs and leaves. Young saplings, which form a major food source, are bitten off, stepped on or broken off with the horns (these behaviours may also be used to mark territory boundaries). Favoured foods are mangoes, figs, bamboo and many plants found in secondary growth forest. Animals inhabiting the forest fringes may also raid nearby cultivated crops (Groves and Kurt, 1972; Khan, 1989; Nowak, 1999; van Strien, 1985).

The Sumatran rhino is divided into 3 sub-species. The most common, *Dicerorhinus sumatrensis sumatrensis*, is currently found in Peninsular Malaysia (70-100 animals), Sumatra (100-150 animals) and possibly Thailand (if so, no more than 10 animals). Thirteen representatives of this sub-species are maintained in captivity at sites in Peninsular Malaysia, Sumatra and the USA. *D. s. harrissoni* is the sub-species of Borneo and is represented by 50 to 70 wild animals in 3 sites in Sabah and Sarawak. Two representatives of this sub-species are maintained in captivity in Sabah. The third sub-species, *D. s. lasiotis*, was formerly found in India, Bangladesh and Burma but is now designated extinct by the IUCN, although it has been speculated that there may still be 6 to 7 animals in Burma. The persons in charge of the captive breeding programs are working to preserve the two extant sub-species (Bernstein, 1997; Foose, pers. comm., 2000; Foose, 2002; Foose and van Strien, 1997; Khan, 1989; Nowak, 1999).

Endocrinology and Reproduction in the Sumatran Rhinoceros

Breeding Sumatran rhinos has proven to be even more difficult than breeding Indian rhinos. This is no doubt due, at least in large part, to the fact that almost nothing is known about the reproductive biology of the Sumatran rhinoceros. Little information is available about natural reproduction because of the scarcity of *in situ* studies and the difficulties involved in observing this shy, solitary animal in its dense rainforest home. Detailed knowledge of captive reproduction is limited by high mortality (for example, 40 animals were caught between 1984 and 1994, but only 15 currently survive), failure to reproduce in captivity (only two calves have been born in captivity, and the first of these was bred in the wild) and by the fact that only one female has been extensively studied (Foose, 2002; Foose and van Strien, 1997; Roth et al., 2001). The Sumatran female in Roth et al's (2001) study was polyestrous with a reproductive cycle of 21 days in length. Van Strien (1985) found that Sumatran rhinos are seasonal breeders - most of the births at his study site in northern Sumatra occurred during the time of heaviest rainfall, which is October to May. The gestation period of the Sumatran rhinoceros is thought to be 17 to 18 months or 510 – 550 days and the inter-birth interval 3 to 4 years (Fouraker and Wagener, 1996; van Strien, 1985). Van Strien (1985) reported that calves remain with their mothers for 16 to 17 months after birth and that neither sex breeds until 7 or 8 years of age.

Recently two new factors have been recognized as contributing to the problem of breeding Sumatran rhinos. The first involves the pathology of the female reproductive

tract. In the past, rectal ultrasound examinations of the 12 Sumatran females maintained in captivity had revealed a high incidence of cysts and tumours in the reproductive tract (Schaffer et al., 1994). Ultrasounds conducted during 2000 have shown that not only have many of these pathologies continued to worsen but also that some previously unaffected females are now developing problems (Schaffer, pers. comm., 2000).

The second difficulty arises from the possibility that Sumatran females are induced ovulators, which would make them the first reported induced ovulators within the Perissodactyla order. An adult female housed at the Cincinnati Zoo in Ohio has been the subject of exhaustive study by Roth et al. (2001). Roth has used fecal and serum progesterone analyses, serum luteinizing hormone (LH) analysis and rectal ultrasound to determine that this female is indeed an induced ovulator. Ultrasound examinations revealed the formation of anovulatory, hemorrhagic follicles when the animal was not bred. These follicles underwent varied levels of luteinization, resulting in irregular fecal progesterone profiles. When, however, the female was breeding regularly the fecal and serum progesterone profiles reflected a regular 21-day cycle. Serum LH levels were baseline prior to breeding, increased up to 30-fold within 1-2 hours after copulation and then returned to baseline within 22 hours. Ovulation occurred within 46 hours after copulation.

Indian, Black and White rhinos are known to be spontaneous ovulators (Bellem and Goodrowe, unpublished data; Czekala and Callison, 1996; Hindle and Hodges, 1990; Hindle et al., 1992; Kasman et al., 1986; Kassam and Lasley; 1981), so until the results of

Roth et al.'s (2001) study were revealed it had been assumed that Sumatran rhinos would follow the same pattern as the rest of their Family (it should be noted that the reproductive physiology of the Javan rhino has not been studied at all). Sumatran rhinos, however, are evolutionarily ancient compared to the other four rhino species (Foose and van Strien, 1997; Roth et al., 2001), so it is perhaps not surprising that they are different from the rest of their genus. It has been hypothesized (Roth, pers. comm., 1999; Schaffer, pers. comm., 1999) that once Sumatran females have reached the age of sexual maturity they may need to breed on a fairly regular basis, in order to stimulate ovulation and a regular estrogen cycle and thereby maintain the health of their reproductive tracts. This possibility dramatically increases the already intense pressure to breed these animals.

Hodges (pers. comm., 2000) and Agil (pers. comm., 2000) conducted high-pressure liquid chromatography followed by immunoreactivity (EIA) analysis of fecal samples from a male Sumatran rhino. They found that epiandrosterone/5α-dihydrotestosterone (these metabolites were eluted in the same fraction) and testosterone were major metabolites (~ 880 pg/fraction and ~ 600 pg/fraction, respectively). In contrast, similar analysis of urine from the same male revealed that the above metabolites each measured only ~ 160 pg/fraction.

Purpose of Study

This project was originally intended to study behaviour and reproductive hormones in Sumatran rhinos in the same manner as was done for the Indian rhinos. Before research with Sumatran rhinos could proceed, however, it was necessary to first develop an EIA for use in monitoring fecal progestogens of female Sumatran rhinos. Although analysis of fecal progestogen metabolites by radioimmunoassay (RIA) had previously been found to be useful in monitoring the reproductive cycle of Black, White and two Sumatran female rhinos (Berkeley et al., 1997; Patton et al., 1999; Radcliffe et al., 1997; Roth et al., 2001), few of the facilities that house Sumatran rhinos are equipped to conduct assays involving radioactivity (i.e. RIA). For facilities with limited research budgets and laboratory space, enzyme immunoassay (EIA) offers an affordable alternative to RIA while still permitting accurate assessment of reproductive activity. Besides low cost, EIA has several other advantages over RIA, for example, ease and speed of performance, easy reading of end point and avoidance of the dangers and difficulties associated with the acquisition, use and disposal of radioactive isotopes (Bellem et al., 1995; Hodges, 1986; Hodges and Green, 1989; Kirkpatrick et al., 1990; Kirkpatrick et al., 1991). For these reasons it was deemed necessary to develop an EIA, preferably one comparable to the existing RIA, before beginning any studies of Sumatran rhinos. Such an EIA was developed with the assistance of Astrid C. Bellem at the Smithsonian Institution's National Zoological Park/Conservation and Research Center (CRC) in Front Royal, VA, USA, where the above mentioned RIA was already in use.

MATERIALS and METHODS

Experimental Animal

The study subject was an 8-year-old, nulliparous female Sumatran rhinoceros housed at the Cincinnati Zoo and Botanical Garden in Ohio. This female was wild-caught in 1991 at approximately 2 years of age, raised at the Los Angeles Zoo in California and transferred to the Cincinnati Zoo in 1995. She previously had been shown to exhibit regular ovarian cyclicity, as evidenced by rectal ultrasound, RIA analysis of fecal progestogens, EIA analysis of serum LH and breeding activity (Roth et al., 2001).

Housing and Care of Animals

This female was kept with two other Sumatran rhinos, a non-cycling female and a male who had previously bred the study subject without producing any offspring. The three animals were kept apart (except during breeding introductions) but within auditory, olfactory and, occasionally, visual range. During the summer the animals were displayed in outdoor exhibits (2 to 2.5 km^2), but had access to individual stalls within a barn. When the weather was colder the animals were kept off display in the heated barn (21 °C) and allowed access to their yards for an hour each day. Access to the outdoor yards was denied during heavy snow fall, severe wind chill or when temperatures dropped below 5° C. During the winter the barn was lit artificially from 0700 hours to 2000 hours (Roth et al., 2001).

The rhinos were fed 30 to 50 kg per day of fresh browse consisting of 10 types of Ficus and, occasionally, Kaffir plum. They also received 2-3 flakes of hay (40% alfalfa, 60% orchard grass) and 1.8 kg of grain (ADF16 by Mazuri®, St. Louis, MO, USA) daily. (An average flake of alfalfa hay weighs 5.5 kg, grass hay 3 kg). This diet was supplemented by 6 ml vitamin E per day (Emcelle Tocopherol, 500 IU/ml, Stuart Products Inc., Bedford, TX, USA) and fresh produce consisting of 15 bananas, 15 apples and 4 sweet potatoes per animal per day. The animals also had free access to a mineral block and fresh water (Roth et al., 2001).

Sample Collection

Fecal samples (n = 170) were collected from the study subject over a period of 182 days (September 5, 1997 to March 6, 1998). The samples were collected soon after voiding, sealed in 50 ml polystyrene tubes and frozen at –20° C. Later they were packed in dry ice and shipped to the CRC for analysis.

Fecal Steroid Extraction – Progestogen (P) Metabolites

Fecal samples were dried using a rotary evaporator, pulverized and then extracted twice with 5 ml of 90% EtOH: dH_2O. For the first extraction 0.09-0.11 g of fecal powder was weighed into numbered 16 x 125 mm glass tubes. Next 0.5 ml EtOH and 0.5 ml dH_2O were added to each tube and the tubes were vortexed briefly.

To monitor procedural losses (i.e. the proficiency of the person performing the extraction), 100 µl of [^{3}H] estradiol tracer (equal to 1000 dpm or depreciation per minute) were added to the first 62 sample tubes and two controls which contained only the tracer and 10 ml of scintillation fluid. Extraction proceeded as described below, and when finished the dpm of each sample was counted in a Beckman LS5801 counter and compared to the dpm of the two controls. Recovery was 70% (i.e. 30% procedural loss) with a CV of 12%. This loss was deemed acceptable so this step was omitted for the remainder of the samples.

An additional 4.0 ml EtOH were added to each tube, which were then vortexed briefly. The tubes were boiled in a water bath at approximately 90° C for 20 minutes, (EtOH was added as necessary to prevent the samples from drying out), and after boiling EtOH was used to raise the volumes to approximately to pre-boil levels (5 ml). The samples were centrifuged at 1500 rpm for 20 minutes. After centrifugation the supernatants were poured into an identical set of numbered glass tubes.

For the second extraction the pellets which remained after centrifugation were re-suspended in 4.5 ml EtOH and 0.5 ml dH_2O, vortexed for 30 seconds and centrifuged again at 1500 rpm for 15 minutes. The second set of supernatants was combined with the first, and then the combined solutions were dried down under air in a warm water bath (37°C). Once all of the tubes were completely dry 1 ml of MeOH was added to each and they were vortexed briefly then placed in an ultrasonic glass cleaner for 15 minutes. For each sample 200 µl of the extract were mixed with 1800 µl of PBS (phosphate-buffered

solution) Buffer (1:10 dilution) in labelled (animal's name and sample date) 12 x 75 mm plastic tubes. Appendix III contains recipes for PBS Buffer and all other stocks and solutions. Extracts were stored in the plastic tubes at -20° C until required for analysis.

Assay Development - P Enzyme Immunoassay

The RIA, previously described by Brown et al. (1994), used a monoclonal progestogen (P) antibody (Ab) which was produced against 4-pregnene-11-ol-3,20-dione hemisuccinate:BSA. This Ab was provided by J. Roser, University of California, Davis, CA, USA. The major cross-reactivities (refer to Appendix II for a complete list) were as follows: progesterone 100%, allopregnanolone 96%, 5α-pregnane-3α-ol-20-one 36%, pregnanolone 15%, 17ß-hydroxyprogesterone 15%, pregnenolone 13%,5ß-pregnane-3α-ol-20-one 7% and 5ß-pregnane-3α,17α-diol,20α-one 5% (Brown et al., 1994; Grieger et al., 1990 and Wasser et al., 1994).

Unfortunately the P Ab was suspended in ascites fluid, which contains a variety of large molecules including many antibodies to the P Ab's carrier protein complex, hemisuccinate:BSA. According to C. Munro (pers. comm., 1998) ascites contains approximately 10% steroid Ab, 90% carrier protein Ab, and although this mixture is acceptable for RIA, in EIA the hemisuccinate:BSA Ab out-competes the P Ab for binding sites on the microtitration plate. Additionally, the other large molecules in the ascites interfere with the binding of both antibodies.

An attempt was made to purify the P Ab by a process called ammonium sulfate (AmS) stripping (C. Munro, pers. comm., 1998). One ml of ascites, 1 ml of Saline Solution and 0.5 ml of double-distilled water (DDW) were combined in a glass beaker. While this mixture was stirring quickly on a stirplate, AmS Solution was added drop-by-drop with the aim of producing a milky colour to indicate that the AmS had bound to the P Ab. Despite adding an excess of AmS (1 ml instead of the called for 0.5 ml) the mixture did not change colour, nevertheless a decision was made to continue with the process and monitor the results. After addition of AmS Solution the mixture was left to stir for 3 hours, and then poured into glass test tubes. The tubes were centrifuged at 3500 rpm and 20° C for 30 minutes, then the supernatants were discarded, the precipitates were combined in a glass beaker and Saline Solution was added to bring the volume up to 1 ml (the original volume of ascites). The above described process was repeated to further purify the precipitated Ab, this time adding Saline Solution to bring the volume up to 0.5 ml. The final step in the AmS stripping was dialysis of the combined precipitate solutions to isolate the purified P Ab. A large glass beaker containing 500 ml of Borate-Buffered Saline (BBS) was placed on a stirplate to stir at the lowest speed. The precipitate solution was poured into a dialysis membrane and placed in the beaker, which was then left to stir for 3 days at 4° C.

Once the dialysis was completed, fecal extracts from the study subject were analyzed using the purified P Ab in a previously established progesterone EIA (Matsuda et al., 1996). This progesterone EIA was conducted several times using different Ab

concentrations, but always failed to produce a colour response to indicate that a reaction had taken place between the P Ab and the enzyme-labelled antigen. It was assumed that AmS stripping had failed to remove the interfering molecules from the ascites, therefore the next step was to try an EIA with a double-layer of Ab, such as the assay previously described by Schwarzenberger et al. (1996 and 1998). The double-layer protocol uses a non-specific Coating or 2° Ab (e.g. goat anti-mouse immunoglobulin) to bind to the plate, then the 1° Ab (P Ab) binds to the 2° antibody. Once the 1° Ab is in place the assay proceeds in a similar manner as the EC, PdG and T assays described in Chapter 1. (For an illustration of the double-layer protocol, refer to Fig. 2 in the General Introduction).

The first trial of the double-layer P EIA assayed various concentrations of progesterone standard – these concentrations, chosen based on the standard curve of the aforementioned progesterone EIA, were as follows: 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.039, 0.195 and 0 ng/ml. It was decided to extend the range of the progesterone EIA at both ends, so 200, 100, 0.098 and 0.049 ng/ml were also tested. This first trial produced what looked like the beginnings of a standard curve, but the colour was not sufficiently developed. The next step in assay development was to adjust reagent concentrations (in particular, Coating or 2° Ab, 1° Ab and enzyme-labelled hormone) so that the '0' wells (which contained no hormone or 1° Ab) developed to an optical density of 1.0 (acceptable range 0.9 to 1.1). For maximum efficiency in testing different concentrations a "checkerboard" pattern of titration (Fig. 30) was used - this allows the concentrations of two reagents to be varied in tandem so that the best combinations can determined. Once

	R1-1	R1-2	R1-3	R1-4	R1-5	R1-6	R1-7	R1-8	R1-9	R1-10	R1-11	R1-12
R2-1	R1-1 & R2-1	R1-2 & R2-1	R1-3 & R2-1	R1-4 & R2-1	R1-5 & R2-1	R1-6 & R2-1	R1-7 & R2-1	R1-8 & R2-1	R1-9 & R2-1	R1-10 & R2-1	R1-11 & R2-1	R1-12 & R2-1
R2-2	R1-1 & R2-2	R1-2 & R2-2	R1-3 & R2-2	R1-4 & R2-2	R1-5 & R2-2	R1-6 & R2-2	R1-7 & R2-2	R1-8 & R2-2	R1-9 & R2-2	R1-10 & R2-2	R1-11 & R2-2	R1-12 & R2-2
R2-3	R1-1 & R2-3	R1-2 & R2-3	R1-3 & R2-3	R1-4 & R2-3	R1-5 & R2-3	R1-6 & R2-3	R1- & R2-3	R1-8 & R2-3	R1-9 & R2-3	R1-10 & R2-3	R1-11 & R2-3	R1-12 & R2-3
R2-4	R1-1 & R2-4	R1-2 & R2-4	R1-3 & R2-4	R1-4 & R2-4	R1-5 & R2-4	R1-6 & R2-4	R1-7 & R2-4	R1-8 & R2-4	R1-9 & R2-4	R1-10 & R2-4	R1-11 & R2-4	R1-12 & R2-4
R2-5	R1-1 & R2-5	R1-2 & R2-5	R1-3 & R2-5	R1-4 & R2-5	R1-5 & R2-5	R1-6 & R2-5	R1-7 & R2-5	R1-8 & R2-5	R1-9 & R2-5	R1-10 & R2-5	R1-11 & R2-5	R1-12 & R2-5
R2-6	R1-1 & R2-6	R1-2 & R2-6	R1-3 & R2-6	R1-4 & R2-6	R1-5 & R2-6	R1-6 & R2-6	R1-7 & R2-6	R1-8 & R2-6	R1-9 & R2-6	R1-10 & R2-6	R1-11 & R2-6	R1-12 & R2-6
R2-7	R1-1 & R2-7	R1-2 & R2-7	R1-3 & R2-7	R1-4 & R2-7	R1-5 & R2-7	R1-6 & R2-7	R1-7 & R2-7	R1-8 & R2-7	R1-9 & R2-7	R1-10 & R2-7	R1-11 & R2-7	R1-12 & R2-7
R2-8	R1-1 & R2-8	R1-2 & R2-8	R1-3 & R2-8	R1-4 & R2-8	R1-5 & R2-8	R1-6 & R2-8	R1-7 & R2-8	R1-8 & R2-8	R1-9 & R2-8	R1-10 & R2-8	R1-11 & R2-8	R1-12 & R2-8

Fig. 30 Microtitre plate map for checkerboard titration.
R1-1 to **R1-12** = Reagent 1 at twelve successive concentrations; **R2-1** to **R2-8** = Reagent 2 at eight successive concentrations.

the ideal reagent concentrations (see next section) had been chosen the progesterone standards were analyzed again to establish the P EIA standard curve. The appropriate range of the curve was determined to be 200 to 0.78 ng/ml. Finally, tests for parallelism and extraction efficiency were performed (described below).

Protocol for P Enzyme Immunoassay

The Coating or 2° Ab, supplied by Sigma-Aldrich (refer to Appendix IV), was Goat Anti-Mouse Immunoglobulin G (IgG). The working Ab solution was prepared by adding 50 µl of Coating (2°) Ab stock to 25 ml of Coating Buffer (Appendix III). NUNC MaxiSorp microtitration plates (Appendix V) were coated with 250 µl/well of the working Ab solution (all wells). Each plate was then covered with an acetate plate sealer and incubated overnight (minimum 12 hours) at room temperature.

After overnight incubation, the contents of the plates were removed, and 300 µl of 2nd Coating (Blocking) Buffer were added to each well. The plates were re-covered and again incubated overnight at room temperature (at this stage, plates can be used after 3 hours or left for up to 4 weeks).

Immediately prior to plate loading, the progesterone standards, samples, 1° Ab and biotin-labelled hormone (BL) were prepared in 12 x 75 mm glass culture tubes. The primary standard stock had been made by mixing 0.5 mg of progesterone with 5 ml of ethanol (EtOH) for a 100,000 ng/ml solution. The secondary stock had been prepared by doing a 1:100 dilution of the primary stock in reagent grade EtOH (secondary stock is

therefore 1000 ng/ml). Finally, a 1:5 dilution of the secondary stock in reagent grade EtOH produced the top standard (200 ng/ml). All standards were stored at -20° C. Just before plate loading, the top standard was diluted 1:2 in Assay Buffer to yield a progesterone standard curve with concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 and 0 ng/ml ('0' wells contained only Assay Buffer). The functional sensitivity of the assay, based on the lowest standard dilution, was 0.781 ng/ml of fecal extract.

Fecal extracts were diluted in Assay Buffer according to the 50% binding concentrations determined by parallelism (refer to Assay Validation – Parallelism, below). The primary antibody (P Ab) stock had been prepared by a 1:1000 dilution in PBS Buffer and stored at -20° C until needed. Just prior to plate loading, the working Ab solution (1:50,000) was prepared by a 1:50 dilution of the primary stock in Assay Buffer. The working Ab solution was then incubated for 20 minutes before use. To prepare the working BL solution (1:500,000), the BL secondary stock (1:5000 – refer to Appendix III) was diluted a further 1:100 in Assay Buffer. The working BL solution was also incubated for 20 minutes before use.

Before loading, the plates were washed at least 3 times with cold (4° C) Wash Solution, then tipped upside-down and banged against a paper-towel-covered surface. (Plates were washed by hand, but this could also be done by a plate washer, as was described for the assays in Chapter 1). The loading step had to be completed within a maximum of 10 minutes. The first two wells of the first column (A1 and A2), designated

non-specific binding or NSB wells, each received 50 µl of Assay Buffer. Fifty µl each of standards (in triplicate) and samples (in duplicate) were then added to the appropriate wells. Next 50 µl of 1° Ab and then 50 µl of BL were added to each well (except 1° Ab was not added to NSB wells). The plates were re-covered and incubated for 3 hours at room temperature.

Immediately before the end of the 3-hour incubation period, the working Streptavidin-_-peroxidase (Streptavidin-POD) solution (1:1.2 x 10^7) was prepared by adding 5 µl of the Streptavidin-POD secondary stock (1:5000) to 12 ml of Assay Buffer (1:2400 dilution). This Streptavidin-POD solution was mixed on a stirplate for a few minutes before use. When ready, the plates were washed at least four times with cold Wash Solution, shaking for 30 seconds on a flat rotator between each wash. After washing, 100 µl of working Streptavidin-POD solution were added to each well. The plates were then re-covered and incubated for 45 minutes (at 4° C) while shaking on the flat rotator.

The substrate solution was prepared immediately before use by combining 500 µl of 0.4% TMB (Appendix III), 100 µl of 0.6% H_2O_2 and 12 ml Substrate Buffer (Appendix III has the recipes for these 3 ingredients). This substrate solution was mixed on a stirplate for a few minutes before use. After incubation the plates were washed at least 4 times with cold Wash Solution, shaking between washes, and then 100 µl of the substrate solution was pipetted into each well. The plates were re-covered and incubated, while being shaken, for 45 minutes (depending on colour intensity) at 4° C. During this

last incubation, the substrate turned blue (due to a chemical reaction between the substrate and the Streptavidin-POD). When the plates had reached a maximum development ('0' standards were darkest) of approximately 1 optical density unit, 50 µl of Stop Reagent (2 M H_2SO_4) were added to each well to prevent further colour development (the blue colour then turned to yellow). The plates were read (test filter 450 nm, reference filter 620 nm) using a PC linked to a plate reader (Dynex Technologies MRX Plate Reader). Plates could be read immediately after adding the Stop Reagent, or within two hours.

Protocol for P Radioimmunoassay

A description of the P RIA protocol can be found in Brown et al. (1994). Roth et al. (2001) reports on parallelism and quality controls established using fecal samples from the same Sumatran rhino female who was the subject of this study. The P RIA used the same 1° Ab (monoclonal progestogen Ab provided by J. Roser) and the same progesterone standard stock as did the P EIA, as well as an ^{125}I-labelled progesterone tracer (ICN Biomedical, Inc., Costa Mesa, CA).

Assay Validation – Parallelism (P EIA)

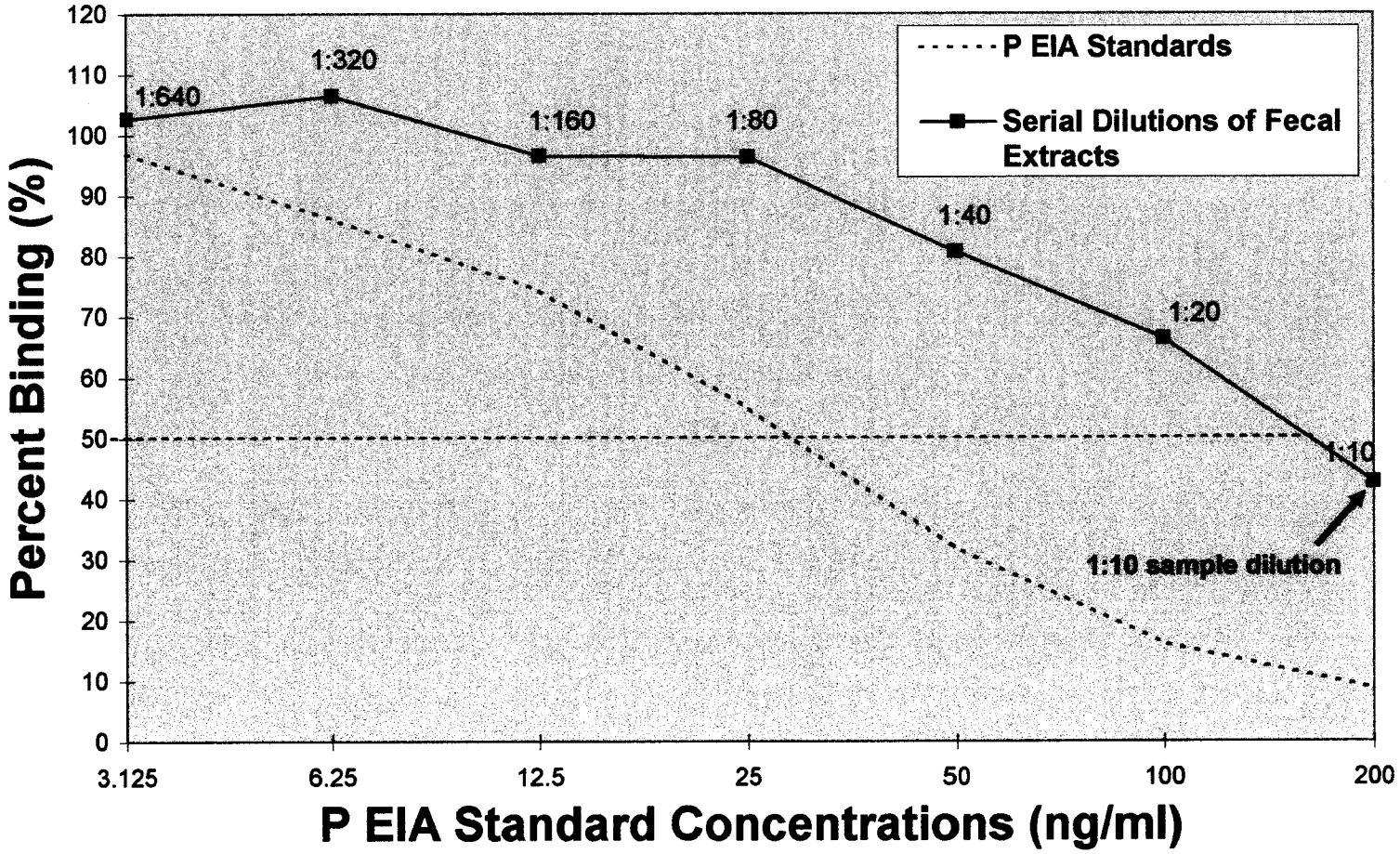
As a preliminary validation of the P EIA, parallelism was determined between serial dilutions of pooled Sumatran female fecal extracts and the P EIA's standard curve. Aliquots from sixty-two extracts (collected between January 3 and March 6, 1998) were

pooled together then serially diluted in Assay Buffer to yield the following range of dilutions: neat, 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560 and 1:5120. The parallelism was conducted on the P EIA, with both the dilution curve and the standard curve pipetted in triplicate. A parallel response of the sample dilutions and the P standard curve indicated that the appropriate progestogens were indeed present in the feces and being measured by the assay (Fig. 31). A sample dilution of 1:10 was chosen for this female, based on approximately 50% binding from the parallelism results (remembering that the extracts were already diluted 1:10 in PBS Buffer, for a total dilution of 1:100).

Assay Validation - Extraction Efficiency (P EIA)

To assess the method of fecal steroid extraction described above, as well as the ability of the P EIA to measure P in the feces of Sumatran rhino females, increasing concentrations of exogenous progesterone (100, 50, 25, 20, 10 ng/ml) were added to five 0.5 g aliquots of a fecal sample from a 20^{+}-year-old , non-cycling Sumatran female housed at the Cincinnati Zoo (this female had regularly exhibited extremely low levels of progesterone, as determined by RIA analysis of fecal progestogens - Roth et al., 2001). These concentrations were chosen based on the P EIA's standard curve. A sixth aliquot of feces was left unspiked to determine the quantity of endogenous hormone in the spiked aliquots.

Fig. 31
Parallelism between P EIA Standard Curve and Serial Dilutions of Fecal Extracts



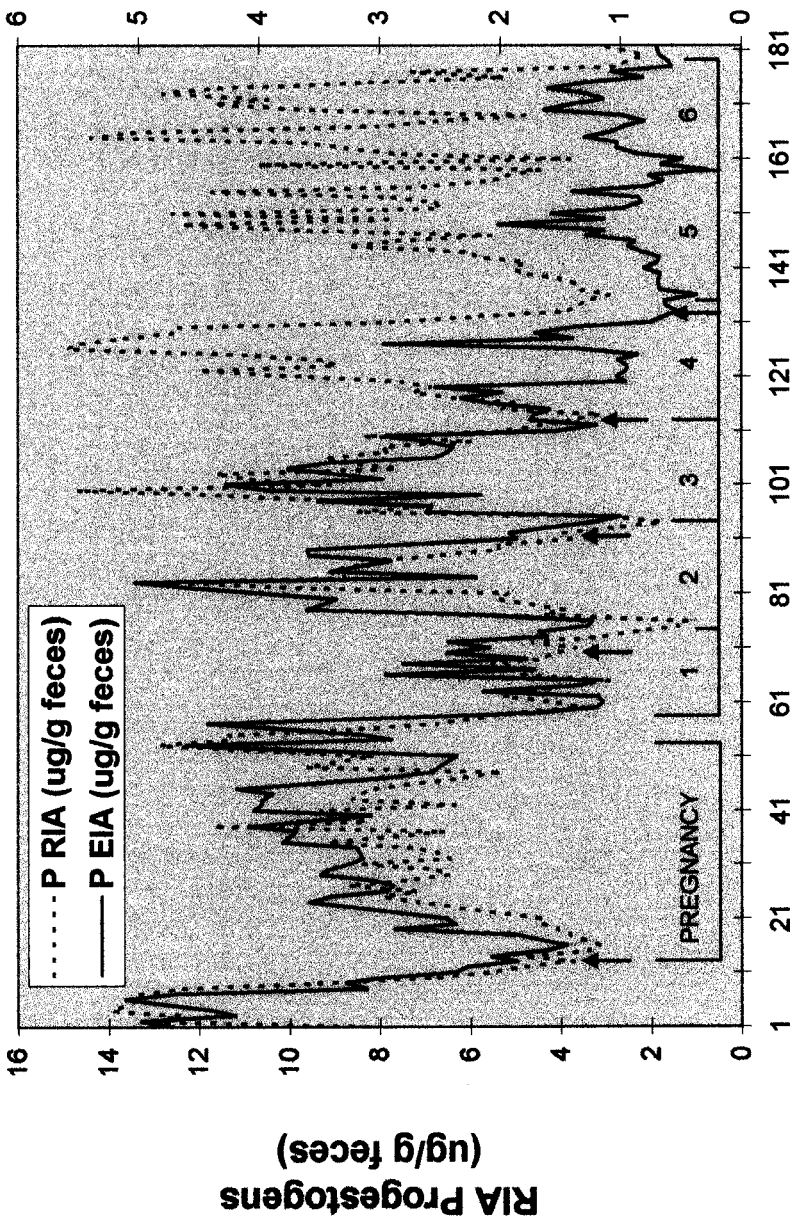
The volumes of progesterone needed for spiking (0.5 ml for the highest concentration) were significant, so the progesterone was measured into five glass extraction tubes which were then (along with a sixth, empty tube) vortexed and dried down under air in a warm water bath (37° C). Once the tubes were completely dry the fecal aliquots were added, then the tubes were vortexed, sonicated, and vortexed again. Finally the aliquots were extracted, and then the extracts were analyzed in duplicate on the P EIA. Extraction efficiency was calculated (see Chapter 1) to be 115%.

RESULTS and DISCUSSION

Fig. 32 shows the results of analysis of the Sumatran rhino fecal samples using both the P RIA and the P EIA. Over the sampling interval, the profile initially reflected the end of a luteal phase, followed by a sharp drop in progestogen levels, at which time the female was observed breeding (Day 13). Dates of breeding are marked by black arrows in Fig. 32. After this breeding progestogens were elevated for a prolonged time, and rectal ultrasound revealed that the female was pregnant. Pregnancy loss was detected on Day 54, after 37 days of elevated progestogens (Roth et al., 2001). Six estrous cycles, identified by gradual increases in progestogens followed by gradual decreases, followed the pregnancy loss. All but the first and sixth cycles were delineated by breeding (Days 71, 91, 112 and 133) at the beginning of each cycle, just before or on the day that progestogens from both assays reached nadir values. After each breeding ovulation was confirmed by rectal ultrasound and/or by a surge of serum LH (Roth et al., 2001). No such data are available for the first or sixth cycles.

Although the first and sixth cycles in Fig. 32 were not confirmed by breeding, ultrasound and/or LH data as were the other four cycles, they do fit the pattern of a gradual increase in progestogens followed by a gradual decrease, and the cycle lengths (16 and 18 days) were comparable to 21 days reported by Roth et al. (2001) and 25 days reported by Heistermann et al. (1998). Based on the intervals between nadir values in the

Fig. 32
Progestogen Assay Comparison - P RIA vs P EIA



P EIA profile, the average cycle length was 20.2 days (range 16-24 days, n = 6). Based on the intervals between the four breedings, the average cycle length was 20.7 days (range 20-21 days, n = 3).

As can be seen in Fig. 32, the P EIA and the P RIA both track essentially the same cycles. The progestogen profiles generated by the two assays over the entire sampling period had a correlation coefficient (r) of 0.47 ($p < 0.001$). It should be noted, however, that after Day 116 the P EIA values tended to drop, widening the discrepancy between the two profiles and thus lowering the value of r. Analysis of the profiles in two sections resulted in better correlations: $r = 0.75$ ($p < 0.001$) for Days 1 to 116 and $r = 0.71$ ($p < 0.001$) for Days 117 to 182. There are two possible explanations for the drop in the P EIA values after Day 116. The samples were run in two batches, separated by over a month, so it may be that the lower values (Days 117 to 182) were from the second batch and represent the effects of hormone breakdown in the samples. Unfortunately, there is no record of the dates that individual samples were run, so this explanation cannot be established. The second possible explanation is technician error – these data were produced during the author's first attempts at using the new EIA, so the drop in values may have been due to an unintentional change in lab technique.

As was discussed earlier, Roth et al. (2001) suggested that the cycling female in their study (this same female also provided samples for the author's study) was an induced ovulator. When she was breeding regularly the female's fecal and serum progestogen profiles reflected a regular 21-day cycle. When she was not breeding,

however, these profiles were erratic, and ultrasound exams revealed the formation of anovulatory, hemorrhagic follicles which underwent varied levels of luteinization. Given Roth et al.'s (2001) results it is difficult to explain why the first and sixth estrous cycles in Fig. 32, which were not preceded by breeding, were still so clearly defined and so similar to the other cycles. This is especially true of the first cycle, which occurs just after a pregnancy loss. In the case of the sixth cycle it is possible, though it seems unlikely, that breeding must be discontinued for more than one cycle before the formation of anovulatory follicles is reflected in the fecal progestogen profile. Obviously, further investigation of more females will be necessary before anyone can claim that all female Sumatran rhinos are induced ovulators.

CONCLUSIONS

Analysis of Sumatran rhino fecal samples using both the P RIA and the P EIA developed in this study clearly demonstrated that an EIA with an appropriate broad-spectrum progestogen antibody can be used to monitor the reproductive cycle of the female Sumatran rhinoceros. Unfortunately, time restrictions as well as financial and political constraints made continuation of the Sumatran rhino study impossible, and no behavioural data could be collected. The development of the P EIA will, however, greatly simplify endocrine analysis of fecal samples collected from captive animals and will also allow field researchers to monitor the reproductive cycles of animals in conditions where it is impossible to gain access to laboratories equipped to handle radioactivity.

GENERAL DISCUSSION

The Indian rhino study clearly demonstrated that relationships exist between the female estrous cycle, male testosterone levels and the behaviour of female and male Indian rhinos. The results supported the hypotheses which were being tested, namely that certain female and male behaviours occurred only (or more frequently) when female estrogen levels were high, while others occurred only (or more frequently) when male testosterone levels were high. These findings afford further insight into the reproductive biology of this unusual, and often puzzling, mammal by developing a more complete understanding of courtship behaviours and the physiological mechanisms by which they are governed.

The practical goal of this study, to establish that behavioural observations can be used to recognize sexual receptivity, was satisfied. In total, 22 behaviours were identified as relating to EC – Defecating, Walking, Trot/Canter, Weaving, Pacing, Charging, Side-to-Side, Sniff/Taste Urine/Feces, Flehmen, Urine Spraying, Penis Extended, Mooing, Blowing, Whistling, Whistle-Blowing, Whistle-Barking, Teeth Grinding, Banging, Bleating, Snorting, Bellowing and Barking. Two behaviours, Head Bobbing and Blow-Snorting, were identified as relating to T. Animal managers can use the occurrence of these behaviours to time breeding introductions, thereby eliminating or reducing the need for endocrine analysis, reducing the chance of fights and injuries during introductions and ultimately improving reproductive rates.

Future research on Indian rhinos must include more animals of both sexes, in order to establish a larger database and also to ascertain whether some of the behaviours in the repertoire established by this study are more useful indicators of sexual receptivity than others. In addition, more attention must be paid to male reproductive hormones, and the behavioural responses of both males and females to these hormones.

The Sumatran rhino study clearly demonstrated that an EIA with an appropriate broad-spectrum progestogen antibody can be used to monitor the reproductive cycle of the female Sumatran rhinoceros. The development of the P EIA will greatly simplify endocrine analysis of fecal samples collected from captive animals and will allow field researchers to monitor the reproductive cycles of animals in conditions where it is impossible to gain access to laboratories equipped to handle radioactivity.

The next logical step would be to study the behaviour of Sumatran rhinos using the same protocol as was used with the Indian rhino, in order to determine whether relationships exist between the female estrous cycle and the behaviour of female and male Sumatran rhinos. Since only one female Sumatran rhinoceros has been extensively studied, future research must involve more animals observed over a longer period of time, in order to determine whether this female is representative of the species. In addition, it will be important to develop the technology necessary to study male reproductive hormones and the potential relationships between male hormones and the behaviour of both females and males. Hopefully, such research will make possible the breeding of Sumatran rhinos in captivity.

Why Do Rhinos Make Breeding So Difficult?

The results of this study help to describe the means by which sexually receptive male and female rhinos locate one another and communicate their sexual condition. It is difficult to understand, however, why animals which exist in virtually impenetrable habitats with low visibility would evolve lifestyles in which they are solitary and widely dispersed. It is also hard to comprehend the benefits of courtship rituals which often involve serious aggression, both in captivity and in the wild.

The evolution of social organization in ungulates (hoofed mammals of the orders *Proboscidea*, *Artiodactyla* and *Perissodactyla*, which includes the family *Rhinocerotidae*) has been the subject of several studies, all of which emphasized the relationship between ecology and social organization (Geist, 1974; Jarman, 1974; Laurie, 1982; Owen-Smith, 1975; Owen-Smith, 1977). Social organization varies considerably among ungulate species, due in large part to the wide variety of habitats occupied by ungulates, and differences in habitat parameters such as temporal and spatial fluctuations in plant productivity, habitat stability, food dispersion and the three-dimensional structure of habitats. These parameters can be linked, via natural selection, with the species' differing modes of communication, frequency and severity of aggression, sociability and group structure, movement patterns of groups and individuals, home-range configuration, territoriality and reproductive strategies (Geist, 1974; Laurie, 1982; Owen-Smith, 1975).

Sociability and Group Structure

The five species of rhinoceros roughly fit into Jarman's (1974) scheme for classifying African antelopes based on ecological, social and behavioural characteristics. Each of Jarman's (1974) five classes of antelope uses a different feeding style, defined by the dispersion and availability of food eaten. The relatively sociable White rhino is found in short grass plains with a uniformly distributed, abundant food supply of grass and herbs. Population density is 0.6 to 5.7 rhinos per km^2, and breeding males defend exclusive territories of 0.8 to 2.6 km^2. Female home ranges are 4 to 15 km^2 (Laurie, 1982; Owen-Smith, 1975). Black rhinos are also fairly sociable and live in a wide-range of habitat types, from semi-desert to montane forest, feeding on leaves, twigs, shrubs and herbs. Population density is 0.02 to 0.90 rhinos per km^2, and males can be territorial or nomadic, depending on habitat type and availability of water. Female home ranges are 2.6 to 90 km^2, depending on habitat type (Laurie, 1982; Schenkel and Schenkel-Hulliger, 1969).

In contrast, the mostly solitary Sumatran and Javan rhinos live in rain-forest where visibility is poor and food is widely scattered and not quickly renewable. The population density of Sumatran rhinos is 0.02 to 0.05 per km^2. Individual rhinos tend to maintain large, permanent home ranges which include a natural salt lick. Males are not truly territorial – their home ranges average 30 km^2 and overlap extensively, although each range does seem to contain an exclusive core area. Female ranges are smaller but overlap less, except in the vicinity of salt licks. The data available for the Javan rhino is

extremely limited. Natural population density is thought to be greater than 0.3 rhinos per km^2, with current density less than 0.15 per km^2. Males are not thought to be territorial and female home ranges are quite small (Laurie, 1982; van Strien, 1985).

Indian rhinos are in the middle of the social structure spectrum. Population density is 0.4 to 13.3 rhinos per km^2. Breeding males have some range exclusivity (2 to 8 km^2) but no true territoriality. Female ranges are 2 to 10 km^2 in size. Poor visibility and the relatively unpredictable distribution of resources in time and space have perhaps selected against a territorial mating system. High population densities are associated with high diversity of vegetation types and ease of access to water and agricultural land (Dinerstein and Price, 1991; Laurie, 1982; Laurie et al., 1983; Nowak, 1999).

Breeding White rhino males, with access to a uniformly distributed, abundant food supply, are able to defend territories in which they maintain exclusive mating rights (Laurie, 1982; Owen-Smith, 1975). Such a mating system would not be feasible for the Indian rhino since dramatic seasonal changes in the distribution of resources mean that males cannot restrict their movements to small areas. In the rapidly changing habitats of the Indian rhino, males have adopted wider ranging habits and thus increase their chances of finding both new food sources and females already feeding on them (Laurie, 1982).

Courtship and Mating

Although courtship in ungulates often contains elements of aggressive behaviour, and the male commonly drives the female, unmitigated aggression is unusual. Indian rhino courtship chases and fights are extremely noisy, and the loud vocalizations of the

female advertise the courtship over a radius of at least one kilometre, with the result that breeding males in the vicinity are attracted to the courting pair. Laurie (1982) found that some adult males, and occasionally sub-adult and adult females, showed particular interest in sounds of fighting and moved off in the direction of their origin. Similar but less violent behaviour has been noted in Javan, Sumatran and Black rhinos but not in White rhinos (Laurie, 1982; Owen-Smith, 1975).

The differences between the peaceful courtship of the White rhino and the noisy, aggressive courtship of the Indian rhino are probably related to differences in habitat structure and visibility. In a habitat with low visibility in which males do not hold territories, an estral female may increase her chances of being mated by the strongest available male if she advertises her readiness for copulation with loud vocalizations and resists copulating with the first male to appear. Laurie (1982) suggested that, apart from directly testing the male's strength, one of the "functions" of the long, noisy chases and fights of the Indian rhino's courtship could be to ensure that the female is eventually mated by the strongest male. Males could obtain more matings by following the sounds of fighting between another male and a female. It is also possible that adult females in estrus may approach a consort pair, being attracted by both smell and vocalizations, as a male with a female is likely to be a strong male.

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APPENDIX I – RHINO ETHOGRAM[1]

Observations should be made at roughly the same time each day, for a minimum of one hour per session.

Externally Directed Behaviour – make note of any instance in which the rhino's behaviour seems to be directed towards or interrupted by outside influences (e.g. rhino Walks towards barn when keeper appears at barn gate; rhino Canters away from running lawnmower).

Not Visible - note whenever the rhino is out of view (study design should be such that this doesn't happen very often).

Continuous Behaviours - every 5 minutes note which behaviour is occurring.

Note whenever behaviour occurs in wallow or pool.

1. Standing – head up and eyes open, head, ears and tail may move occasionally, weight may shift .
2. Standing at Rest – head down (possibly resting on object), eyes closed or partially closed.
3. Passive Exploration – rhino stands still but moves it head repeatedly and/or sniffs the air (with or without raising its head), generally appears to be investigating its environment.

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[1] **REFERENCES**:

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b) Personal communication (2000) with the following people:
   - Marcellus Adi, Sumatran Rhino Sanctuary, Lampung, Indonesia.
   - Aidi Mohamad, Sumatran Rhino Conservation Centre, Selangor, Malaysia.
   - Steve Romo, Center for Research of Endangered Wildlife, Cincinnati Zoo and Botanical Garden, Ohio, USA.
   - Nico van Strien, International Rhino Foundation, Ohio, USA.

c) Bennett, C., R. Hodgden and J. Piazza. Ethogram: African Rhinos. Dallas Zoo, Texas, USA. Unpublished paper.

d) UK Cat Behaviour Working Group (1995). An Ethogram for Behavioural Studies of the Domestic Cat (*Felis silvestris catus* L.), UFAW Animal Welfare Research Report No. 8. Universities Federation for Animal Welfare, Herts., UK.

4. Drinking
5. Eating – hay, grain, vegetation, etc.
6. Lying Down – either lying flat on side or recumbent on sternum; head may be raised or resting on ground/object.
7. Walking – continuous forward movement, two feet on ground at all times. Rhino travels without obviously investigating its environment.
8. Intermittent Walking – as above but movement is discontinuous, walking is punctuated by brief periods of Standing.
9. Exploring – rhino walks slowly, sniffing at objects and otherwise investigating its surroundings. Forward travel may be punctuated by brief periods of Passive Exploration.
10. Back Up – movement is backwards.
11. Patrol – rhino walks around sniffing at objects and watching other rhinos, human observers, etc., periodically stopping and scent marking. Rhino may circle the entire enclosure or a smaller area repeatedly.

Discrete Behaviours - record each occurrence of the behaviour and its duration.

Auditory Signals

1. Moo – similar to the "moo" emitted by a cow, i.e. a short sound that varies in intensity but is usually easily audible. Made by both males and females. Often related to agitation (e.g. often heard when rhinos are soon to be fed or put outside).
2. Whistle – similar to a human whistle but often fairly soft; short, single-pitch sound.
3. Blow – short, sharp exhalation through the nostrils (occasionally slightly longer and softer, similar to a human "sigh").
4. Bark (Honk) - cross between a Moo and a cough, short and fairly loud.
5. Snort – similar to a horse's snort; produced by the expulsion of air in a series of quick bursts through the lips and nostrils.
6. Whistle-Blow – Whistle followed immediately by a Blow.
7. Whistle-Bark - Whistle followed immediately by a Bark.
8. Whistle-Snort - Whistle followed immediately by a Snort.
9. Blow-Snort – Blow followed immediately by a Snort. Often emitted when another rhino is approaching.

10. Bellow (Roar) - very loud, low-pitched sound.
11. Whine – penetrating, high-pitched auditory signal similar to a dog's whine. Varies in loudness and in duration. Often heard from a rhino who is hungry or wants to go into/out of an enclosure, or when one rhino is running away from another.
12. Grumble/Growl – low-pitched sound uttered with an open-mouth, varies in duration. Reportedly heard from a female Sumatran rhinoceros after she has been licking/chewing on a salt block.
13. Teeth Grinding - act of rubbing molars together, produces a grinding sound. Often associated with stress or annoyance.
14. Bleat – similar to a goat's bleat, but usually shorter in duration.

Female and Male Behaviours

1. Banging – rhino hits part of its body against a solid object, usually done by hitting the head against a wall or enclosure bars. Associated with stress, impatience, aggression.
2. Trotting - two-time gait, legs move in diagonal pairs.
3. Cantering - three-time gait, one hind leg moves forward, then a diagonal pair, and finally the remaining front leg.

Note: In the author's study trotting and cantering were lumped together as one behaviour (Trot/Canter), as rhinos often alternated one for the other rapidly and frequently during a course of movement.

4. Galloping - four-time gait, as for Cantering but there is a moment of suspension when all four legs are off the ground.
5. Stereotypic Behaviour – Pacing - repetitive pattern of travel within a restricted area (i.e. rhino moves in a circle or back and forth along the same route). Usually done at the walk, but may be faster. Note where and at what speed pacing occurs.
6. Stereotypic Behaviour – Weaving - rhino stands still except for swinging its head from side to side, shifting its weight from one front leg to another as its head moves. Head is usually lowered. Note where behaviour occurs (weaving is often performed at an enclosure gate). Associated with stress, agitation or boredom.
7. Stereotypic Behaviour - Rubbing Horn while Weaving - note where this occurs.
8. Approach - one rhino moves (at a walk or trot) into a zone not greater than two body lengths from another animal or a human that is stationary or moving towards the rhino. Approaching rhino must be looking at the animal/human it is approaching.

9. Charge - as above for Approach, but charging rhino moves at a canter or gallop.
10. Startle – rhino jumps or moves suddenly, usually in response to loud noise or a sudden movement. Often accompanied by a Snort or Blow, animal may pause and then leave the area quickly.
11. Stereotypic Behaviour – Side-to-Side – while standing still rhino shifts weight from one side to the other, sometimes picking up one foot on the side that is not weight bearing. Associated with stress and agitation.
12. Investigation - rhino pays special interest to one particular area. Note whether another animal or food, etc. has been in the area recently and whether the area is sniffed or tasted.
13. Sniff/Taste Urine/Feces - note whether sample is only smelled or actually taken into the mouth. After sampling rhino will often stand still with its head raised or level with its back; nostrils may flare and/or contract. This behaviour often leads to Flehmen (see below).
14. Mouthing – lips are used to investigate a surface or object (e.g. the ground, a rock, salt/mineral lick, scent mark, etc.). Objects may be taken into the mouth for further investigation.
15. Licking – use of the tongue outside of the mouth to investigate a surface or object (e.g. the ground, a rock, salt/mineral lick, scent mark, etc.).
16. Flehmen - rhino stands with its head raised, mouth slightly open and upper lip curled back, often holding this pose for several seconds. Usually occurs after investigating another rhino's urine (or feces) - note whether this is the case.
17. Object Rear – rhino stands up on its hind legs with its forelegs against an object. Usually done while foraging for high vegetation, or to see over the wall of an enclosure.
18. Urination - rhino passes a large volume of urine all at once. Usually discharged in one full stream, although may occur in successive spurts.
19. Urine Spraying - brief, quick spurts of small amounts of urine. Note whether urination is directed against an object, another rhino, etc.
20. Pseudo-Urination/Pseudo-Urine Spray – rhino assumes its usual position for Urination or Urine Spraying (e.g. rhino may stand with hind legs wide apart and/or may lift its tail) but no urine is produced. May be impossible to tell the difference between Pseudo-Urination and Pseudo-Urine Spray.
21. Defecation - note whether rhino defecates in the "toilet area", after sniffing another animal's feces, on top of another rhino's feces, etc.

22. Foot Scrape – rhino scrapes its feet (usually hind feet) backwards one after another, displacing vegetation, soil or other material. Often performed by males after urination or defecation.
23. Foot Stamp – rhino stamps one foot (usually front foot), often repeatedly. May be in response to irritation from insects, etc., but also may be a sign of anger or annoyance.
24. Digging – rhino scrapes the ground with its nose or a front foot. May be done for the purpose of creating a wallow, especially in muddy/swampy areas.
25. Marking Behaviour – saplings are bitten, stepped on or broken with the horns; saplings may be bent or broken and then twisted around. Bark, twigs and leaves may be severely bruised by the rhino's teeth or horns or by rubbing (see Body Rub and Horn Rub below – these behaviours may serve the purpose of marking). Urine Spraying is often used for marking.
26. Body Rub – rhino rubs any part of its body (with the exception of the horn, the penis or the vulva – see Horn Rub or Contact Masturbation below) against an object or surface. May serve the purposes of scratching, grooming and/or marking.
27. Horn Rub – rhino rubs horn against an object or surface. May serve the purposes of marking or of wearing down the horn. If the horn is rubbed while the rhino is swinging its head from side-to-side, see Weaving above.
28. Yawn – rhino opens mouth wide. Not directed towards another animal; not associated with an aggressive situation or food. (If the "yawn" appears to be aggressive or is outwardly directed, Open Mouth or Tusk Display below.
29. Tail Lift/Flick – rhino lifts or flicks its tail straight up or to the side, once or repeatedly. May be a sign of annoyance, e.g. a response to irritation by insects. Excludes tail lifting done immediately prior to defecation, urination, or urine spraying, also tail lifting done by a female when she is in close proximity to a male (two body lengths or less apart) and her rear is pointing towards him.
30. Lip Curl - act of flexing lips to expose lower tusks. Associated with aggression.
31. Open Mouth – rhino turns to face another animal or human and opens its mouth wide. Associated with aggression and often accompanied by Snorting or Barking.
32. Tusk Display – rhino turns to face another animal or human and opens its mouth wide while curling the lips back to bare its tusks. Associated with aggression and often accompanied by Blow-Snorting or Bellowing.
33. Head Bobbing - rapid movement of head up and down for several repetitions, often while backing up. Associated with fear and stress, often done in response to aggression from another rhino.

34. Head Wavering - circular movement of head associated with stress.
35. Playing - investigating/manipulating object or environment in an apparently playful manner. Object may be kicked or butted with nose or horns.

Sexual Behaviours - Female

1. Contact Masturbation – rubbing of the vulva (exposed by a lifted tail) against any object or surface.
2. Winking - rapid contractions of vulva, usually done with the tail held up or to one side. Indicative of estrus.
3. Ignoring calf - refusing to let calf nurse, ignoring calf's calls, etc. May be difficult to judge unless the observer has seen the female's usual behaviour towards her calf. Some females reportedly begin to ignore their calf whenever they enter estrus.
4. Aggression towards calf - female engages in any of the following potentially aggressive behaviours towards her calf: pushing, butting with head or horn, biting, Charging. Some females reportedly become aggressive towards their calf when beginning estrus.
5. Vaginal Discharge – not a behaviour per se, but discharge is potentially related to stages of the estrous cycle. Record discharge on a scale of 0 to 3, 0 being no discharge and 3 being the maximum amount seen (you will have to observe several estrous cycles before being able to make this distinction).

Sexual Behaviours - Male

1. Penis Extended - the penis is exposed but not erect.
2. Penis Erect - penis is exposed and in erect (stiff) position.
3. Non-Contact Masturbation - the male swings his penis back and forth, often touching the abdomen. May or may not be accompanied by Climax (see below).
4. Contact Masturbation - the male rubs his penis against an object or surface, may also swing it back and forth. May or may not be accompanied by Climax (see below).
5. Climax without Ejaculation (occurs after masturbation) - male humps back and drops hindquarters, hindquarters often shake or shiver, may be accompanied by Blowing or Snorting. A few drops of semen may be visible on the tip of the penis.
6. Climax with Ejaculation (occurs after masturbation) – as for above, but followed by ejaculation.

4. Contact Masturbation - the male rubs his penis against an object or surface, may also swing it back and forth. May or may not be accompanied by Climax (see below).
5. Climax without Ejaculation (occurs after masturbation) - male humps back and drops hindquarters, hindquarters often shake or shiver, may be accompanied by Blowing or Snorting. A few drops of semen may be visible on the tip of the penis.
6. Climax with Ejaculation (occurs after masturbation) – as for above, but followed by ejaculation.

APPENDIX II – ANTIBODY CROSS-REACTIVITIES[1]

Estrone Conjugates Antibody (R522) – EC

estrone-3-glucuronide	100%
estrone-3-sulfate	66.6%
estrone	23.8%
estradiol-17ß	7.8%
estradiol-3-glucuronide	3.8%
estradiol-3-sulfate	3.3%
estradiol-17-sulfate	< or = 0.1%
estradiol-3-disulfate	< or = 0.1%
ethinyl estradiol-17ß	< or = 0.1%
estriol	< or = 0.1%
progesterone	< or = 0.1%
pregnanediol	< or = 0.1%
androsterone	< or = 0.1%
cortisol	< or = 0.1%
testosterone	< or = 0.1%

Pregnanediol-Glucuronide Antibody (P70) – PdG

pregnanediol-3-glucuronide	100%
20a-hydroxyprogesterone	60.7%
20ß-hydroxyprogesterone	25%
pregnanediol	7.3%
17a-hydroxyprogesterone	< 0.1%
11a-hydroxyprogesterone	< 0.1%
progesterone	< 0.1%
pregnenolone	< 0.1%
androstenedione	< 0.1%
cortisol	< 0.1%
estradiol-17ß	< 0.1%
estrone	< 0.1%
testosterone	< 0.1%

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[1] Cross-reactivities for the EC and PdG antibodies taken from Bellem et al. (1995) and Munro et al. (1991). Cross-reactivity for the T antibody taken from Walker (1999). Cross-reactivity for the P antibody taken from Brown et al. (1994), Grieger et al. (1990) and Wasser et al. (1994)

Testosterone Antibody (R156/7) - T

testosterone	100%
5a-dihydrotestosterone	57.37%
androstenedione	.0.27%
androsterone	0.04%
dehydroepiandrosterone	0.04%
cholesterol	0.03%
ß-estradiol	0.02%
progesterone	<0.02%
pregnenolone	<0.02%
hydrocortisone	<0.02%
cholic acid	<0.02%
chenodeoxycholic acid	<0.02%
cholic acid methyl ester	<0.02%
dehydrocholic acid	<0.02%
deoxycholic acid	<0.02%
lithocholic acid	<0.02%
glycholic acid	<0.02%
taurodeoxycholic acid	<0.02%
taurochenodeoxycholic acid	<0.02%
glycochenodeoxycholic acid	<0.02%
taurocholic acid	<0.02%

Progestogen Antibody – P (also used in P4 RIA)

progesterone (4-pregnene-3,20-dione) 100%
allopregnanolone (5a-pregnane-3ß-ol-20-one) 96%
† 5-P-3OH (5α-pregnane-3α-ol-20-one 36%
pregnanolone (5ß-pregnane-3ß-ol-20-one 15%
17ß-hydroxyprogesterone 15%
pregnenolone (3ß-hydroxy-5-pregnen-20-one) 13%
‡ 5ß-pregnane-3α-ol-20-one 7%
5ß-pregnane-3α,17α-diol, 20α-one 5%
pregnanediol-3-glucuronide ..... <1%
androstenedione (4-androstene-3,17,-dione) ..... <1%
testosterone (17ß-hydroxy-4-androsten-3-one) ..... <1%
estradiol (1,3,5(10)-estratriene-3,17ß-diol) ..... <1%
estrone (3-hydroxy-1,3,5(10)-estratrien-17-one ..... <1%
estriol (1,3,5(10)-estratriene-3,16α,17ß-triol ..... <1%
21-hydroxyprogesterone ..... <1%
20α-hydroxyprogesterone ..... <1%
cortisol (11ß,17,21-trihydroxy-4-pregnene-3,20-dione ..... <1%

† Identified by Heistermann et al. (1998) as being useful for monitoring follicular and luteal activity in the Sumatran rhinoceros.

‡ Identified by Heistermann et al. (1998) as being one of the three most abundant progestogen metabolites in the feces of the female Sumatran rhinoceros.

APPENDIX III – RECIPES FOR ASSAY STOCKS AND SOLUTIONS

EC, PdG & T EIA Stocks and Solutions - used in the TO Zoo lab

Coating Buffer

Na_2CO_3 ..... 1.59 g
$NaHCO_3$ ..... 2.93 g
dH_20 ..... 1000 ml

- pH solution to 9.6 with 1 N HCl or 1 N NaOH
- store at 4° C

1 N HCl

HCl (37%) ..... 100 ml
DDW ..... 900 ml

- store at room temperature

1 N NaOH

NaOH pellets ..... 40 g
DDW ..... 1000 ml

- store at room temperature

EIA Assay Buffer

Stock A ..... 195 ml
Stock B ..... 305 ml
NaCl ..... 8.7 g
BSA ..... 1.0 g
dH_2O ..... 500 ml
Stock A: 0.2 M NaH_2PO_4 ..... 27.8 g/1000 ml dH_2O
Stock B: 0.2 M Na_2HPO_4 ..... 28.4 g/1000 ml dH_2O

- pH solution to 7.0 with 1 N HCl or 1 N NaOH
- store at 4° C
- no solution containing BSA should ever be shaken to the point that it foams

Wash Solution Concentrate (10x)

NaCl ..... 87.66 g
Tween 20 ..... 5.0 ml
dH_2O ..... 1000 ml

- store at 4° C

Wash Solution (for use in Plate Washer, dilute concentrate 1:10)
Wash Solution Concentrate ..... 100 ml
dH_2O ..... 900 ml
- leftover solution can be left in Plate Washer (at room temperature) for future use

Substrate Buffer
anhydrous citric acid ..... 9.61 g
dH_2O ..... 1000 ml
- pH solution to 4.0 with 5 N NaOH
- store at 4° C

5 N NaOH
NaOH pellets ..... 200 g
DDW ..... 1000 ml
- store at room temperature

40 mM ABTS
ABTS powder ..... 0.55 g
dH_2O ..... 25 ml
- pH solution to 6.0 with 1 N HCl or 1 N NaOH
- store at 4° C
- keep ABTS powder and 40 mM ABTS protected from light at all times (use brown glass or aluminum foil for storage)

0.5 M H_2O_2
30% H_2O_2 ..... 500 µl
dH_2O ..... 8 ml
- store at 4° C

<u>Fecal Extraction and P EIA Stocks and Solutions</u> - used in the CRC lab

PBS Buffer
DDW ..... 1000 ml
NaCl ..... 8.8 g
Na_2HPO_4 ..... 4.6 g
NaH_2PO_4 ..... 2.71 g
NaN_3 ..... 0.1 g
- stir on a magnetic stirplate until dissolved
- pH to 7.0 with 1 N HCl or 1 N NaOH
- store at 4° C

Ammonium Sulfate (AmS) Solution

DDW ..... 100 ml
$(NH_4)_2SO_4$ ..... 77 g

- heat while mixing on a stirplate to dissolve the $(NH_4)_2SO_4$
- pH should be 7.8 (measure pH while solution is at room temperature)
- if pH needs to be adjusted, stir solution while adding 1 N HCl or 1 N NaOH (most likely NaOH will be required)
- addition of NaOH may cause the $(NH_4)_2SO_4$ to precipitate, but it will re-dissolve

Saline Solution (for AmS Stripping)

DDW ..... 1000 ml
NaCl ..... 8.7 g

BBS Solution (for AmS Stripping)

DDW ..... 500 ml
Boric Acid ..... 3.09 g
Borax ..... 4.77 g
NaCl ..... 2.19 g

- combine 25 ml of the BBS Solution with 475 ml of the above described Saline Solution and stir quickly without heat
- BBS takes a very long time to dissolve

1 N HCl

HCl (37%) ..... 100 ml
DDW ..... 900 ml

- store at room temperature

1 N NaOH

NaOH pellets ..... 40 g
DDW ..... 1000 ml

- store at room temperature

Coating (2°) Antibody Stock

Coating (2°) Ab (goat anti-mouse IgG) ..... whole vial (1 mg)
NaCl (0.135 M) ..... 1 ml

0.135 M NaCl = 7.89 g of NaCl in 1000 ml DDW

- store at - 20° C

Coating Buffer

Na_2CO_3 ..... 1.59 g
$NaHCO_3$ ..... 2.936 g
DDW ..... 1000 ml

- pH to 9.6 with 1 N HCl (~ 10 ml, or use 5 N HCl)
- store at 4° C

5 N HCl

HCl (37%) ..... 492 ml
DDW ..... 508 ml

- store at room temperature

2nd Coating (Blocking) Buffer

TRIS ..... 3.146 g
NaCl ..... 23.3 g
BSA ..... 13 g
NaN_3 ..... 1.3 g
DDW ..... 1300 ml

- pH to 7.5 with 1 N HCl (~ 40 ml, or use 5 N HCl)
- vacuum filter using RAININ Nylon-66 filters (pore size 0.2 μm, diameter 47 mm)
- store at 4° C
- no solution containing BSA should ever be shaken to the point that it foams

Assay Buffer

TRIS ..... 2.42 g
NaCl ..... 17.9 g
BSA ..... 1 g
Tween 80 ..... 1 ml
DDW ..... 1000 ml

- pH to 7.5 with 1 N HCl (~17 ml, or use 5 N HCl)
- filter as described above for 2nd Coating (Blocking) Buffer
- store at 4° C
- no solution containing BSA should ever be shaken to the point that it foams

Wash Solution

Tween 20 ..... 500 μl
DDW ..... 2500 ml

- store at 4° C

Biotinylated Label (BL) Primary and Secondary Stock (1:5000)

- for each new vial, follow specific instructions from F. Schwarzenberger – instructions may vary dependent on dilution of original stock
- add 10 µl MeOH to the vial of lyophilized BL for a 1:10 dilution
- sonicate briefly
- do a further 1:100 dilution by adding 990 µl Assay Buffer to the vial (primary stock 1:1000)
- take 100 µl from the vial, mix with 400 µl Assay Buffer for a 1:5 dilution (secondary stock – 1:5000)
- store both primary and secondary stocks at -20° C

Streptavidin-POD Conjugate Primary and Secondary Stock

- add 1 ml DDW water to the lyophilized Streptavidin for a 1:1000 dilution (primary stock 500 U conjugate/ml)
- primary stock is stable for up to 6 months at 4° C
- do not freeze
- prepare secondary stock by a 1:5 dilution - add 100 µl of primary stock to 400 µl DDW (1:5000)
- store at 4° C – do not freeze

0.4% TMB

TMB ..... 0.1 g
DMSO ..... 25 ml

- store at room temperature
- protect from light

0.6% H_2O_2

H_2O_2 (30%) ..... 100 µl
DDW ..... 5 ml

- store at 4° C
- stir on a magnetic stirplate for a few minutes before use

Substrate Buffer

Sodium Acetate ($CH_3COONa·3H_20$) ..... 1.36 g
DDW ..... 1000 ml

- pH to 5.0 with 5% Citric Acid (5 g anhydrous citric acid in 100 ml DDW, store at room temperature)
- store at 4° C

Stop Reagent (2 M H_2SO_4)

H_2SO_4 (95-97%)..... 200 ml
DDW ..... 800 ml

- store at room temperature

APPENDIX IV – LIST OF CHEMICALS USED

Store all chemicals at room temperature unless otherwise noted

Fecal Extraction and Cr, EC, PdG & T EIA Chemicals – used in the TO Zoo lab

ABTS (or 2,2'-azino-bis)..... Fisher Scientific, Nepean, ON, Canada
(3-ethylbenzthiazoline-6-sulfonic acid)
- protect from light using brown glass or aluminum foil

Aluminum Oxide..... 0536-05, J.T. Baker
(Al_2O_3).....a division of Mallinckrodt Baker, Inc., NJ, USA

Bovine Serum Albumin, Fraction V
(BSA) .....A-7906, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada
- store at 2-8° C in a dessicator

Citric Acid, anhydrous.....C-0759, Sigma-Aldrich
($C_6H_8O_7$)

Estrone Conjugates Polyclonal Antibody (R522)
(EC Ab) ..... C. Munro, University of California, Davis, CA, USA
- store at -20° C

Estrone-ß-D-glucuronide (for EC standards) ..... E1752, Sigma-Aldrich
- store at 0° C

Estrone-glucuronide-Horseradish Peroxidase (E-HRP)..... C. Munro
- store at -20° C

Ethanol (Reagent Grade Alcohol)..... A 962-4, Fisher Scientific
(CH_3CH_3OH)

Hydrochloric Acid (36.5-38%, ~ 10 M) ..... 9535-02, J.T. Baker
(HCl)

Hydrogen Peroxide (30%) .....Fisher Scientific
(H_2O_2)
- store at 4° C

Methanol (reagent grade, min. 99.8%)..... ACS531-82, BDH, Toronto, ON, Canada (CH_3OH)

Pregnanediol-Glucuronide Polyclonal Antibody (P70)..... C. Munro (PdG Ab)
- store at -20° C

4-pregnen-20-α-ol-3-one (for PdG standards) ..... P6288, Sigma-Aldrich

Pregnanediol-glucuronide-Horseradish Peroxidase (P-HRP)..... C. Munro
- store at -20° C

Sodium Carbonate, anhydrous.....S263-500, Fisher Scientific (Na_2CO_3)

Sodium Chloride (NaCl).....B30123, BDH

Sodium Hydrogen Carbonate (Sodium Bicarbonate).....ACS 804, BDH ($NaHCO_3$)

Sodium Hydroxide pellets (NaOH) ..... 3722-01, J.T. Baker

Sodium Phosphate Monobasic (monohydrate) ..... 3818-01, BDH ($NaH_2PO_4 \cdot H_2O$)

Sodium Phosphate Dibasic (anhydrous) ..... 3828-01, BDH (Na_2HPO_4)

Testosterone (for T standards)..... A6950, Steraloids, NH, USA (17ß-hydroxy-4-androsten-3-one)

Testosterone Antibody (R156/7) ..... C. Munro (T Ab)
- store at -20° C

Testosterone-Horseradish Peroxidase (T-HRP)..... C. Munro
- store at -20° C

Tween 20 (Polyoxyethylenesorbitan Monolaurate) ..... Sigma-Aldrich

<u>Fecal Extraction and P EIA Chemicals</u> – used in the CRC lab
Chemicals are listed only if different from those used in the TO Zoo lab

Ammonium Sulfate (1 M in H_2O).....A-2939, Sigma Chemical/Sigma Diagnostics
($(NH_4)_2SO_4$)..... St. Louis, MO, USA

Biotin-Labelled Steroid (Biotinylated Label)
lyophilized enzyme label #57 (5α-pregnane-3ß-ol-20-one pentylamin)
..... F. Schwarzenberger
.....Institut fur Biochemie, Veterinärmedizinische Universität Wien, Vienna, Austria
- store at -20° C

Borax (anhydrous).....B-0127, Sigma Chemical/Sigma Diagnostics
($Na_2B_4O_7$)

Boric Acid (ACS Reagent) .....B-0394, Sigma Chemical/Sigma Diagnostics
(N_3BO_3)

Bovine Serum Albumin (BSA).....A-4503, Sigma Chemical/Sigma Diagnostics
- store at 2-8° C in a dessicator

Citric Acid (anhydrous).....C-0759, Sigma Chemical/Sigma Diagnostics
($C_6H_8O_7$)

Coating (2°) Antibody
Whole Molecule Affinity Purified Goat Anti-Mouse IgG (1 mg)
..... M-8645, Sigma Chemical/Sigma Diagnostics
- avoid repeated freezing and thawing

Dimethylsulfoxide (DMSO).....Fluka 41641

Hydrochloric Acid (36.5-38%, ~ 10 M)..... 9535-02, J.T. Baker
(HCl)

Hydrogen Peroxide (30%)..... Fisher Chemical/Fisher Scientific, Fair Lawn, NJ, USA
(H_2O_2)

Progesterone (99% pure steroid hormone, no conjugates)
(for P EIA standards)..... P-0130, Sigma Chemical/Sigma Diagnostics

Progestogen (P) Ab (1° Ab) .....J. Roser, University of California, Davis, CA, USA
- original stock is diluted 1:1000 in PBS Buffer
- store at -20° C

Sodium Acetate crystals ..... S209-500, Fisher Chemical/Fisher Scientific ($CH_3COONa\ddot{i}3H_20$)

Sodium Azide (NaN_3) ..... $S227^I$-25, Fisher Chemical/Fisher Scientific

Sodium Carbonate, anhydrous .....S263-500, Fisher Scientific (Na_2CO_3)

Sodium Hydrogen Carbonate (Sodium Bicarbonate).....ACS 804, BDH Inc. ($NaHCO_3$)

Streptavidin-POD conjugate, 500 U (of conjugate)
(Streptavidin-_-peroxidase).....1 089 153, Boehringer Mannheim, GmBH
- store at 4° C
- do not freeze

Sulfuric Acid (95-97%, 10 M).....A300-225, Fisher Chemical/Fisher Scientific (H_2SO_4)

TMB..... T-2885, Sigma Chemical/Sigma Diagnostics
(3,3',5,5'-tetramethylbenzedine, $C_{16}H_{20}N_2$)
- protect from light

TRIS (reagent grade, min. 99%) ..... T-1503, Sigma Chemical/Sigma Diagnostics
(Tris[hydroxymethyl]aminomethane or "Trizma Base")
($C_4H_{11}NO_3$)

Tritiated Estradiol ($[^3H]E_2$)..... New England Nuclear, Boston, MA, USA

Tween 20..... Sigma Chemical/Sigma Diagnostics
(Polyoxyethylenesorbitan Monolaurate)

Tween 80..... P-1754, Sigma Chemical/Sigma Diagnostics
(Polyoxyethylenesorbitan Monooleate)

APPENDIX V – LIST OF EQUIPMENT USED

Fecal Extraction and Cr, EC, PdG & T EIA Equipment – used in the TO Zoo lab

Durex™ Borosilicate Disposable Glass Culture Tubes (12 x 75 mm)..... .....60825-467, VWR Scientific Products, PA, USA

Dynatech Ultrawash II 96-Well Microplate Washer ..... VWR Can Lab

Dynex Technologies MRX Plate Reader .....Dynex Technologies, Chantilly, VA, USA

Extraction Tubes, Glass (16 x 125 mm)..... 14-930A, Fisher Scientific

Nunc-Immuno™ Plates, F96 Cert. MaxiSorp™ Surface (for EC, PdG, T and P)..... ..... 439454, NUNC™ Brand Products, Nalge Nunc International, Roskilde, Denmark

Platelet Mixer, Model 348 .....Fisher Scientific, NO LONGER AVAILABLE

Polypropylene Tubes (12 x 75 mm)..... T400-3A, Simport, PQ, Canada

Revelation Version 4.21, Copyright© 1999 ..... Dynex Technologies (software for interfacing plate reader with PC)

Rotator American Instruments R4140 (flat rotator for shaking plates).... VWR Can Lab

Fecal Extraction and P EIA Equipment – used in the CRC lab
Equipment is listed only if different from that used in the TO lab

Fecal Collection Tubes – Falcon 50 ml Polystyrene Conical Tubes..... ..... Becton Dickinson Labware, NJ, USA

Vacuum Filtration Equipment..... .....#38-111, RAININ Nylon-66 filters (pore size 0.2 μm, diameter 47 mm)