THE ENDOCRINOLOGY OF MUSTH IN THE MALE ASIATIC ELEPHANT (*Elephas maximus*): SERUM ESTRADIOL, SERUM LH AND SERUM, FECAL AND URINARY TESTOSTERONE

A Dissertation

by

CRAIG JOSEPH TURCZYNSKI

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Approved as to style and content by:

Duane C. Kraemer
(Chair of Committee)

Paul G. Harms
(Member)

Larry Johnson
(Member)

James G. Anderson
(Member)

Ronnie L. Edwards
(Head of Department)

May 1993

ABSTRACT

The Endocrinology of Musth in the Male Asiatic Elephant (*Elephas maximus*): Serum Estradiol, Serum LH and Serum, Fecal and Urinary Testosterone. (May 1993)

Craig Joseph Turczynski, B.S., Iowa State University

Chair of Advisory Committee: Dr. Duane Kraemer

Efforts to breed the endangered Asian elephant in captivity have historically been difficult because of our primitive understanding of musth. Long-term endocrinological studies of musth are needed; however, it is often difficult and dangerous to collect serum from the musth male.

Serum, urine and fecal samples were collected along with behavioral data from 11 male Asiatic elephants. Methods of testosterone analysis were developed, validated and subsequently performed on all serum, urine and fecal material. Serum samples were additionally analyzed for estradiol and LH. All endocrine data were analyzed for significant differences during behavioral musth for the purpose of developing techniques valuable for the study and understanding of this phenomenon.

Animals ranging from 10-30 years of age experienced musth but older elephants experienced a greater incidence of musth. Significantly more testosterone was secreted in the serum by animals in musth and by musth animals 21-30 years old then by non-musth and musth animals 15-20 years old respectively. Serum estradiol concentrations were significantly elevated during musth and were correlated with serum testosterone and aggressive behavior. Serum LH begins to rise 4 weeks prior to musth symptoms and quickly drops after musth symptoms have started.

Concentrations of urinary and fecal testosterone were significantly elevated during musth and positive linear relationships were observed between serum, urinary and fecal testosterone values. After injection of ^{14}C testosterone into one male Asiatic elephant,

57% of the radioactivity was excreted in the feces, 7% in the urine and 36% was not recovered. Testosterone excretion in the feces peaked 48-58 hours post-injection whereas the majority of urinary testosterone was excreted within 24 hours. Nearly 100% of steroids in the feces and 30% of steroids in the urine were unconjugated. In-vivo metabolized ^{14}C testosterone was separated by HPLC and demonstrated five distinct peaks of radioactivity. One of these peaks co-eluted with testosterone standard, however, the largest peaks were less-polar metabolites.

In conclusion, Serum testosterone, estradiol and LH concentrations can be used to potentially quantitate or predict musth and non-invasive fecal and/or urinary testosterone assay procedures can be used successfully to monitor and further study musth.

DEDICATION

I wish to dedicate this manuscript to my family; my parents, Tom and Evelyn, who have equipped me with the personal skills necessary to pursue and accomplish my life goals, my wife, Rosa, who has supported, encouraged and motivated me and at the same time demonstrated unending patience, and finally to my children, Seth Andrew and our gestating child, may they someday read this and be inspired to pursue their dreams with vigor.

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INTRODUCTION

A relationship between man and the elephant is known to have existed for well over 4000 years. Elephant's have served man for agricultural, logging, war and general transportation purposes. They are important to many cultures for religious ceremonies, sporting events and displays of wealth and power. They have entertained us in circuses and fascinated millions in zoological parks. There are few other animals in which man has fostered such a useful relationship.

Today, the continued existence of the elephant and the elephant's relationship with man are threatened. Free-ranging populations of elephants are being forced to survive in smaller, fragmented geographical areas and poaching for ivory has become a threat to the viability of many of the remaining populations. Consequently, the Asian elephant *(Elephas maximas)* is listed as endangered and the African elephant *(Loxodonta africana)* as threatened under the U.S. Endangered Species Act and both species are listed as appendix I under The Convention on International Trade in Endangered Species of Wild Fauna and Flora. Man has always considered the wild populations to be a renewable resource for animals to serve as beasts of burden. Therefore, we have gained very little information on the reproductive physiology of these species and have produced few offspring from captive bred animals (Tuttle, 1991). In order for man to continue to enjoy and use this unique mammal, the current captive populations must serve as ambassadors and educators to man. Man in turn must commit to scientific study of this animal with the intent of gaining knowledge useful for reproductive management of the species. It is likely that in addition to producing the information needed to successfully maintain a viable genetic base, reproductive research of the elephant will produce new information on comparative reproductive biology. Nowak, (1991) believes the total

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number of mammalian taxonomic categories to be 21 orders, 135 families, 1,116 genera and 4,444 species. If we use a generous estimate of 100 species of which man has a reasonable understanding of the reproductive physiology, this would represent 2.3% of the total mammalian fauna. We have found both subtle and major differences in the physiology of this 2.3% and while research has been conducted, we have discovered information useful for other areas of food and health science. It may be argued that comprehensive study of a new species could be justified for that reason alone.

Efforts to breed elephants in captivity have been hampered by our primitive understanding of the male elephant's behavior, especially during the period of musth. In the United States, particularly in zoological institutions, adult males destroy expensive facilities and are a considerable risk to their keepers. In Asian countries, where the elephant is still used for work, the unpredictable behavior of an adult male may render the animal useless and dangerous for several weeks (Jainudeen et. al., 1972b: Shrestha, personal communication). The term "musth" was derived from the Hindi word "mast" meaning intoxicated and although documentation of musth is dated back millenniums (Strabo, 63 BC-21 AD; Darwin, 1871, Sanderson, 1876), it's significance is not well understood. Musth is a unique phenomenon possessed only by the African and Asian elephant. It is characterized by heightened aggressive behavior, hyperactivity of the temporal glands, abnormal urination and elevated concentrations of circulating testosterone (Jainudeen et al., 1972a; 1972b; Rasmussen et al., 1984; Hall-Martin and Van der Walt, 1984; Poole et al., 1984; Poole 1987a; Hall-Martin, 1987; Cooper et al., 1990; Niemuller and Liptrap, 1991). Although it has been compared to and is similar to the rut observed in many of the seasonally polyestrous ungulates, it differs in several important ways; 1) the elephant has been determined to be nonseasonal, except when nutritional factors are altered by climatic changes (Jainudeen et al., 1972a; 1972b; Hall-Martin and Van der Walt, 1984; Poole et al., 1984; Poole 1987a; Hall-Martin, 1987), 2)

musth is a variable phenomenon but appears to be cyclic in older males with each individual possessing his own interval of musth, (Jainudeen et al., 1972a; 1972b; Rasmussen et al., 1984; Hall-Martin and Van der Walt, 1984; Poole et al., 1984; Poole 1987a; Hall-Martin, 1987; Cooper et al., 1990), 3) male elephants will breed while in or out of musth (Jainudeen et al., 1972a; 1972b; Hall-Martin and Van der Walt, 1984; Poole et al., 1984; Poole 1987a; Hall-Martin, 1987; Cooper et al., 1990; Niemuller and Liptrap, 1991).

Establishing a means of monitoring and controlling musth offers obvious advantages for captive management of both species of elephant. Eliminating or suppressing the musth period would result in a more useful and dependable animal as well as protecting facilities and human life. The control of musth may also be beneficial in field conditions if planned mating becomes necessary. Since musth males are more successful in guarding and breeding estrous females and estrous females prefer musth males, we could manipulate mating and ensure genetic diversity by suppressing or inducing musth. The same mechanisms which are used to control musth may also be found useful for contraception in male elephants, offering an alternative to culling in some circumstances.

Although control of musth is our long-term goal, a greater understanding of this phenomenon is a prerequisite to any logical attempt to control or manipulate aggressive behavior in male elephants. Further study into the endocrinology of musth may help with determination of the triggering mechanisms involved. The cascade of endocrinological events involved with musth may have physiological significance important to the health and fecundity of the animal. One of the biggest obstacles affecting the progress of studying musth, however, is the inability to acquire consistent serum samples for long-term and intensive investigation. An alternative method of endocrine study must be developed.

While this research has focused on the areas which need further investigation, the confines of establishing appropriate scientific design for this species has dictated the type

of hypotheses tested. Investigation has targeted the Asian species but information has been extrapolated and compared to literature from both species of elephant.

The objectives of this dissertation were to develop non-invasive fecal and urinary testosterone assay protocols useful for monitoring and studying musth and to determine the serum concentrations of testosterone, estradiol and LH from musth and non-musth male elephants.

LITERATURE REVIEW

Review of musth in the male Asian and African elephant

The literature on musth was filled with observational reports offering a diversity of opinions (McGaughey, 1963) until 1972 when the first systematic and experimental investigation of musth was reported (Jainudeen et al., 1972a). After acquiring case histories on 80 male Asian elephants along with behavioral observations and experimental breeding results from an additional 8 males, Jainudeen et al., (1972a) reported the sex, age and seasonal distribution as well as the duration and recurrence of musth for this species. Musth occurred only in males and was confined to animals over the age of 15. Duration ranged from 2 weeks to 5 months with a mean of 2 to 3 months. There was a slight seasonal distribution with peaks occurring concurrent to maximum rainfall and nutrient availability. Musth showed a cyclic pattern repeating on a yearly basis. Factors influencing musth included nutrition, physical condition and exposure to females in estrus. Sickness, overwork and inadequate nutrition all tended to suppress musth while sexual activity induced musth. They felt that the similarity of musth and rut was striking and hypothesized that musth is a vestige of a true seasonal rut which may have existed in the elephant in earlier years.

Also in 1972b, Jainudeen et al., reported on a parallel study in which musth was categorized into four periods; pre-musth, full-musth, post-musth and non-musth. Plasma samples where obtained from 11 males, age 21-45, so that all four periods would be represented. Plasma samples were subsequently analyzed for testosterone using a competitive protein binding assay. Testosterone concentrations ranged from 0.2 to 1.4 ng/ml during non-musth and post-musth, 4.3 to 13.7 ng/ml during pre-musth and 29.6 to 65.4 ng/ml during full-musth. Thus an endocrine basis of musth similar to that of the rut was demonstrated. In their discussions, Jainudeen et al., (1972a, b) suggested the use of

chemical compounds such as estrogen, antiandrogens or GnRH inhibitors for controlling the aggressive nature of musth bulls.

Interestingly, in 1962, West et al., and again in 1984, Siegel attempted to induce musth like behavior in Asian elephants by administering lysergic acid diethylamide (LSD). The animal responses during these studies were not comparable to musth behavior and their work provided little information useful for understanding this phenomenon.

It was not until more recently that the behavioral manifestations of musth in the African elephant were documented. Poole and Moss, (1981) observed and collected behavioral data from 7 male elephants in Amboseli National Park, Kenya for 3 years. They believed that the pronounced enlargement and copious secretion of the temporal glands, increased aggression, urine dribbling and green penis color observed was identical to the reported symptoms of musth in Asian males. This was both substantiated by and added validity to the hypothesis presented by Buss and Johnson (1967) that the African bull elephant experiences a cycle of sexual activity. Free-ranging male elephants of different age and social status were shot and Leydig cell characteristics and intra-testicular testosterone data were evaluated. A large variation in testosterone concentration between animals old enough to be post-pubertal was observed and behavior such as searching for estrous females was related to higher testosterone levels.

The occurrence of musth in the African elephant was questioned by Rasmussen et al., in 1984. After obtaining intermittent serum and temporal gland secretions from both sexes and species of elephant, the authors reported testosterone and dihydrotestosterone concentrations in these two types of biological samples. The only samples collected during behavioral musth were from two Asian males. The highest concentrations of serum testosterone and dihydrotestosterone were obtained from the male Asian elephants during musth, 40 and 1.52 ng/ml respectively. Of considerable interest was that androgen concentrations were up to 10 times higher in temporal gland

secretion than in serum however, as with serum, the highest testosterone and dihydrotestosterone concentrations were found in the Asian musth males, 286-810 and 201-560 ng/ml respectively. Behavioral musth records collected for 10 years for one Asian male and 3 years on another bull introduced to the same facility, demonstrated the individual variability of musth. During the first 7 years, bull #1 exhibited sporadic musth activity ranging in duration and intensity. Following the introduction of bull #2 to the same facility (the last two years of data collection, #1-21 yrs. #2-20 yrs old.) musth activity for both bulls occurred between February and July. In fact, during the last year of data collection, musth activity in the two bulls was nearly perfectly synchronized. Several reasons were given for why they believed that what Poole and Moss (1981) had reported was not analogous to musth in Asian elephants. They stated that aggressive behavior was inadequately described, temporal gland secretion is present in African elephants during various social situations and musth had never been documented in captive African bulls.

These comments had influenced elephant biologists and several studies of musth in African bull elephants were soon published. Hall-Martin and Van Der Walt, (1984) compared plasma testosterone levels in single samples from three African bulls of known musth status. Non-musth bulls had testosterone levels of 3.48 and 9.47 ng/ml whereas the musth bull had a testosterone concentration of 19.8 ng/ml. Poole et al., (1984) demonstrated that urinary testosterone levels in musth males (241.3 $\pm$ 78.4 ng/mg Cr.) exceeded concentrations of testosterone in nonmusth males (77.7 $\pm$ 39.7 ng/mg Cr.). Brannian et al., (1989) employing urinary hormone analysis on one animal 37 years of age, was the first to document that musth occurs in captive African elephants. Urinary androstenedione was significantly higher during musth (737 $\pm$ 134 vs 103 $\pm$ 8 ng/mg Cr.) They also demonstrated that urinary LH levels were increased and urinary creatinine levels were decreased during musth. Following these studies it was clear that both species exhibit the characteristic inflamed and hypersecreting temporal glands, urine

dribbling, aggressive behavior and increasing concentrations of circulating testosterone indicative of musth.

Intensive behavioral studies of musth in the African elephant published by Poole in 1987a, b and 1989a, b, further defined musth and again suggested a comparison to the rut. Males in musth were more frequently aggressive and in association with females than non-musth males. The occurrence and duration of musth was observed to be age-related with older males exhibiting longer more predictable musth annually and younger animals experiencing shorter and more sporadic musth. The sporadic musth activity in younger animals was attributed to a suppression by higher ranking bulls. Seasonality was not apparent, however, frequency of musth was greatest during ideal weather and nutrient availability when the majority of females were observed in estrus. During musth the male's dominance relative to other males changed allowing him to have greater success competing for estrous females. A variety of visual, olfactory and vocal signals were also found to be unique to musth males. Visual signals included a specific form of ear flapping, posture while walking and head oscillations. Musth males would also tusk the ground, hurling clods of dirt, grass and logs and males in musth frequently rubbed their temporal glands with their trunk. Olfactory behavior included constant urine dribbling and spraying and an increase in marking temporal gland secretions on trees. A specific pattern of low frequency rumbling was also characteristic to the musth male elephant. Hall-Martin, (1987) in an independent study of a separate elephant population validated many of the same behaviors. The author hypothesized that the reproductive strategy of musth was to promote gene flow and ensure out-breeding.

In 1988, Rasmussen reported the chemosensory responses of African and Asian elephants to constituents of temporal gland secretion and musth urine. This study revealed the chemosensitvity of the elephant and the potency of olfactory cues on eliciting certain behaviors. Of most interest, was that specific fractions of Asian musth urine separated by HPLC revealed a dramatic avoidance reaction by African female

elephants and a less intense reaction by Asian females. From chemical analysis of temporal gland secretions, the author concluded that there was an inverse relationship between levels of (E)-farnesol and testosterone and when African elephants were exposed to synthetic mixtures containing low concentrations of (E)-farnesol, the response was 50% the response to musth urine.

Rasmussen et al., (1990) presented further information on the chemical analysis of serum and temporal gland secretions during musth from a bull Asian elephant. The serum testosterone levels reported had a much greater range than had been previously reported (12.71-125.7 ng/ml). In temporal gland secretions, testosterone and dihydrotestosterone levels were highest during the first half of musth (921.6 and 1007.4 vs 162 and 177.1 ng/ml respectively). Higher T/DHT ratios in serum and temporal gland secretions were found during musth and significantly higher ratios were found during heaviest musth in temporal gland secretions. When the authors compared these levels to other mammals, the T/DHT ratio was elevated similar to rutting males yet during musth, the high/high situation was found to be unique to the elephant. They concluded that the existence of certain volatiles in temporal gland secretions indicates the chemocommunicative function of the gland, furthermore, these volatiles appeared to be controlled by the endocrine status during musth.

In 1990, Cooper et al., published the results of the first long-term investigation of serial serum testosterone concentrations in one captive Asian and one captive African elephant. The data were consistently collected over a five year period. The male Asian elephant, as early as 13, experienced a consistent annual pattern of serum testosterone elevation and musth with the elevation in testosterone preceding temporal gland secretion and other symptoms of musth. The African bull did not show symptoms of musth until age 17 when serum testosterone levels were significantly higher than previous years, 18.0 ± 2.7 vs 4.4 ± 8.8 ng/ml. Testosterone concentration in the Asian male ranged from a mean value of 7.6 ± 1.0 ng/ml during non-musth to a mean value of 41.2 ± 2.8

ng/ml and peak values exceeding 80 ng/ml during musth. Nutritional restriction in the Asian male resulted in a quantitatively less intense behavioral musth, delayed onset of musth and a shift in the temporal relationship between elevated androgen and temporal gland secretion. This reversed relationship suggested that in addition to increased production of testosterone, other factors were involved in the activation of temporal gland activity and perhaps the triggering of musth. The results of the study also suggested that musth occurs much earlier in captivity due to beneficial nutritional and/or social factors.

In 1990 and 1991, Niemuller and coworkers published two articles on studies of 8 captive Asian elephants, 4-35 years of age. All hematological and biochemical analytes except for alkaline phosphatase, gamma glutamyl transferase and creatinine remained constant in musth and non-musth samples (Niemuller et al., 1990). The levels of all three of the non-constant biochemical analytes were statistically higher during musth. The authors speculated that the high levels of alkaline phosphatase and gamma glutamyl transferase during musth were indicative of liver malfunction and are similar to what is observed in humans receiving androgen injections. They also speculated that the increase in serum creatinine levels indicated a change in renal function. This postulate is supported by the findings of decreased urinary creatinine levels observed in African elephant musth urine (Brannian et al., 1989). Furthermore, musth male elephants frequently exhibit a persistent urine dribbling and are known to reduce their food and water intake, possibly causing dehydration and hypoproteinemia. Among other factors, dehydration and hypoproteinemia do affect glomerular filtration rate (Ganong, 1987).

Niemuller and Liptrap (1991) also demonstrated a reversal in the ratio of androstenedione to testosterone in favor of testosterone during musth in 6 of the 8 Asian male elephants experiencing musth periods. Testosterone mean values during musth displayed a marked difference between animals (152.53 to 15.97 ng/ml) with the older males demonstrating greater mean values than younger males. Severity of musth was

also greater in older males. Using a heterologous bovine radioimmunoassay and employing intensive sampling of two animals (every 15 min for 12 hrs) during musth and nonmusth, the authors demonstrated that although significantly more LH was secreted during musth, pulse frequency was not significantly different.

Although a considerable amount of information has been published on musth, the data must be interpreted with caution due to the limited number of animals available for consistent sampling. To date, only small segments of the endocrinological events have been elucidated. Further study into the hypothalamic-pituitary-gonadal control of musth as well as replication of the above work are needed to understand how this phenomenon is triggered and how it can be manipulated.

Comparisons of musth to rut

The term rut, Latin for *rugire,* meaning roar, describes the period of intense breeding behavior characteristic of male deer and other seasonally polyestrus ungulates. Like musth, it is characterized by increased aggression and interest in breeding (Espmark, 1964; Lent, 1965; Lincoln and Short, 1980), elevated levels of circulating testosterone (Lincoln and Short, 1980; Asher et al., 1989; Fennessy et al., 1988; Asher and Peterson, 1991) and exaggerated sexually dimorphic expression (Espmark, 1964; Lent, 1965; Lincoln and Short, 1980). Males also display a variety of visual, olfactory and vocal advertising behaviors (Espmark, 1964; Lent, 1965; Lincoln and Short, 1980). Unlike musth, rut is limited to a precise period, for all males, which has been attributed to fluctuations in daylength (Lincoln and Davidson, 1977; Lincoln and Short, 1980). Most rutting males experience a drop in fertility and libido during the non-breeding season (Lincoln and Short, 1980) but it has not been determined if the musth male elephant experiences this same change (Jainudeen et al., 1971; 1972b; Howard et al., 1984; Rasmussen et al., 1984; Hall-Martin, 1987; Poole, 1987a; Schmidt, 1988; Cooper et al., 1990). In fact, it is quite well documented that male elephants will breed while in or out of musth (Eisenberg et al., 1971; Scheurmann and Jainudeen, 1972; Hall-Martin,

1987; Poole, 1987a; 1989a; Cooper et al., 1990), Numerous authors have compared the behavioral manifestations of musth and rut with the majority of investigators favoring the similarity (Benedict, 1936; Eisenberg et al., 1971; Jainudeen, 1972a; Scheurmann and Jainudeen, 1972; Poole, 1987a). Although limited work has been devoted to the comparison of the two at the hypothalamic-pituitary-gonadal level, the information available on rut may be valuable for understanding the mechanism involved in musth.

Lincoln and Short, (1980) concluded that during the non-breeding season, one or more of a number of factors, including photoperiod, are relayed to the hypothalamus via chemically active neurons from higher centers of the brain. The signals work to alter the episodic release of GnRH from the hypothalamus resulting in low circulating levels of LH and to some degree FSH. The neuronal and molecular mechanisms involved in GnRH and gonadotropin secretion are known to be complex. Halasz et al., (1989) reviewed the evidence related to the organization of the GnRH nerve cell and the chemically identified neurons synapsing with it. Immunoreactive catecholaminergic, serotoninergic, GABA-ergic, opiod peptidergic, substance-P and CRF axons have all been shown to synapse with GnRH neurons. In addition, GnRH neurons appear to innervate higher centers of the brain and may be involved in short-loop regulation of GnRH (Negro-Vilar et al., 1988; Halasz et al., 1989). Negro-Vilar et al., (1988) also indicated that testosterone plays a key role in GnRH neuronal activity and may actually stimulate GnRH synthesis and release. GnRH directly alters the pulse frequency, amplitude and baseline levels of LH but only basal levels of FSH appear to be altered. In the case of FSH, inhibin and activin are additionally involved in regulation of secretion (Negro-Vilar et al., 1988). GnRH is released from the median eminence in a pulsatile manner (Knobil et al., 1980). Pituitary gonadotrophs respond by releasing LH in a similar ultradian rhythm (Levine et al., 1982). Circulating LH is transported to the Ledig cells of the testis and binds to the LH-adenylate cyclase coupled receptors on the plasma membrane (Hall, 1988). Second messengers (ie Ca, cyclic AMP) are then

activated which control the cellular processes responsible for testosterone production. FSH has a similar mechanism of action on the Sertoli cells and in concert with intra-testicular androgens, the cells of the seminiferous tubules are activated to carry-out spermatogenesis (Griffin and Wilson, 1985). Therefore, in the rutting animal, the result of low circulating levels of LH and FSH is a decrease in testicular function (Lincoln and Short, 1980). During the transition into the breeding season, suppression is reversed resulting in an increase in circulating levels of LH and FSH. Testicular function is regained resulting in high circulating levels of testosterone and renewed spermatogenesis (Lincoln and Short, 1980).

Studying the seasonal fluctuations of LH and testosterone in male fallow deer, Asher et al., (1989) and Asher and Peterson, (1991) found that during the transition from the non-breeding to breeding season, (February) there is a marked increase in the frequency of LH and testosterone secretion. In March and April (prerut and rut) there is a decrease in LH pulse rate, decreasing basal LH concentrations and huge surges in testosterone secretion. Following the rut, levels of both LH and testosterone decrease dramatically. Other authors have reported similar data for the red deer stag (Suttie et al., 1984; Fennessy et al., 1988). In contrast, the limited data presented by Niemuller and Liptrap, (1991) for the elephant showed that LH and testosterone increased significantly during musth but pulse frequency was not significantly different from non-musth.

Exogenous treatment with GnRH during various times of the breeding period is useful in determining the change in pituitary and testicular sensitivity throughout the rut (Lincoln and Short, 1980; Illius et al., 1983; Fennessy et al., 1988). Fennessy et al., (1988) found that pituitary response to a single 95 ug injection of GnRH was greatest during full velvet antler (period preceding the rut) and significantly reduced during rut. Testosterone response, however was greatest during the rut. Lincoln and Short, (1980) reported evidence that LH pulse amplitude in response to intravenous injection of 1 ug of GnRH was greatest during gonadal regression (periods preceding and following the rut)

but during the rut, the duration of LH secretion was greater. During the non-breeding season there is a decrease in the response of the pituitary as well as the gonad to exogenous treatment of GnRH and LH (Lincoln and Short, 1980). There have not been any reports of the use of this procedure in male elephants although it has been shown that exogenous treatment of 1 and 10 mg but not 0.01 and 0.1 mg of GnRH to one female Asian elephant after estrone pretreatment will elicit a measurable LH response (Chappel and Schmidt, 1979). Brown et al., (1991) also demonstrated a measurable response in both LH and FSH with 0.5 mg of GnRH given intravenously in female Asian elephants.

Noninvasive endocrine analysis

Encountering difficulties studying the endocrinology of many non-domestic species, investigators have turned to the measurement of hormones excreted in urine and feces (Lasley, 1985; Lasley and Kirkpatrick, 1991; Bamberg et al., 1991). The majority of early work performed to describe gonadal function from analysis of urine in non-domestic mammals was accomplished by measuring two groups of metabolites, estrone conjugates and pregnanediol-3-glucuronide (Loskutoff et al., 1987, Lasley, 1985). These assays are commonly used today in a variety of mammalian species (Kirkpatrick et al., 1991a; Monfort et al., 1991; Knox et al., 1992). The assays utilize direct measurement of metabolites in urine without extraction. Correction for differential water clearance is performed by indexing the sample per mg of urinary creatinine. This method assumes that creatinine clearance is constant (Lasley, 1985). Steroids can also be measured in urine after solvent extraction. Extraction of steroids in the urine usually includes a step to first liberate the steroid from its water soluble conjugated form. This is accomplished by either incubating the urine at precise temperature and pH with digestive enzymes isolated from *Helix pomatia* or chemically solvolyzing the sample by acidifying it and extracting with low polarity solvents (Jacobsohn and Lieberman, 1962).

Two methods have been used to measure androgens in the urine of African elephants. Poole et al., (1984) used an extraction method; 0.2ml urine were enzymatically

hydrolyzed by incubating at 37° C and pH 5 with *B*-glucuronidase-arylsulphatase. Following this hydrolysis procedure, which is effective on both sulfates and glucuronosides, the sample was extracted with diethyl ether. In contrast, Brannian et al., (1989) measured urinary androstenedione directly in 0.02 ml of unextracted urine.

Much of the earliest work devoted to fecal hormone analysis in domestic and non-domestic mammalian species came out of one laboratory (Bamberg et al., 1984; Mostl et al., 1984; Safer-Hermann et al., 1987). These researchers presented useful information for pregnancy detection using fecal estrogen determination in a variety of mammals. In their published work, several different methods were used to extract steroids from the feces. In Bamberg et al., (1984), one gram of feces was suspended in 3 ml of water and extracted twice with a 10 ml mixture of pet/diethyl ether (9:1). After separating and evaporating the organic layer, the residue was re-extracted with chloroform and sodium hydroxide. The alkaline phase was then acidified with acetic acid, extracted with diethyl ether, evaporated, reconstituted in buffer and brought to assay. Recovery of added radioactive estradiol was 50 $\pm$ 5%. Mostl et al., (1984) extracted 0.5 grams of feces with 0.5 ml chloroform and 1.5 ml 2N NaOH. After centrifugation, 0.5 ml of the supernatant were extracted with 5 ml of petroleum ether and the organic layer was separated, dried under nitrogen gas and brought to assay. Recovery of added radioactive estradiol was 50 $\pm$ 5%. Safar-Hermann et al., (1987) also used 0.5 grams of feces, however the extraction was performed using 1ml distilled water and 8 ml of petroleum ether/methanol (1/1). The methanol phase (1 ml) was evaporated and reconstituted in buffer at pH 5 and subsequently hydrolyzed with *B*-glucuronidase-arylsulphatase. After hydrolysis the samples were re-extracted with pet/diethyl ether (9/1), the organic layer separated, dried under nitrogen gas and brought to assay. Extraction recovery of the entire procedure was 69 $\pm$ 6%. Both an enzyme assay method (Bamberg et al., 1984) and an RIA (Mostl et al., 1984; Safer-Hermann et al., 1987) were used for detection of

estrogen after these extraction methods and differences in pregnant and nonpregnant animals were successfully detected.

Today, fecal hormone analysis in mammals has become popular among wildlife and exotic animal researchers as evidenced by several review articles (Bamberg 1991; Lasley and Kirkpatrick, 1991). The holding of The First International Symposium on Fecal Steroid Monitoring in Zoo Animals on February 28-29, 1992 also illustrates this point. During this symposium, several papers were presented on the utilization of fecal steroid monitoring in mammals which demonstrated the diversity of procedures used, especially as it relates to sample preparation and extraction. To summarize these proceedings, in 4 of the 11 papers presented on mammalian fecal steroid analysis, the investigator dried the fecal sample (Flood et al., 1992; Gross, 1992; Hodges et al., 1992; Wasser et al., 1992). Flood et al., (1992) and Hodges et al., (1992) additionally pulverized the sample after drying. In 3 of the papers presented, the investigators used hydrolysis to include measurement of any conjugated steroids found in the fecal extract (Hodges et al., 1992; Hoppen et al., 1992; Gross, 1992).

Three types of methods were used to extract fecal samples, the investigators either solubilized/homogenized the fecal sample in an aqueous solution (Kirkpatrick et al., 1992), directly extracted the sample with an organic solvent (Buiter et al., 1992; Holtz, 1992; Mostl, 1992; Pryce and Dobel, 1992; Shaw et al., 1992) or combined both procedures (Flood et al., 1992; Gross, 1992; Hodges et al., 1992; Hoppen et al., 1992; Wasser et al., 1992). Wasser et al., (1992) and Kirkpatrick et al., (1992) methods differed most from those of the other investigators detailed above. After drying fecal samples in a lyophilizer or rotary evaporator, 0.18-0.2 grams of feces were placed in a 50 ml centrifuge tube. The sample was then boiled with 10 ml of ethanol for 20 minutes, centrifuged 15 minutes at 2000g and the supernatant decanted. The supernatant was evaporated to 6 ml, combined with 2 ml water and further evaporated until 2 ml remained. The sample was then extracted twice with dichloromethane, evaporated and

brought to assay (Wasser et al., 1992). Kirkpatrick et al., (1992) used 0.5 grams of wet feces and solubilized/homogenized it in 5 ml distilled water, centrifuged for 10 minutes at 2500 rpm and took 0.02 ml to assay for steroid conjugates. While the papers presented at this symposium clearly demonstrated that improvements are needed in the methods of analysis, the data collectively reveal that fecal steroid analysis is a useful procedure for monitoring mammalian endocrinology.

Only one paper was presented on fecal analysis in the elephant. Hoppen et al., (1992) reported data from 3 female and 1 male Asian elephants. Fecal samples (0.5 grams) were first solubilized/homogenized in 2 ml sodium acetate (pH 4.8), hydrolyzed with *B*-glucuronidase/arylsulfatase and extracted with ethyl acetate. The evaporated ethyl acetate fraction was subjected to a phenolic extraction using chloroform and NaOH. The neutral fraction, containing progesterone and testosterone, was evaporated, dissolved in 70% methanol and defated with n-hexane. The remaining methanol fraction was then evaporated and brought to assay. The phenolic fraction (above) was adjusted to pH 6.5 with HCL, extracted with ethyl acetate, defated and assayed for estrogens. Extraction efficiency was reported as 83% for progesterone, 92% for testosterone and 70% for estradiol. Fecal progesterone was not strongly correlated with plasma progesterone and fecal estrogen was not correlated with plasma values. Fecal testosterone data for the male elephant were reported to be significantly correlated with plasma testosterone. During the presentation however, the author indicated that attempts to duplicate the fecal testosterone assay had failed (Gross, personal communication). Also reporting data from a collaborating laboratory, the authors stated that fecal 20-alpha dihydroprogesterone was highly correlated with plasma progesterone but fecal pregnanediol-3-glucuronide was not.

Although steroid structure is relatively conserved among the animal kingdom (Lasley and Kirkpatrick, 1991), the excretory patterns of steroidal hormones have been shown to exhibit a great deal of variability between species (Lasley, 1985; Lasley et al., 1989;

Loskutoff et al., 1987; Hindle and Hodges, 1990; Shille et al., 1990). Circulating steroids go through a series of peripheral, hepatic, renal and intestinal metabolic reactions before being excreted in the urine and/or feces. The majority of steroids excreted in the urine are conjugated, however considerable amounts of enzymatic hydrolysis and/or reconjugation can occur before steroids are excreted in the feces. (Musey et al., 1979). Before accurate measurement of gonadal function can be obtained, appropriate assay validation must be performed (Lasley and Kirkpatrick, 1991). Choice of an antiserum which will not cross-react with compounds deleterious to accurate interpretation of results is critical. Further assay validation should be carried out in several ways; 1) HPLC co-chromatographic separation of a pooled sample from the species of concern with a known radiolabeled standard. Fractions are collected during the chromatographic separation and split for measurement of the radioactive standard and measurement of the unknown immunoreactive substances (Lasley, 1985). 2) Comparison between a dose response curve of the sample and a cold standard. The slopes of the two curves should be statistically and significantly parallel (Yallow, 1985). If difficulties are encountered in producing parallelism, the standard hormone can be diluted with charcoal striped serum or urine (Brown et al., 1991; Gross, personal communication). 3) Comparison of fecal or urine steroid concentrations with behavioral/physiological indicators and/or with circulating steroids in matched samples (Lasley and Kirkpatrick, 1991). 4) Acceptable precision in repeated measurement of the same sample (Yallow, 1985).

Radiolabeled infusion studies have been used to study the metabolism of steroids in a variety of species. Assuming that the injected hormone follows hepatic, renal and intestinal metabolism similar to the endogenous steroid, investigators have used this procedure to determine the relative percentages of fecal and urinary excretion. (Loskutoff et al., 1987; Hindle and Hodges, 1990; Shille et al., 1990; Czekala et al., 1992). The authors were also able to determine the proper metabolite useful for

describing gonadal function in the species of concern. Hindle and Hodges, (1990) injected 50 uCi each of ^{14}C estradiol and progesterone into an adult female white rhinoceros. Sixty-one percent of the total activity injected was recovered, 25% in the urine, 36% in the feces. Ninety percent of excreted activity in the urine and 48% of the activity in the feces was released during the second day following injection. Progesterone metabolites predominated in the urine and estrogen metabolites predominated in the feces. The majority of steroids recovered in the feces were unconjugated whereas approximately half of the urinary steroids were conjugated. Progesterone and estradiol (17 alpha and beta) were the only detectable metabolites excreted in the feces. In the urine, progesterone and estrone were the most abundant unconjugated steroids and 4-pregnen-20-alpha-ol-3-one and conjugated estrone were the most abundant conjugated steroids. Czekala et al., (1992) injected 500 uCi of ^{3}H estradiol into the ear vein of one Asian female elephant and collected urine and feces for 24 hours. No activity was recovered in the feces, however, urine radioactivity peaked within 2 hours of injection and had stabilized after 16 hours. Injection of 1.5 mg unlabeled estradiol I.V. into a second animal indicated that estradiol is rapidly converted to conjugated form and cleared from circulation through the urine. The fact that fecal collection was only continued for 24 hours made it difficult to make conclusions about intestinal clearance of estrogens.

There have been no reports of the use of this procedure in the male elephant, however, studies on testosterone metabolism and urinary excretion in the horse have demonstrated complex metabolism (Houghton and Dumasia, 1979). Twenty-two uCi of ^{14}C testosterone were injected intramuscularly into two gelded horses. Within 96 hours, 45% of the administered dose was excreted in the urine with only small amounts detected afterwards up to 200 hours. For the two horses, 83% and 78% of urinary ^{14}C was in the conjugated form. Sulfates made up 66% and glucuronides made up 20% of the conjugated steroids. The identifiable metabolites included; testosterone, 3beta-hydroxy-

5alpha-androstane-17-one, 5alpha-androstane-3beta,17alpha-diol, 5alpha-androstane-3beta,17beta-diol, 3,17-dihydroxyandrostan-16-one, 3,16-dihydroxyandrostan-17-one and two isomers of androstan-3,16,17-triol.

The metabolites of testosterone in man include estradiol and dihydrotestosterone, 17-ketosteroids such as androsterone and etiocholanolone and polar diol, triol and conjugated metabolites (Griffin and Wilson, 1980).

EXPERIMENTAL DESIGN

Temporal patterns of musth--age and environmental factors

Hypothesis. The captive environment contributes to an earlier onset of musth symptoms, the musth pattern will be sporadic and short in younger animals, more cyclic and/or prolonged in more physiologically mature animals and significant differences in serum testosterone will be detected in animals of different maturity.

Rationale. Jainudeen et al., (1972b) reported that over 90% of Asian males experienced musth in a cyclic pattern every year and that irregularities in the cyclic pattern were most often observed in younger animals. In 1984, Rasmussen et al., presented the behavioral musth pattern for one Asian male elephant over a ten year period. The animal experienced a highly variable musth pattern starting at age 12 until age 19 and 20 when musth became prolonged and occurred during the same time of the year. The authors speculated that the change in musth pattern was due to the stimuli produced by the addition of a second bull elephant. Poole, (1987a) utilizing behavioral data from 26 free-ranging male African elephants, reported that only males 24 years and older exhibited symptoms of musth and duration of musth periods were positively correlated with male age. In a subsequent publication (Poole, 1989a) the author postulated that the sporadic nature of musth observed was due to a suppression by higher ranking bulls. Rank was reported to be determined by size. The data presented by Cooper et al., (1990) demonstrated a consistent pattern of musth behavior for 5 years in one captive male Asian elephant beginning at age 13. Data presented on 3 bull elephants by Niemuller and Liptrap (1990) demonstrated variable patterns of musth, one bull exhibited synchronous annual musth periods, one bull biannual and one bull experienced musth at completely different periods during the two year study period. Collectively, these data have produced the following question. Is the sporadic pattern of musth observed in some

animals an effect of environmental and/or social factors, individual variability or sexual maturity?

Much of the literature on musth supports the concept that environmental and/or social factors affect the pattern of musth. These factors include nutrition (Jainudeen et al., 1972a; Poole, 1987a; Cooper et al., 1990), male-male interactions (Rasmussen et al., 1984; Poole et al 1989a) and exposure to estrous females (Jainudeen et al., 1972a; Poole, 1987a; Brannian et al., 1989). There is no published evidence to support or refute the effect of other sources of stress, photoperiod and/or ambient temperature on musth.

Sexual maturity, or the attainment of adult testicular histology, was reported to occur between 7 and 18 years of age based on histological evaluation of the testes in culled African elephants (Johnson and Buss, 1967). Yet Poole, (1987a) reported that free-ranging African elephants do not experience musth until after the age of 24 and male elephants >30 years old are responsible for the majority of breeding in the wild (Moss, 1983). Puberty is the transitional period between the sexually mature and immature state. It includes growth, the appearance of secondary sexual characteristics, the attainment of fertility and psychological changes (Reiter and Grumbach, 1982). Reiter and Grumbach (1982) proposed that the reproductive endocrine changes characterizing puberty are part of a continuum extending from sexual differentiation and the ontogeny of the H-P-G system in the fetus, to fertility and sexual maturity and ultimately to senescence. Are there degrees of musth which are actually part of a continuum of physiological development? Are the variable patterns of musth reported in male elephants, static windows of experimental observation representing degree of physiological development? If so, would inhibiting or suppressing musth during this development be deleterious to normal physiological and psychological reproductive function?

If the musth pattern observed in some elephants is due to endocrine immaturity then younger animals will experience variable musth patterns even in the absence of other male elephants and animals of greater age will exhibit a less sporadic pattern. The serum

testosterone concentrations will also reflect differences in maturity, thus supporting the hypothesis. If environmental and/or social factors are exclusively responsible for the sporadic pattern of musth then male elephants being feed a consistent diet, housed in the absence of male-male interactions and having exposure to estrus females would exhibit consistent patterns of musth independent of age, thus refuting the hypothesis. If sporadic patterns of musth are observed in animals independent of age, then it is due to other factors, perhaps genetically controlled variability or other environmental factors and thus would also refute the hypothesis.

Experiments and methods of analysis. Serum, fecal and/or urine samples were obtained from 11 male elephants ranging from 4-30 years of age for 1-4 years. All males were fed a similar diet and were housed with limited access to female elephants. Testosterone concentrations in the three biological samples were measured by RIA. Behavioral and physiological data as they relate to musth were collected concurrently. Serum, fecal and urine endocrine profiles were combined with the behavioral musth profiles for each individual and presented graphically. The frequency of musth, duration of musth periods and duration of intermusth periods for the group were presented graphically. Serum data were also categorized into three groups according to the animals age. Group 1, 4-14 years old; group 2, 15-21 years old and group 3, 21-30 years old. The serum testosterone data during musth and during nonmusth were analyzed for a group affect on means using one-way ANOVA and Student Newman Keuls test (Ott, 1988). Tests were considered significant at $p \leq 0.05$.

Circulating levels of estradiol--musth and aggression

Hypothesis. Serum levels of estradiol will be highly correlated with musth and aggressive behavior in male elephants.

Rationale. The most consistent results observed in all of the previous investigations of musth are that musth is associated with aggressive behavior and increased level of circulating androgens (Jainudeen et al., 1972a; 1972b; Rasmussen et al., 1984; Hall-

Martin and Van der Walt, 1984; Poole et al., 1984; 1987; Hall-Martin, 1987; Cooper et al., 1990; Niemuller and Liptrap, 1991). In-fact, Jainudeen et al., (1972a) discussed the similarities of musth and the androgen induced aggressive behavior of rats, finding them to be striking. A direct correlation between these three characteristics, however, has never been presented. If androgens are responsible for the increase in aggressive behavior during musth, by what mechanisms are androgens mediating this aggressive behavior?

Testicular androgens are known to be involved in male sexual and aggressive behavior (Brain, 1979). Testosterone can be converted by 5-alpha reduction to dihydrotestosterone and/or by aromatization to estradiol which is an important part of androgen action in the vertebrate brain (Callard et al., 1978). Both of these metabolites have been shown to induce masculine behavior in mammals (Brain, 1979), however the relative importance of testosterone, dihydrotestosterone and estradiol at the level of the CNS requires further investigation (Crichton et al., 1991).

In the elephant, circulating levels of both androstenedione and testosterone (aromatizable androgens) as well as dihydrotestosterone (non-aromatizable androgen) are increased during musth (Rasmussen et al., 1984; Niemuller and Liptrap, 1991). Estrogen's have not been investigated. Since estradiol is known to be a mediator of masculine behavior it is reasonable to ask if circulating estradiol levels in the male elephant are correlated with musth activity. Because aromatase activity occurs in neural tissue, circulating levels may not be a true reflection of estradiol's link to aggressive behavior (Rissman, 1991). If aromatase activity is involved in mediating the behavioral manifestations of musth and serum estradiol is a true reflection of neural and/or peripheral aromatization of androgens then estradiol levels will be significantly increased during musth and will be highly correlated with aggressive behavior, thus supporting the hypothesis.

Experiment and method of data analysis. Serum samples were obtained from 8 male elephants ranging from 4-30 years of age for up to 4 years, including periods of musth and non-musth. Behavioral observations were recorded concurrently on a daily basis. The concentration of estradiol was determined in selected samples by RIA. Serum estradiol concentrations obtained during musth and non-musth were compared using Student's t-test assuming independence, normal distribution and equal variance and by the Wilcoxen rank sum test assuming non-parametric data. Estradiol vs testosterone concentrations were analyzed by simple linear regression and Pearson's correlation. Serum testosterone and estradiol were compared to the intensity of aggression and presented as a time series plot ($n=3$ animals). Significance was assumed at $p \leq 0.05$.

Pituitary luteinizing hormone stimulation of testosterone secretion and musth

Hypothesis. Serum levels of immunoreactive LH will exhibit a strong positive correlation with serum levels of testosterone independent of musth status and mean levels of LH will be greater during behavioral signs of musth.

Rationale. Only limited data on LH concentrations and patterns associated with musth in male elephants have been presented. Brannian et al., (1989), using 21 urine samples collected from 1 African male, demonstrated a significant positive correlation between urinary LH and androstenedione concentrations. Niemuller and Liptrap (1990) collected serum samples every 15 minutes for 12 hours from 2 animals during musth and 4 animals during non-musth and indicated that LH pulse amplitude, area and mean levels increased during musth. These studies suggest the pituitary's involvement in the regulation of musth, however, several questions remain. Are increases in serum testosterone consistently associated with concurrent increases in serum LH? How strongly are serum LH and testosterone coupled? At what point during the transition into and/or the transition out of musth do serum concentrations of LH change?

Studies of rut indicate that a more significant change in pituitary LH secretion occurs during the transition period than during the true rut period (Asher and Peterson, 1991).

It appears that during the rut while testosterone secretion is at its peak, LH concentrations may actually be decreasing (Suttie et al., 1984; Asher and Peterson, 1991). None of the studies reporting levels of LH in the elephant have included data during the transition period, nor have they presented data on serial sampling throughout the musth cycle. In the absence of samples collected frequent enough to describe pulsatal patterns of LH, circulating levels of LH do provide useful information on the relationship between pituitary and gonadal hormone secretion (Lincoln and Short, 1980). If testosterone secretion and musth are dependent on pituitary regulation then circulating levels of immunoreactive LH will exhibit a strong positive correlation to serum testosterone and to behavioral symptoms of musth, thus supporting the hypothesis.

Experiment and method of data analysis. Serum samples were obtained from 8 male elephants ranging from 4-30 years of age for up to 4 years, including periods of musth and non-musth. Collection frequency varied from daily to bimonthly. Behavioral observations were recorded concurrently on a daily basis. Immunoreactive LH was determined by RIA. Data for individual animals were graphed against serum testosterone and behavioral musth. Data representing the transition into musth for all animals ($n=10$ musth periods) were combined into one graph to illustrate the pattern of testosterone and LH during this period. Serum LH vs testosterone concentrations were tested for strength and significance of positive correlation by simple linear regression and Pearson's correlation. Significance was determined at $p \leq 0.05$.

Noninvasive methods to monitor musth

Hypothesis. Following hepatic, renal and intestinal metabolism, significant amounts of testosterone are excreted in its parent and/or metabolized form in the male elephant's urine and feces, excreted levels of these hormones can be extracted, measured and used to reflect the high circulating levels of testosterone indicative of musth.

Rationale. The size, strength and aggressive nature of the male elephant, makes blood collection from many individuals difficult or even impossible. Without the ability to

perform long term consistent sampling for hormonal analysis, musth can not be sufficiently studied. Furthermore, the stress of handling animals unaccustomed to the riggers of frequent blood collection is known to affect the normal temporal variations in hormone concentration (Sitarz et al., 1977). To successfully develop non-invasive endocrine techniques in the male elephant several questions need to be answered. Is testosterone or a direct metabolite of testosterone being excreted in the urine and/or feces of male Asian elephants? If testosterone or a metabolite of testosterone is being excreted in the urine and/or feces can it be quantified? Do levels of the hormone(s) reflect systemic concentrations of testosterone or musth behavior?

If testosterone is being excreted in a free or conjugated form in the urine and/or feces then following proper chemical extraction testosterone can be chromatagraphicly separated and detected by specific RIA. If testosterone can be quantified by RIA in the urine and/or feces then repeated measurements of the same sample will produce values of acceptable precision and dose response curves of the material will be parallel to standard testosterone. If urinary and/or fecal testosterone reflect circulating levels of testosterone and musth behavior then urinary and/or fecal testosterone will be positively correlated with serum testosterone and will be significantly elevated during behavioral musth. Successfully obtaining all three of these outcomes will provide adequate support for the hypothesis.

Experiments and methods of data analysis. C^{14} labeled testosterone was injected intramuscularly into 1 male Asian elephant and all feces and urine voided was collected for 6 days. Using liquid scintillation counting, the percentage and kinetics of radioactivity released in the urine and feces were calculated and presented graphically. Ratios of conjugated vs unconjugated steroids were determined by chemical extraction and hydrolysis and presented as mean $\pm$ standard error. Using high pressure liquid chromatography the metabolism of testosterone (based on the number and retention of radioactive peaks) was determined. Carbon 14 peaks were defined as the highest

consecutive value (cpm) after a rise of greater than 40% from a preceding value and tritiated peaks were defined as a 400% increase.

Fecal and urine samples collected from 8 male Asian elephants were chemically extracted for testosterone before quantifying by RIA and comparing to serum values. Fecal, urinary and serum testosterone concentrations for the entire group were tested for strength and significance of positive correlation by simple linear regression and both Pearson's and Spearman's rank order correlation. Serum vs fecal testosterone concentrations for each individual were analyzed by simple linear regression and Pearson's correlation. Fecal and urinary testosterone concentrations during musth and non-musth were analyzed for differences using Student's t-test assuming independence, normal distribution and equal variance and the Wilcoxen rank sum test assuming non-parametric data. Validation of the testosterone antiserum specificity was performed by sephadex and high pressure liquid chromatography and presented graphically. Assay quantification was determined by comparing serial diluted doses of extracted feces and urine to doses of standard testosterone. Slopes of the regression lines for each of the dose responses were compared for parallelism to the standard curve using a multiple regression equation. All tests assumed significance at $p \leq 0.05$.

MATERIAL AND METHODS

Animals, facilities and funding

The elephants used for this research were housed and cared for in 7 different zoological institutions. They include, the Fort Worth Zoological Park, Fort Worth, TX; the Tulsa Zoo and Living Museum, Tulsa, OK; the Dickerson Park Zoo, Springfield, MO; the Miami Metrozoo, Miami, FL; the Burnet Park Zoo, Syracuse, NY; the Chaffee Zoological Gardens of Fresno, Fresno, CA and the Calgary Zoo, Botanical Garden & Prehistoric Park, Calgary, Alberta, Canada. All of these institutions are members of the Association of Zoological Parks and Aquariums and contribute to the Asian elephant Species Survival Plan (SSP). The SSP is made up of zoo and university representatives having expertise in various areas of elephant management. They compile breeding, genetic and other information on the entire North American elephant population and make recommendations to the individual members. Recommendations include animal acquisition and disposition for breeding purposes, health and nutrition, research and management policies.

A proposal was sent to the elephant SSP prior to initiation of this project to obtain the necessary endorsement. Following this endorsement, the SSP assisted in acquiring the needed involvement from individual members holding male Asian elephants. Data were obtained from 11 Asian male elephants ranging from 4-30 years of age. Permits were required and therefore obtained from the United States Department of Agriculture's Animal and Plant Health Inspection Service to facilitate clearance of blood and urine samples from Canada through customs. All animals had olfactory and visual contact with female Asian elephants and were fed similar rations of hay and grain. Table 1. lists the animal identification, location, age during the sampling period and duration of data collection. Funding was obtained through the Institute of Museum Services (IMS), an

Table 1. ID., location, age and duration of data collection for experimental subjects.

Animal ID	Location	Age	Data collection
Thong-trii	Chaffee Zoo	10-12	11/89-7/90
Sammy	Fort Worth Zoo	22	1/89-10/89
Groucho	Fort Worth Zoo	19-22	7/89-6/92
Sneezy	Tulsa Zoo	17-20	3/89-6/92
Maverick	Tulsa Zoo	4-6	3/90-6/92
Onyx	Dickerson Park Zoo	24-30	6-10/86, 5/89-3/91
Kuhn-chorn	Dickerson Park Zoo	11-14	3/89-1/92
Dalip	Miami Metrozoo	23-25	5/89-3/90
Spike	Miami Metrozoo	8	6/89-3/90
Indy	Burnet Park Zoo	18-21	7/89-6/92
Bandara	Calgary Zoo	15-16	1/90-10/91

independent agency within the Executive Branch established by Act of Congress in 1976. The IMS provides support to the nations museums so that they may be better able to conserve our cultural, historic and scientific heritage. The IMS is governed by a 15 voting member presidential appointed committee called the National Museum Services Board. The nations zoological parks are considered living museums.

The Fort Worth zoo served as the principle applicant for this funding with the other institutions functioning as participants. One grant was obtained in 1988 for $25,000.00 and an additional $25,000.00 grant was obtained in 1990. The grants were awarded on a 50% matching funds basis. Matching funds were provided by in-kind contributions and were documented for each zoological institution and for Texas A&M University.

Sample collection

A sample collection protocol was sent to all participating institutions specifying that

each institution will need to develop their own method of sample collection based on personnel, facilities and animal temperament. These factors dictated how the collections were performed with safety and intra-institution method and time of collection the most important considerations. The samples that were collected included blood, urine and feces. Blood (1-10 ml) was collected from an ear or leg vein with 15 ml vacutainer tubes. Blood was allowed to sit at room temperature for 20 minutes, centrifuged and the serum decanted into storage vials. Samples were immediately labeled with the animal's ID, date and time of collection and subsequently frozen at -20^{o} C. Fecal samples (5-10 grams) were collected weekly, placed into whirl pack bags, labeled and frozen at -20^{o} C. Urine (1-10 ml) was collected midstream during urination or aspirated from the concrete floor. The samples were placed into plastic vials, labeled and frozen at -20^{o} C. Samples were shipped to Texas A&M via Federal Express packed in styrofoam containers with 5 lbs dry ice. Table 2. contains a summary of the type of samples collected and frequency of collection from each animal.

Behavioral and physiological data were recorded onto standardized forms daily by selected elephant keeper staff at each institution. These data were analyzed by a single investigator (Bert Castro, Tulsa Zoo) for a parallel study. Two parameters were selected for correlation with endocrine data obtained during this project, musth (defined as the presence of secreting temporal glands) and aggressive behavior. Aggression, which was focused on zoo personnel, the public, other elephants or inanimate objects was recorded by intensity. It included behavior such as throwing spit, rocks or other objects, hitting walls or doors with their trunk, head butting, grabbing objects with their trunk and spinning quickly while intensively staring at a potential victim.

Quantification of hormones in serum

Serum testosterone concentrations were determined by RIA using a modification of the procedures of Abraham et al., (1971). A 308 sample batch was run in 8 assays from 6-5-90 to 7-17-90 and a 281 sample batch was run in 2 assays from 10-1-92 to 10-7-92.

Table 2. Type and frequency of samples collected from each animal.

Animal ID.	Serum	Urine	Feces
Thong-trii	none	weekly	weekly
Sammy	none	weekly	weekly
Groucho	none	weekly	weekly
Sneezy	weekly	none	weekly
Maverick	periodically	none	none
Onyx	daily to weekly	weekly	weekly
Kuhn-chorn	weekly	weekly	weekly
Dalip	every 3 days	none	none
Spike	every 3 days	none	none
Indy	periodically	weekly	weekly
Bandara	weekly	weekly	none

In batch 1, serum aliquots (0.01-0.1 ml) were extracted with 4 ml anhydrous ethyl ether (Fisher Scientific, Houston, TX.) in 16 x 125 mm glass culture tubes, vortexed for 30 seconds and allowed to settle for 10 minutes at room temperature. Samples were first extracted at 0.1 ml. If concentrations were too high, the volume was progressively reduced to achieve binding between 20-85%. After the aqueous (serum) and organic (ether) layers had separated, the aqueous layer was frozen for 12 seconds in liquid nitrogen. The organic layer was immediately decanted into 16 x 125 mm glass culture tubes and placed into a 37^{o} C water bath under light (approximately 2 psi) nitrogen gas flow for 20 minutes. The samples were then reconstituted in 1 ml acetone (Fisher Scientific, Houston, TX.), vortexed for 15 seconds and two 0.4 ml aliquots were transferred from each sample into 10 x 75 mm glass culture tubes, allowing each sample to be run in duplicate. The acetone was allowed to evaporate overnight at room

temperature. The extraction recovery for this batch was 89.3 $\pm$ 6.6% based on recovery of added ^{3}H testosterone. The following day 0.1 ml of tris-gel buffer (0.05 M tris, 0.1 M NaCl, 0.1% sodium azide and 0.1% gelatin) was added to each tube and incubated at 37^{o} C for 30 minutes. Trace and anti-testosterone (0.1 ml in tris-gel buffer) were then added and the tubes incubated for 18-24 hours at 5^{o} C. The reagents used for this assay were, standard testosterone # T-1268 (Sigma, St. Louis, MO.) at 3.12- 400 pg, ^{3}H testosterone # NET-187 (New England Nuclear, Wimington, DE.) at 5000-7000 cpm/tube and rabbit direct testosterone-HSA antiserum # P1517 (Ventrex, Portland, ME.) at 53.4 $\pm$ 7.3% total binding. Following incubation, the immunoreaction was terminated and the Ab bound/free portions separated by the addition of 1 ml charcoal-dextran suspension (2.5 grams charcoal and .25 grams dextran/500 ml tris-gel buffer) and vortexed. The tubes were incubated for 15 minutes and centrifuged (840 x g) for 15 minutes at 5^{o} C, the supernatant decanted into 8 ml scintillation vials, combined with 4 ml scintillation cocktail (Packard, Downer's Grove, IL.) and counted for 5 minutes in a liquid scintillation counter (Beckman, Fullerton, CA.) on channel 1, 0-400 UL . Counts per minute (cpm) were fed into a 386 IBM compatible computer using data capture software (Beckman, Fullerton, CA) and the data were calculated using Lotus-123 for Windows software on custom made spreadsheets. The testosterone antiserum was listed as having cross-reactivity with dihydrotestosterone 5.16%, 19-nortestosterone 17.8%, 17alpha-methyltestosterone 1.07% and SHBG 0%. Further testing revealed cross-reactivities as follows; dihydrotestosterone 50%, androstenedione 1%, androstanedione 0.6%, estradiol 0.16%, 5alpha-androstane-3beta,17beta-diol 0.08% and testosterone-glucuronide, androsterone, etiocholan-3alpha-ol-17-one and 11beta-hydroxy-androsterone less than 0.01%. The intra and inter-assay coefficients of variation were 2.1 and 11.7% respectively. Sensitivity at 90% binding was 21.2 $\pm$ 2.2 pg and blank values were 15.7 $\pm$ 2.1%.

In batch 2, the following changes were made. Serum aliquots (0.1 or 0.05 ml) were extracted, then after adding 1 ml acetone to ether evaporated extracts, 0.5 and 0.04 ml acetone were transferred to 10 x 75 mm glass culture tubes from each sample. This allowed a greater range of concentrations to be detected for each extraction and eliminated the need to re-extract samples at different volumes. In addition, ^{3}H testosterone was purified before dilution in assay buffer (protocol compliments of Dr. Roy Butcher, West Virginia University) A sephadex LH-20 column was set-up using 0.8 grams sephadex (Sigma, St Louis, MO.) in a 5ml glass pipette. Glass wool was placed into the tip of the glass pipette, then the sephadex, dissolved in 5 ml elute (methylene chloride:methanol, 95:5) was placed on top of the glass wool and allowed to settle. Approximately, 20 uCi (0.02 ml) tritiated testosterone were loaded on top of the sephadex and allowed to enter. The ^{3}H testosterone was then eluted with 3ml of elute, discarding the first 1 ml and collecting the last 2 ml. The 2 ml fraction was subsequently evaporated under nitrogen gas (2 psi) and reconstituted in buffer to give a total count of 5000-7000 cpm. This procedure lowers the counts in the non-specific binding (charcoal) tubes as well as results in a more accurate estimate of extraction recovery. Extraction recovery of added ^{3}H testosterone for this batch was 97 $\pm$ 1%. The intra- and inter-assay coefficients of variation, respectively were 3.1 and 8.0% at 17-21% binding and 6.0 and 12.7% at 56-64 % binding. Sensitivity at 90% binding was 8.1 $\pm$ 2 pg and buffer blank values were 6.24 $\pm$ 1.02 pg. The inter-batch coefficient of variation for the entire testosterone assay was 14.5%.

Validation of the serum testosterone assay was performed by demonstrating dose response (0.1-0.0025ml) parallelism of a male elephant serum pool to standard testosterone diluted in assay buffer. The slopes of the two regression lines were non-different using a multiple regression equation ($F=.33$, alpha$=.05$, $df_1=1$, $df_2=4$).

Random samples from non-musth animals and serum, volume permitting samples from musth animals were used for determination of estradiol ($n=171$ samples). The

estradiol concentrations were determined by RIA in one assay using procedures similar to the testosterone RIA described above. Serum aliquots (1 ml) were extracted twice with 4 ml anhydrous ethyl ether, the aqueous layer separated by freezing in liquid nitrogen for 15 seconds and evaporated under nitrogen gas (2 psi) in a 30° C. water bath for 35 minutes. Each sample was then reconstituted in 0.5 ml acetone, vortexed and 0.43 ml transferred to a single 10 x 75 mm glass culture tube. When serum volume permitted, each sample was extracted in duplicate. The acetone was allowed to evaporate overnight at room temperature and the next morning the assay was completed as described for the testosterone assay. The reagents used for the estradiol assay were, estradiol standard # E-1132 (Sigma, St Louis, MO.) at 1.56-200 pg, ^{3}H estradiol # 07-138026 (ICN Biomedicals, Horsham, PA.) at 6000-7000 cpm/tube and rabbit 17beta-estradiol-3-BSA antiserum #p3642 (Ventrex, Portland, ME.) at 24% total binding. Cross-reactivity data listed for this antiserum were estrone 0.42%, estriol 0.11%, ethinylestradiol 0.0075%, dihydrotestosterone 0.055%, testosterone 0.0021% and both progesterone and cortisol 0.00042%. Extraction recovery of added ^{3}H estradiol for this assay was 96%. The intra-assay coefficients of variation, respectively were 12.4% at 68% binding and 7.5% at 78% binding (n=6). Sensitivity at 90% binding was 3.84 pg and buffer blank values were 2.76 $\pm$ 1.13 pg.

Validation of the estradiol assay was performed by extracting 13, 1ml aliquots of a male elephant serum pool. The extracts were combined in one tube and extracted with a mixture of 2 ml hexane and 1 ml 50% methanol in distilled water. The hexane layer was discarded and the remaining solvent evaporated at 35° C This fraction was then reconstituted in 2 ml acetone and serial diluted 5 times. Acetone (0.85 ml) from each of the 5 tubes were then transferred to 10 x 75 mm glass culture tubes, allowed to dry overnight and brought to assay the following day. This dose response was then compared for parallelism to standard curves diluted in charcoal striped male elephant serum (25 ml serum pool combined with 1 g of charcoal, mixed for 20 minutes and centrifuged for 40

minutes). The slopes of the two regression lines were non-different using a multiple regression equation ($F=0.25$, alpha$=0.05$, $df_1=1$, $df_2=4$).

Concentrations of Luteinizing hormone (LH) were determined by a RIA modified from the methods of Bolt (personal communication) and Forrest et al., (1980). This assay utilized a rabbit anti ovine LH antiserum (GDN #15) and a bovine LH preparation (NIH-LH-B10) for standards. Bovine LH (obtained from Dr. D.J. Bolt, USDA, Beltsville, MD) was radioiodinated by combining a 10 microgram hormone-buffer (0.5 M sodium phosphate) mixture with 0.5 mCi iodine-125 (Amersham, Arlington Heights, IL.) in a 1 ug IODO-GEN (Pierce) plated tube. This reaction mixture was then transferred to a 2.5 g AG 2-X8 (Bio-Rad) anion exchange resin column to separate free iodine-125 from LH bound iodine-125.

This bovine LH assay was validated for quantification of elephant LH by comparing the responses of a serial diluted elephant serum pool to serial dilution of a bovine serum pool and to the standard curve. The slopes of the regression lines were non-different using a multiple regression equation ($F=0.38$, alpha$=0.05$, $df_1=1$, $df_2=4$).

Unknown serum aliquots (0.4 ml) and standards (0.05-20 ng) were diluted to a volume of .5 ml with EW-PBS (0.1% egg white in PBS, pH 7), and combined with 0.2 ml anti-ovine LH (1:40,000; 27% total binding) vortexed and incubated at 4^{o} C for 24 hours. After incubation, 0.1 ml iodine-125 labeled bovine LH (20,000-25,000 cpm) was added to all tubes, vortexed and incubated for 24 hours at 4^{o} C. On day 3, 0.2 ml of sheep anti-rabbit gamma globulin (1:40 dilution with 10% polyethylene glycol-PBS) was added to precipitate the bound antigen, vortexed and incubated for 24 hours at 4^{o} C. On day 4, 1.5 ml ice cold PBS was added and the tubes were centrifuged at 2,000 x g for 40 minutes at 4^{o} C. The supernatant was drained from the tubes for 30 minutes and the radioactivity counted for 2 min/tube with a Micromedic MACC Assay Compucenter. A total of 565 duplicate serum samples were run in one assay. The intra-assay coefficient of variation of a diluted high LH elephant pool was 7.4% at 38% binding and 10% at

56% binding. A low LH elephant serum pool produced a 20% coefficient of variation. The sensitivity at 90% binding was 0.06 ng.

Quantification of testosterone in urine

A 295 sample batch of urine was first assayed for creatinine using the method of Taussky, (1954). Urine (0.05 ml) was added to 16 x 125 mm glass culture tubes, combined with 2.5 ml distilled water, 1 ml 0.75 N NaOH and 1 ml 0.04 N picric acid (Ricca chemical, Arlington, TX.) then vortexed. After 15 minutes the absorbance (515 nm) was determined using a linear absorbance spectrophotometer. The spectrophotometer was first calibrated to 0% absorbance using the same volume of reagents without urine. Control and standard tubes were also assayed using 0.05 ml pooled male elephant urine and 0.05 ml of a 1 mg/ml creatinine standard solution (Fisher Scientific, Houston, TX.) respectively. Absorbance values obtained for the unknown urine samples and controls were divided by the absorbance of creatinine standard to obtain mg/ml creatinine. The inter-assay coefficient of variation for 6 assays was 17.5%. Urine samples obtained from two of the animals from the Fort Worth Zoo were much more dilute then the others and were subsequently rerun at a volume of 0.2 ml.

For the urinary testosterone RIA, only the sample preparation and extraction procedures differed from that of the serum testosterone assay. The assay reagents for urinary testosterone were identical to those described above, however, the antiserum was diluted to provide 32.5 $\pm$ 3.4% total binding.

Experimentation was first performed to determine enzyme hydrolysis efficiency and the relative amounts of conjugated vs unconjugated immunoreactive testosterone in male elephant urine. Aliquots of a male elephant urine pool (0.1 and 0.01 ml) and 2 ng testosterone-glucuronide standard (Sigma, St Louis, MO.) was diluted to a volume of 0.2 ml with 0.05 M sodium citrate buffer (pH 5) in 16 x 125 mm glass culture tubes. These tubes were subjected to 3 treatments, holding at 5^{o} C for 24 hours, holding at 37^{o} C for 24 hours or combining with 0.01 ml beta-glucuronidase-arylsulfatase (Sigma, St Louis,

MO. 1000 ug/hr glucuronidase and 10 umole/hr sulfatase activity) and incubation at 37° C for 24 hours. Decreasing doses (0.1-0.01 ml) from each tube was then extracted with 4 ml ethyl ether and dried under nitrogen gas (2psi) at 37° C. The tubes were reconstituted with 1 ml acetone, 0.4 ml portions transferred to 10 x 75 mm tubes and kept at room temperature overnight. After the acetone evaporated, the tubes were brought to assay as described for the serum testosterone assay above. Validation of the urine testosterone assay was demonstrated during these experiments by obtaining parallelism between standard testosterone and the dose response of hydrolyzed extracted elephant urine.

The unknown urine samples were assayed by first combining 0.01 ml urine with 0.01 ml beta-glucuronidase-arylsulfatase (1000 ug/hr glucuronidase and 10 umole/hr sulfatase activity) with 0.18 ml 0.05 M sodium citrate buffer (pH 5) in 16 x 125 mm glass culture tubes and incubating at 37° C for 24 hours. The entire sample was then extracted with 4 ml ethyl ether, evaporated under nitrogen gas (2psi) at 37° C and reconstituted in 1 ml acetone. Acetone (0.09, 0.1 or 0.25 ml) was then transferred to 10 x 75 mm tubes in duplicate, allowed to evaporate overnight at room temperature and subsequently assayed for testosterone as described. A total of 295 urine samples were assayed in 4 assays. The intra and inter-assay coefficients of variation were 3.9% and 17.6%, respectively. Extraction recovery was 92.8 $\pm$ 2.2%, sensitivity at 90% binding was 8.23 $\pm$ 1.63 pg and blank values were 10.55 $\pm$ 1.2 pg.

Quantification of testosterone in feces

To determine if testosterone immunoreactivity was present in elephant feces, 2 experiments were undertaken. In experiment 1, fecal samples (1 g wet weight) were extracted with a mixture of 1 ml distilled water, 4 ml ethyl ether and 4 ml methanol in 16 x 125 mm glass culture tubes, vortexed for 3 minutes and centrifuged (800 x g) for 10 minutes. One ml of this extract was then transferred to a 16 x 125 mm glass culture tube and evaporated under nitrogen gas (2psi) at 45° C for 45 minutes. This extract was

then reconstituted in 1 ml acetone, vortexed and 0.4 ml volumes transferred to 10 x 75 mm glass culture tubes in duplicate. The tubes were allowed to evaporate overnight at room temperature and were then assayed for testosterone as previously described for the serum and urinary testosterone assay.

In experiment 2, 0.8 grams of lyophilized feces were extracted as described in experiment no. 1. After the solvent extract had evaporated, approximately 1500 cpm ^{3}H testosterone were added and the sample was reconstituted in 1ml benzene:methanol (85:15). This extract was then loaded onto a 3.2 gram sephadex LH-20 (Sigma, St Louis, MO.) column and eluted with 30 ml benzene:methanol (85:15). Thirty, 1ml fractions were collected into 16 x 125 mm glass culture tubes and 0.5 ml from each fraction were immediately transferred to corresponding 10 x 75 mm glass culture tubes. All tubes were allowed to dry overnight at room temperature. Recovery tubes (original 16 x 125 mm tubes) were reconstituted in 1.3 ml tris-gel buffer, incubated for 30 minutes at 37^{o} C combined with 4 ml scintillation cocktail (Packard, Downer's Grove, IL.) in 8ml vials and counted for radioactivity as previously described. The assay tubes (10 x 75 mm tubes) were assayed for testosterone as previously described.

Experimentation was then undertaken to improve the extraction method. Tritiated testosterone (1700 cpm) was added to 0.2 gram portions of dry feces (149^{o} C for 72 hours) and solubilized in 4 replicates of 5 different solutions (8 ml), Tris-gel assay buffer, 0.05 M sodium citrate pH 5, 0.05 M sodium citrate pH 4, absolute ethanol and methanol. Solubilization was performed in 16 x 125 mm glass culture tubes with Teflon coated screw caps (Baxter, Houston, TX.) on a mechanical mixer for 24 hours. After solubilization the samples were centrifuged (800 x g) for 10 minutes and 1 ml aliquots transferred to 8 ml scintillation vials and counted for recovery as previously described.

Absolute ethanol was chosen for further experimentation. Wet feces (0.5 g) was combined with 9000 cpm ^{3}H testosterone in 8 ml absolute ethanol and solubilized on a mechanical mixer for 24 hours. Samples were then centrifuged (800 x g) for 10 minutes,

the ethanol poured off into corresponding 16 x 125 mm glass culture tubes and the residual feces placed under a fume hood to dry. Three, 0.5 ml ethanol aliquots were taken from each sample. One aliquot was combined with 4 ml scintillation cocktail and counted. Another aliquot was transferred to 16 x 125 mm glass culture tubes, evaporated under nitrogen gas (2psi) at 45° C and reconstituted in 0.5 ml tris-gel buffer. After incubating at 37° C for 30 minutes and combining with 4 ml scintillation cocktail in 8 ml scintillation vials the recovery of radioactivity was determined as previously described. The final aliquot was transferred to 16 x 125 mm glass culture tubes, evaporated under nitrogen gas (2psi) at 45° C and reconstituted in 1 ml acetone. Duplicate portions (0.4 ml) were transferred to 10 x 75 mm glass culture tubes and subsequently assayed for testosterone as previously described. The residual feces were oven dried, weighed and used for correction of testosterone values for dry weight.

The effect of lyophilizing fecal samples before assay was evaluated by solubilizing duplicate wet (0.5 g) and dry (0.1 g) samples from two animals and assaying for testosterone as described above. The fecal samples were lyophilized by freezing in liquid nitrogen for 30 seconds and placing them on a Dura dry TM microprocessor control condenser module for 6-7 hours. Testosterone concentrations from wet and dry samples were evaluated for positive correlation.

A total of 372 fecal samples from 7 animals were assayed for testosterone in 9 assays utilizing the following methods. Wet feces (0.5 g) and 8 ml absolute ethanol were combined into 16 x 125 mm glass culture tubes with Teflon coated screw caps and solubilized on a mechanical mixer for 18-24 hours. After solubilization the tubes were centrifuged (800 x g) for 10 minutes and the ethanol poured of into corresponding 16 x 125 mm glass culture tubes. The ethanol portions were vortexed, 0.5 ml transferred to another 16 x 125 mm glass culture tube and evaporated under nitrogen gas (2psi) for 10 minutes at 45° C. The extract was then reconstituted in 1 ml acetone, vortexed and 0.4 ml duplicate portions of acetone transferred to 10 x 75 mm glass culture tubes. After the

10 x 75 mm glass culture tubes had evaporated overnight at room temperature they were assayed for testosterone as described for the serum and urinary testosterone assay. The intra and inter-assay coefficients of variation were 3.69 $\pm$ 2.7% and 20%, respectively. Extraction recovery was 51.3 $\pm$ 5.61%, sensitivity at 90% binding was 7.91 $\pm$ 1.27 pg and blank values were 6.4 $\pm$ 1.25 pg. The Ab titer was run at 32.8 $\pm$ 6.85% total binding.

Quantitative validation of the fecal testosterone assay was performed by solubilizing 0.5 grams of wet feces in 8 ml absolute ethanol on a mechanical mixer for 24 hours. After solubilization the sample was centrifuged (800 x g) for 10 minutes and 2.5 ml of the ethanol transferred to a corresponding 16 x 125 mm glass culture tube. The ethanol was evaporated under nitrogen gas (2psi) for 30 minutes at 45^{o} C and subsequently reconstituted in 2.5 ml acetone. Decreasing amounts (1-0.01 ml) of acetone were transferred to 10 x 75 mm glass culture tubes and allowed to evaporate at room temperature. After evaporation the tubes were assayed for testosterone as described and evaluated for parallelism to the standard curve.

Co-chromatography of fecal and urinary extracts

To determine the antiserum specificity to testosterone and other potential immunoreactive substances present in extracted elephant urine and feces, HPLC co-chromatography was performed. The initial experiments utilized a single solvent metering pump (110a Beckman, Fullerton, CA.) and an isocratic solvent system of methanol and water, 69:31 (Sunde and Lundmo, 1982) at 1 ml/minute solvent flow. The separation was performed on a 5 um fully encapped octadecylsilane reverse phase (250 x 4.6 mm inner diameter, Regis Chemical, Morton Grove, IL.) column and 0.5 ml fractions were collected into 8 ml scintillation vials using a LC 200 fraction collector (Buchler instruments). The column was equilibrated to the mobile phase for 40 minutes at 1 ml/minute prior to injection of the sample. A fecal sample was prepared for injection as follows: 0.5 g wet feces were solubilized in absolute ethanol as previously

described, 2 ml of this extract was then evaporated under nitrogen gas at 45° C for 40 minutes. The extract was reconstituted in 0.2 ml methanol vortexed for 5 minutes and loaded into a C-18 Sep-pak (Waters Associates, Milford, MA.) previously prepared by washing with 10 ml methanol. The fecal steroids were eluted from the Sep-pak with 4 ml methanol, combined with 1000 cpm ^{3}H testosterone and evaporated under nitrogen gas (2psi) at 45° C for 40 minutes. This extract was reconstituted in 0.05 ml methanol and 0.03 ml loaded into the HPLC using a model 7125 (Rheodyne, Cotati, CA.) sample injector with a 0.02 ml sample loop. After injection, 41, 0.5 ml fractions were collected. Aliquots (0.2 ml) from each fraction were immediately transferred to 10 x 75 mm glass culture tubes and placed at room temperature to evaporate. This portion was subsequently assayed for testosterone as previously described. The remaining portion of each fraction was combined with 4 ml scintillation cocktail (Packard, Downer's Grove, IL.) and counted for radioactivity as previously described.

The HPLC system was then further optimized for use of a gradient system. The solvent delivery system (Beckman, Fullerton, CA.) consisted of two Beckman 110a pumps and a 420 gradient system controller. Detection was performed on a UV detector with a 8 ul analytical optical unit at 280 nm (Beckman, Fullerton, CA.) and recording was done on a plotting/recording integrator 3390a (Hewlett Packard, Avondale, PA.). The same column described above was used for reversed phase separation. The gradient system (van der Hoeven, 1984) consisted of two solvents. Solvent A contained 4.8% tetrahydrofuran in distilled water and solvent B contained 4.8% tetrahydrofuran in methanol. The gradient flow was 0.8 ml/minute starting at 40% B from time 0-5 minutes and followed a linear gradient to 75% B from time 5-35 minutes. The column was held at 75% B for 5 minutes before re-equilibrating to 40% B for subsequent injections. A system chromatogram was constructed using a 0.5 mg/ml solution of testosterone, androstenedione, androstanedione, dihydrotestosterone and 0.05 mg/ml estradiol in ethanol. This solution was injected into the HPLC using a 0.02 ml sample loop before

each validation to monitor the consistency of the HPLC system. Prior to injection of the fecal or urine sample, the column flow was diverted to a Frac-200 fraction collector (Pharmacia, Piscataway, NJ.) and fractions collected into 8 ml scintillation vials.

Representative ethanol fecal extracts (solubilized as previously described) from all animals were combined to form a fecal pool. Two replicates utilizing 1.5 ml portions from this pool were evaporated under nitrogen gas (2psi) at 45° C for 45 minutes, reconstituted in 0.02 ml methanol and eluted through a C-18 Sep-pak cartridge with 4 ml methanol. Approximately 1000 cpm ^{3}H testosterone were added to each sample and then both samples were evaporated under nitrogen gas (2psi) at 45° C for 45 minutes. Each sample was then reconstituted with 0.05 ml methanol and consecutively injected into the HPLC gradient system described above. One ml fractions were collected for the first 15 minutes and 0.5 ml fractions for an additional 30 minutes. Aliquots (0.2 ml) from each fraction were immediately transferred to 10 x 75 mm glass culture tubes and placed at room temperature to evaporate. This portion was subsequently assayed for testosterone as previously described. The remaining portion of each fraction was combined with 4 ml scintillation cocktail (Packard, Downer's Grove, IL.) and counted for radioactivity as previously described.

Two aliquots of a urine pool (0.01 ml), representing all animals, were combined with 0.18 ml 0.05 M sodium citrate buffer (pH 5) and 0.01 ml beta-glucuronidase-arylsulfatase (1000 ug/hr glucuronidase and 10 umole/hr sulfatase activity) and incubated for 18 hours at 37° C. Approximately 2000 cpm ^{3}H testosterone were added at this time and each replicate was extracted with 4 ml ethyl ether. The replicates were then reconstituted in 0.06 ml methanol and 0.03 ml from each replicate injected and chromatographicly processed as described for the fecal co-chromatography.

In-vivo metabolism of Carbon 14 testosterone

An ethanol solution (4 ml) containing 233 uCi ^{14}C testosterone, 55.4 mCi/mmol (New England Nuclear, Wilmington, DE.) and 7.5 mg standard testosterone (Sigma, St

Louis, MO.) was loaded into a 5 cc dart and brought to a volume of 5 ml with bacteriostatic water. The purity of the ^{14}C testosterone was first determined by HPLC using the gradient system of van der Hoeven (1984), and collecting fractions and detecting radioactivity in fractions by liquid scintillation counting as previously described. One Asian male elephant, (Groucho) was darted with this mixture and maintained in two 30 x 40 ft concrete stalls for 6 days. Drains were modified with 4 inch diameter PVC pipe to collect all urine. The animal was shifted between the two stalls 3 times/day allowing access to the feces and urine for collection. Feces was shoveled into 50 gallon plastic garbage containers, weighed and approximately 20 kg of feces collected into plastic baggies. Each collection of 20 kg was made up of several random samples of the dung piles present. Urine was mixed, measured and 200-300 ml collected into glass bottles. Urine collected from the drain and from the floor was collected and analyzed separately. Fecal and urine samples were collected as described after each time the animal was shifted for 6 days and frozen at -20^{o} C.

Analysis of the radioactivity in urine was performed by combining triplicate 1 ml urine portions from each collection time and location (floor or drain) with 4 ml scintillation cocktail (Packard, Downer's Grove, IL.) in 8 ml scintillation vials. The vials were allowed to sit for 2 hours to eliminate chemeluminecense and subsequently counted on an LS 1801 liquid scintillation counter at 0-1000 UL. For detection of radioactivity in feces, two fecal samples (1 g) from each collection were solubilized in 8 ml absolute ethanol for 24 hours on a mechanical mixer. One ml from each ethanol extract was then combined with 4 ml scintillation cocktail and counted as for the urine. This procedure was replicated to determine consistency. Extraction recovery was monitored by using identical methods on a non-radioactive fecal ethanol extract after adding 1000-2000 cpm ^{14}C testosterone. The recovery was determined to be 72% and 91% for the first and second replicate, respectively. In addition, a second solubilization was performed on the same fecal portions after removing the ethanol (n=20), to further

evaluate the efficiency of the ethanol solubilization procedure. Data reported as cpm on the liquid scintillation counter were converted to disintegrations per minute (dpm) by first constructing quench curves from fecal and urine material combined with ^{14}C testosterone. Urine and ethanol fecal extracts were serial diluted 8 times with water and absolute ethanol, respectively. Duplicate 1 ml portions of each dilution were transferred to 8 ml scintillation vials and combined with 400,000 cpm ^{14}C testosterone and 4 ml scintillation cocktail. All vials were then counted at 0-1000 UL as described. Each dilution produced a cpm value and a corresponding H#. The cpm obtained for each dilution were divided by the total cpm of ^{14}C added, thus determining the counting efficiency. The H# was graphed against counting efficiency producing a quench curve. The dpm of each unknown sample were then calculated using the following formula, dpm = cpm/counting efficiency. The dpm were then converted to uCi activity based on the formula 1 uCi = 2,220,000 dpm. A Lotus 123 spreadsheet was designed to calculate uCi recovered per collection utilizing the cpm and H# data produced by the liquid scintillation counter as well as the quench curves and the two formulas described above.

To determine the relative percentages of activity present in urine and feces in the conjugated vs unconjugated form, the following experiments were performed. A urine pool made up of the highest activity samples was pooled and counted for radioactivity. One ml aliquots (n=8) of this pool, placed in 16 x 125 mm glass culture tubes, were extracted with 8 ml ethyl ether and the organic and aqueous fractions separated by freezing in liquid nitrogen for 15 seconds. The aqueous phase from each extract was combined with 4 ml scintillation cocktail in 8 ml vials and counted for radioactivity. This aqueous phase represents conjugated steroids. The organic phase was evaporated, reconstituted with 1 ml assay buffer, vortexed and counted for radioactivity. Extraction recovery determined by extracting urine samples combined with 6000-7000 cpm ^{14}C testosterone was 92.1%. To determine enzyme hydrolysis efficiency, 0.25 ml portions of urine (n=16) were combined with 0.25 ml 0.05 M sodium citrate buffer and 0.01 ml

beta-glucuronidase-arylsulfatase (1000 ug/hr glucuronidase and 10 umole/hr sulfatase activity) and subsequently incubated at 37° C for 18 hours. The samples were then extracted 2x with 4 ml ethyl ether and the organic and aqueous fractions separated by freezing in liquid nitrogen for 15 seconds. The aqueous portions were combined to give 4 replicates of 1 ml urine and counted for residual radioactivity. This residual radioactivity represents conjugated steroids resistant to enzyme hydrolysis. Procedural recovery was monitored in duplicate tubes of urine with 7000-8000 cpm added ^{14}C testosterone and was determined to be 96%.

Solvolysis, as reported by Hindle and Hodges, (1990) was also employed to liberate the steroids from their conjugated form. A urine pool were developed containing samples of highest radioactivity and 1 ml portions were counted for radioactivity. Fifty ml of this urine pool were then extracted with 300 ml of ethyl ether and the organic layer was separated and evaporated under nitrogen gas at 37° C. This extract was then reconstituted in 50 ml ethanol and 3, 1ml portions counted for radioactivity. This portion represents free steroids. The extraction efficiency was determined on a separate portion of control urine with added ^{14}C testosterone and yielded 82%. The aqueous portion of the extract was combined with 50 ml phosphate buffered saline (5.38 g monobasic $NaPO_4$, 14.2 g dibasic $NaPO_4$, 9 g NaCL, and 1 g Na azide in 1 liter distilled water), 50 ml saturated NaCL (85.7 ml water, QS to 100 ml with NaCL), 25 ml 5N-sulfuric acid and 200 ml ethyl acetate and incubated at 37° C. After incubation the ethyl acetate was removed and the aqueous phase re-extracted with 200 ml fresh ethyl acetate. The two ethyl acetate fractions were combined, washed with 100 ml distilled water and evaporated under nitrogen gas (2psi) at 50° C for 30 minutes. This extract was then reconstituted in 50 ml ethanol and 3, 1ml portions counted for radioactivity. This portion represents conjugated steroids. Three, 1ml portions of the aqueous phase were counted for radioactivity and represent residual steroids not hydrolyzed by the solvolysis method. Two replicates of the solvolysis procedure were performed.

For determination of the conjugated/unconjugated steroid ratio in feces, 1 ml aliquots of ethanol (n=20), obtained after solubilization of high radioactivity feces, were combined with 0.5 ml distilled water and evaporated to a volume of 0.5 ml under nitrogen gas at 45° C for 40 minutes. The remaining portion was extracted with 4 ml ethyl ether and the organic and aqueous fractions separated by freezing in liquid nitrogen for 15 seconds. The aqueous fractions were then counted for radioactivity and represent non-extractable steroid conjugates. Activity reported as cpm was converted to dpm and background radioactivity was subtracted as previously described. Extraction recovery was monitored by identical methods using a non-radioactive fecal control and adding 7000-8000 cpm ^{14}C testosterone and yielded 95%.

Fecal and urine material corresponding to the time of greatest activity excretion was analyzed by HPLC. The HPLC system used for these experiments is the same gradient system described for the urine and fecal co-chromatography experiments above. Urine samples, after ether extraction (free steroids) and after hydrolysis and ether extraction (total steroids) as reported above for conjugated/unconjugated ratio determination, were prepared for HPLC analysis similar to the methods of Hindle and Hodges, (1990). Evaporated extracts were reconstituted in 0.2 ml methanol and eluted through a C-18 Sep-pak with 4 ml methanol. Approximately 4000 cpm ^{3}H testosterone were added at this time and the sample was evaporated under nitrogen gas (2psi) at 45° C for 40 minutes. This extract was reconstituted in 0.05 ml methanol, injected into the HPLC and 72, 0.5 ml fractions collected as previously described. The fractions were immediately combined with 4 ml scintillation cocktail and counted on the LS-1801 liquid scintillation counter using a dual label program. Each fraction was counted in channel 1, 0-400 UL (tritium energy window) and channel 2, 400-1000 UL (carbon 14 energy window). HPLC of urinary free steroids was performed twice and total urinary steroids 3 times.

Fecal ethanol extracts were evaporated under nitrogen gas (2psi) at 45° C for 40 minutes, reconstituted in 0.2 ml methanol and eluted through a C-18 Sep-pak with 4 ml

methanol. Approximately 4000 cpm ^{3}H testosterone were added at this time and the sample was evaporated under nitrogen gas (2psi) at 45^{o} C for 40 minutes. Chromatography and radioactivity counting were performed as described above for urine extracts. The HPLC analysis of fecal steroids was performed 4 times.

RESULTS

Temporal patterns of musth--age and environmental factors

Testosterone concentrations analyzed by one-way ANOVA revealed that there are significant differences between the 5 groups analyzed. Concentrations listed as means ± s.d. for each group are depicted in table 3. Newman-Keuls multiple comparisons test

Table 3. Concentrations of serum testosterone for age and musth status.

Group 1[a]	Group 2[b]	Group 3[c]	Group 4[c]	Group 5[c]
12 ± 11ng/ml	25 ± 19 ng/ml	2.6± 2.9ng/ml	4.3± 5.6ng/ml	3.6± 6.5ng/ml

groups with different superscript are significantly different.
group 1=musth, 15-20 yrs; group 2=musth, 21-30 yrs; group 3=non-musth, 6-14 yrs; group 4=non-musth, 15-20 yrs; group 5=non-musth, 21-30 yrs.

indicated that musth values were significantly greater than non-musth values, musth values for the 21-30 year old group were significantly greater than musth values for the 15-20 year old group and non-musth values for the three groups were not significantly different. Although the non-musth values for the 3 groups were not significantly different, there was a greater amount of variation about the mean in the groups as they represented increases in age.

Figure 1. represents the frequency of musth, duration of musth periods and duration of intermusth periods for the group based on the occurrence of temporal gland drainage. A clear indication of a changing pattern of musth with increasing age is not discernible. Although it does seem that overall, older animals are in musth more often, younger and older animals alike display a sporadic incidence of musth based on temporal gland secretion. During a 973 day period extending from 7-1-89 to 3-1-92, behavioral and physiological data were collected consistently from 4 animals. For these four animals,

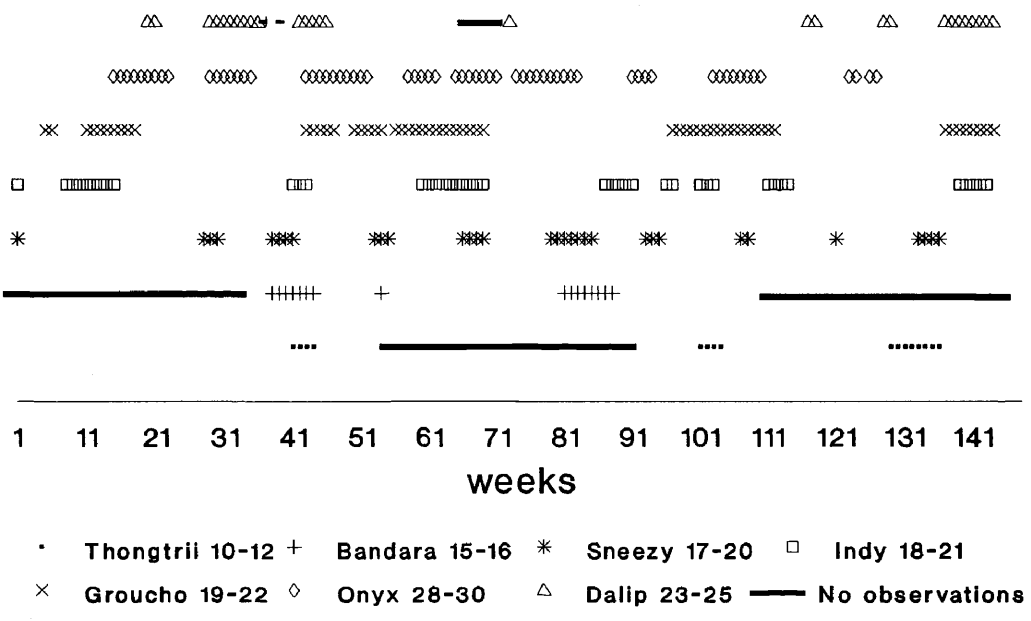

Figure 1. Frequency of musth, duration of musth periods and duration of intermusth periods for captive Asian elephants. Each individual symbol represents a week in which temporal gland secretion was observed. The key illustrates the symbol corresponding to the individual animal. The numbers represent the animal's age during the collection period. Although older animals are in musth more often, younger and older animals alike display a sporadic incidence of musth.

the total number of days in musth increased with age; Sneezy, 17-20 yrs, 208 days; Indy, 18-21 yrs, 248 days; Groucho, 19-22 yrs, 318 days and Onyx, 28-30 yrs, 446 days. The oldest bull was in musth 46% of the entire time period.

The individual time series graphs of serum testosterone plotted against the incidence of musth are depicted in figures 2-9. These graphs demonstrate that testosterone levels begin to rise before physiological signs are evident. They also demonstrate that increased levels of testosterone are not always associated with temporal gland secretion. A trend towards higher peak concentrations of testosterone as the animal increases in age can be noted by observing the Y-axis on individual graphs. In figure 10, the degree of temporal gland secretion is plotted against the daily testosterone concentrations through an entire musth period for one bull. As the bull progressed through this musth period, the peak concentrations of testosterone increased then decreased as did the degree of temporal gland secretion. A considerable amount of daily variation in musth serum testosterone concentration was observed in this animal with values ranging from 1 to 59 ng/ml.

Circulating levels of estradiol--musth and aggression

Serum concentrations of estradiol during musth (19.5 $\pm$ 10.7 pg/ml) were significantly greater than during non-musth (8.7 $\pm$ 3.2 pg/ml). Detectable concentrations of estradiol ranged from 3.94 to 51.97 pg/ml. A significant positive linear relationship was detected between serum testosterone and serum estradiol concentrations with a Pearson's correlation coefficient of 0.91. Figures 11-13 demonstrate the relationship between serum estradiol, testosterone, aggressive behavior and musth. A higher intensity of aggression is shown to be associated with higher testosterone and estradiol concentrations although individual variation is evident. The association is much more evident in figure 13 than in figures 11 and 12. A plot of estradiol concentration against degree of temporal gland secretion reveals less of an association than with testosterone and degree of temporal gland secretion (figure 14).

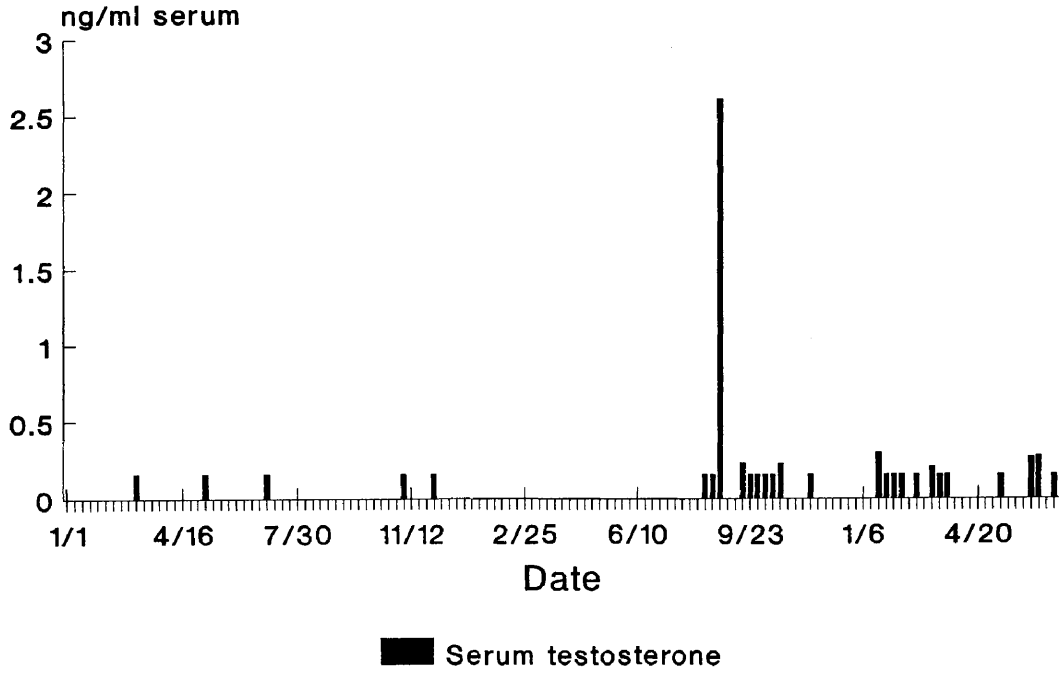

Figure 2. Serum concentration of testosterone for animal "Maverick". Serum samples were collected periodically over a 130 week period. Animal's age 4-6 years. Missing bars represent dates in which serum was not collected. Serum concentrations of testosterone remained low or non-detectable.

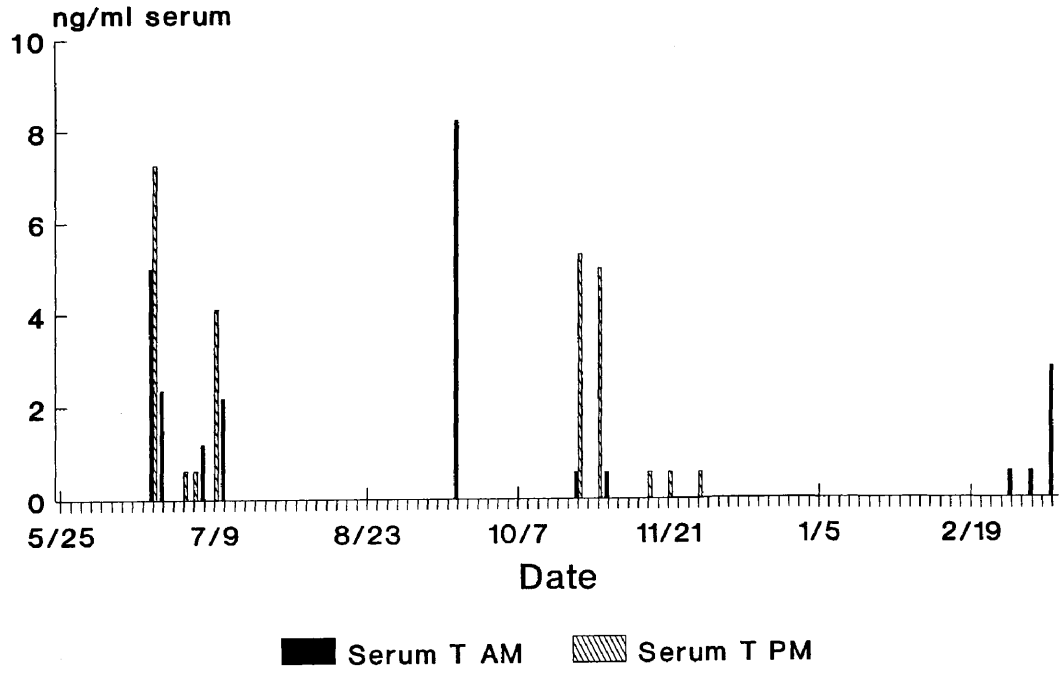

Figure 3. Serum concentrations of testosterone for animal "Spike". Serum samples were collected periodically over a 98 week period. Animal's age 8-9 years. Missing bars represent dates in which serum was not collected. Serum concentrations of testosterone approached 8 ng/ml.

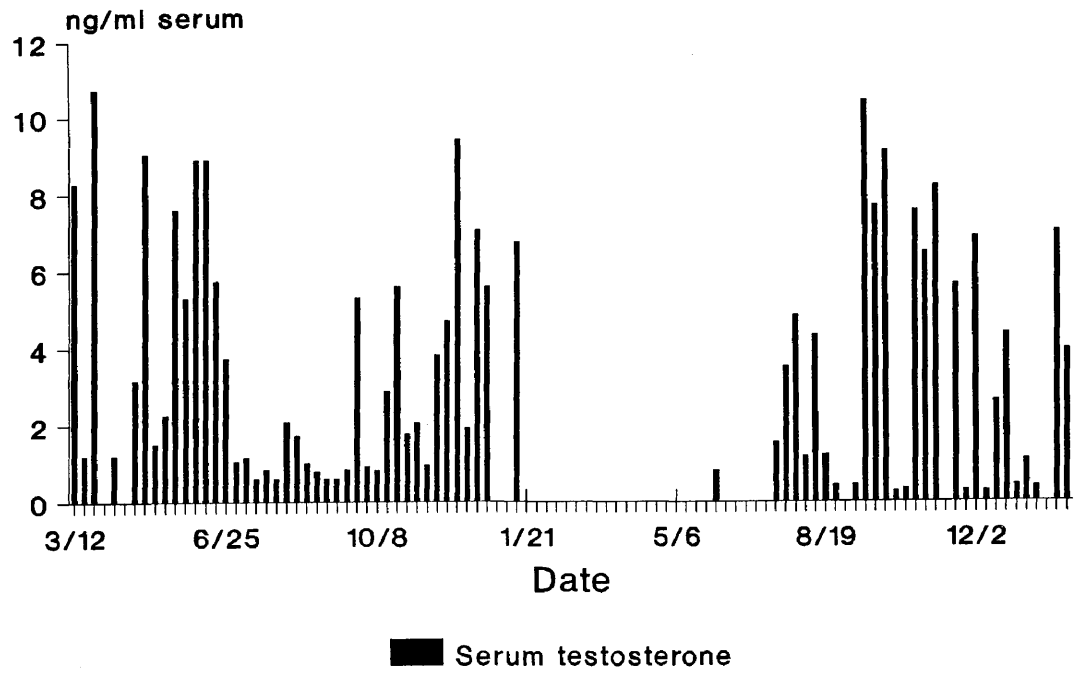

Figure 4. Serum concentrations of testosterone for animal "Kuhn-chorn". Serum samples were collected weekly over a 99 week period. Animal's age 11-13 years. Missing bars represent dates in which serum was not collected. Serum concentrations of testosterone remained less than 12 ng/ml.

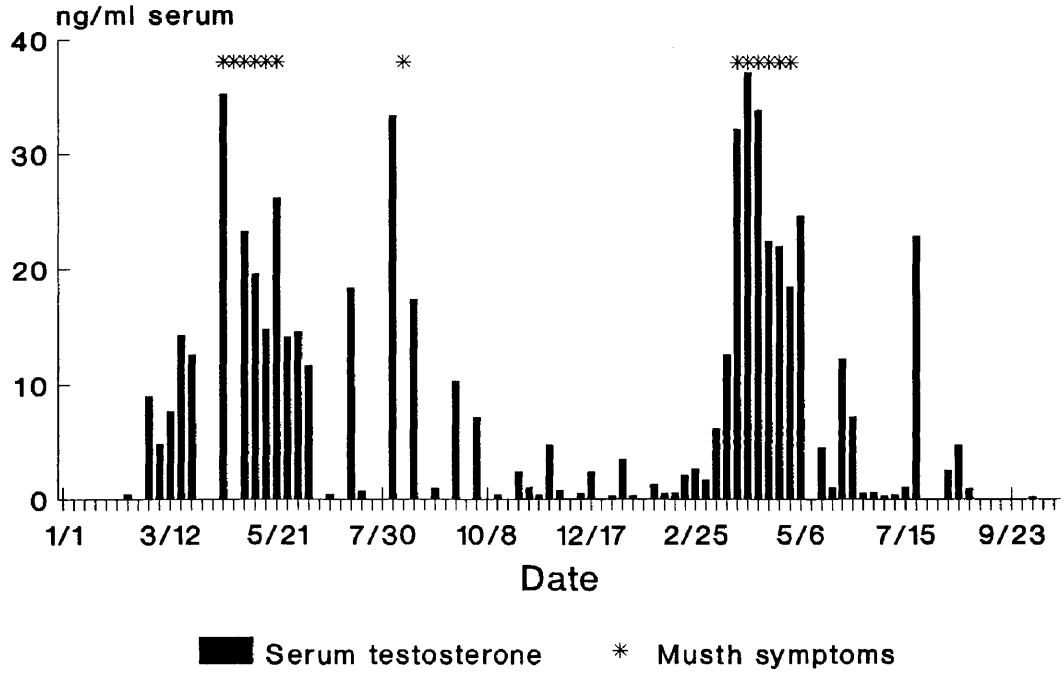

Figure 5. Serum concentrations of testosterone for animal "Bandara". Serum samples were collected weekly over a 90 week period. Animal's age 15-16 years. Missing bars represent dates in which serum was not collected. Each individual asterisk indicates a week in which temporal gland secretion was observed. Note that higher concentrations of testosterone are associated with temporal gland secretion.

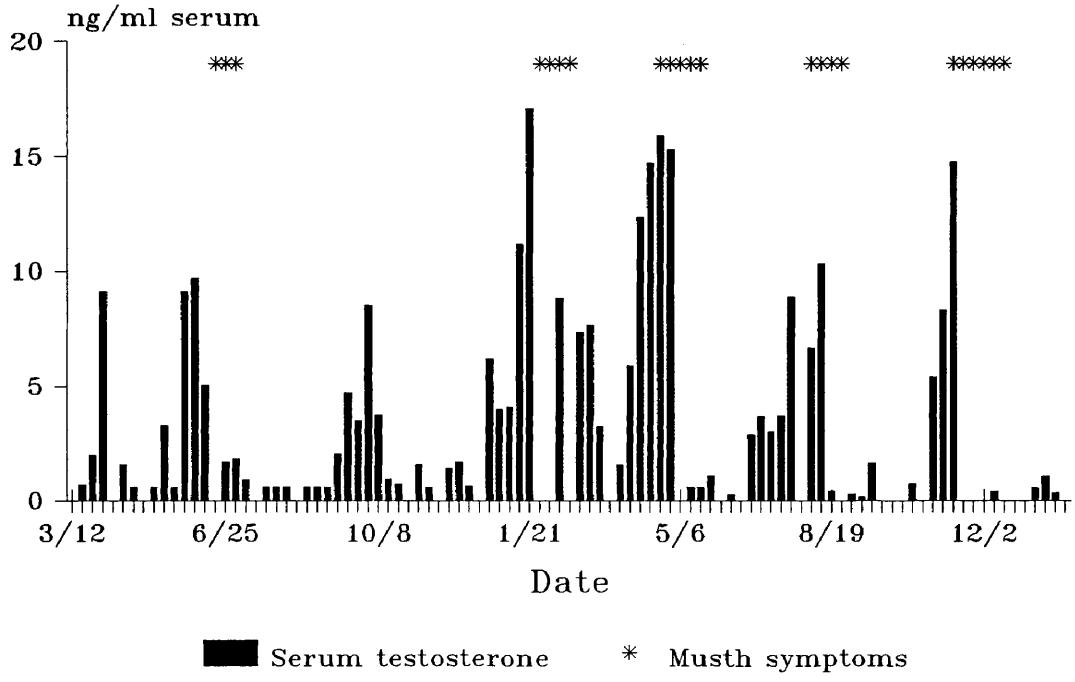

Figure 6. Serum concentrations of testosterone for animal "Sneezy". Serum samples were collected weekly over a 98 week period. Animal's age 17-19 years. Missing bars represent dates in which serum was not collected. Each individual asterisk indicates a week in which temporal gland secretion was observed. A cyclic pattern of testosterone is apparent with higher levels being associated with temporal gland secretion.

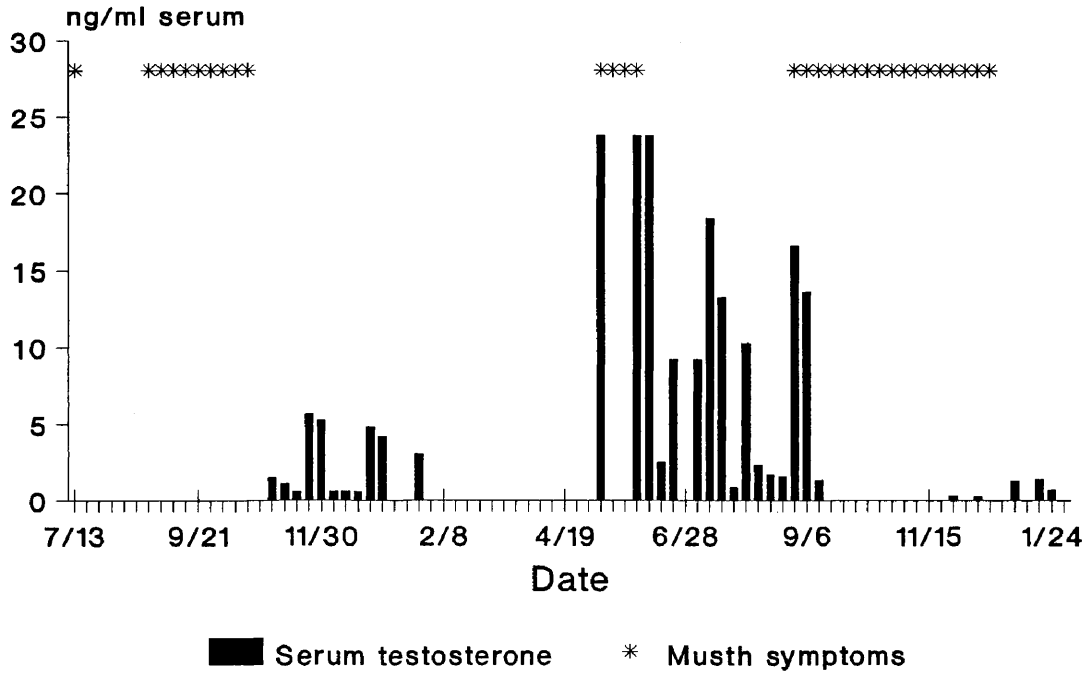

Figure 7. Serum concentrations of testosterone for animal "Indy". Serum samples were collected periodically over a 80 week period. Animal's age 18-20 years. Missing bars represent dates in which serum was not collected. Each individual asterisk indicates a week in which temporal gland secretion was observed. Note that during musth, it was possible to collect only a few serum samples but these samples contained higher concentrations of testosterone.

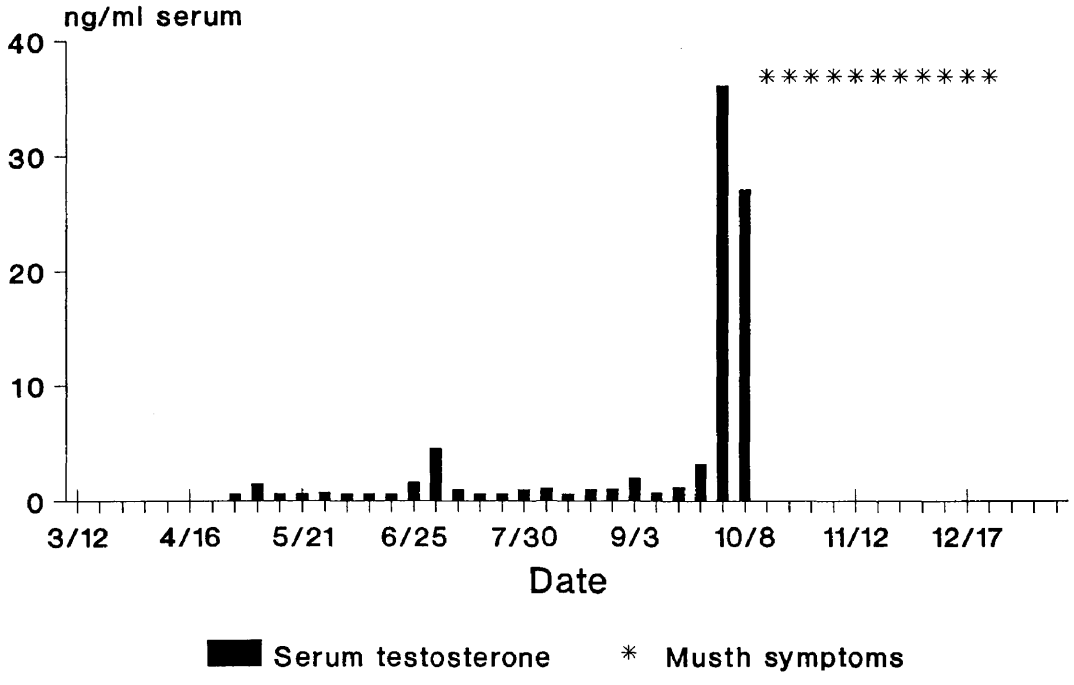

Figure 8. Serum concentrations of testosterone for animal "Onyx" during non-musth. Serum samples were collected weekly over a 25 week period. Animal's age 30 years. Missing bars represent dates in which serum was not collected. Each individual asterisk indicates a week in which temporal gland secretion was observed. Note that when musth symptoms occurred serum collection was not possible.

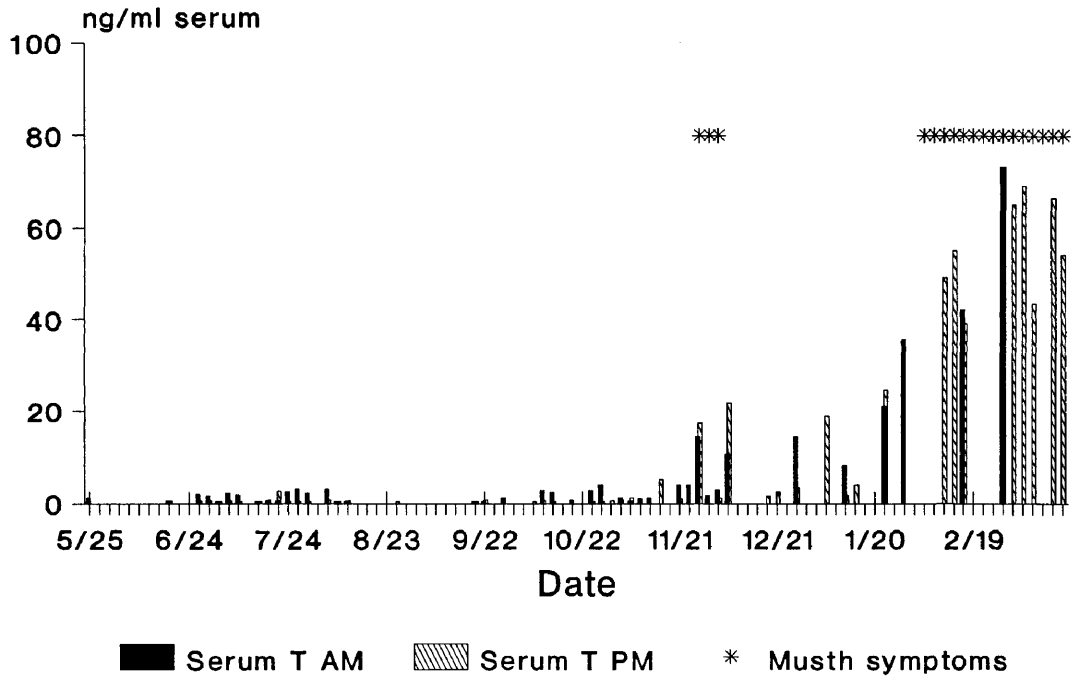

Figure 9. Serum concentrations of testosterone for animal "Dalip". Serum samples were collected approximately every 3 days in the morning and afternoon over a 43 week period. Animal's age 23-24 years. Missing bars represent dates in which serum was not collected. Each individual asterisk indicates a week in which temporal gland secretion was observed. Note the magnitude of change in testosterone levels during musth.

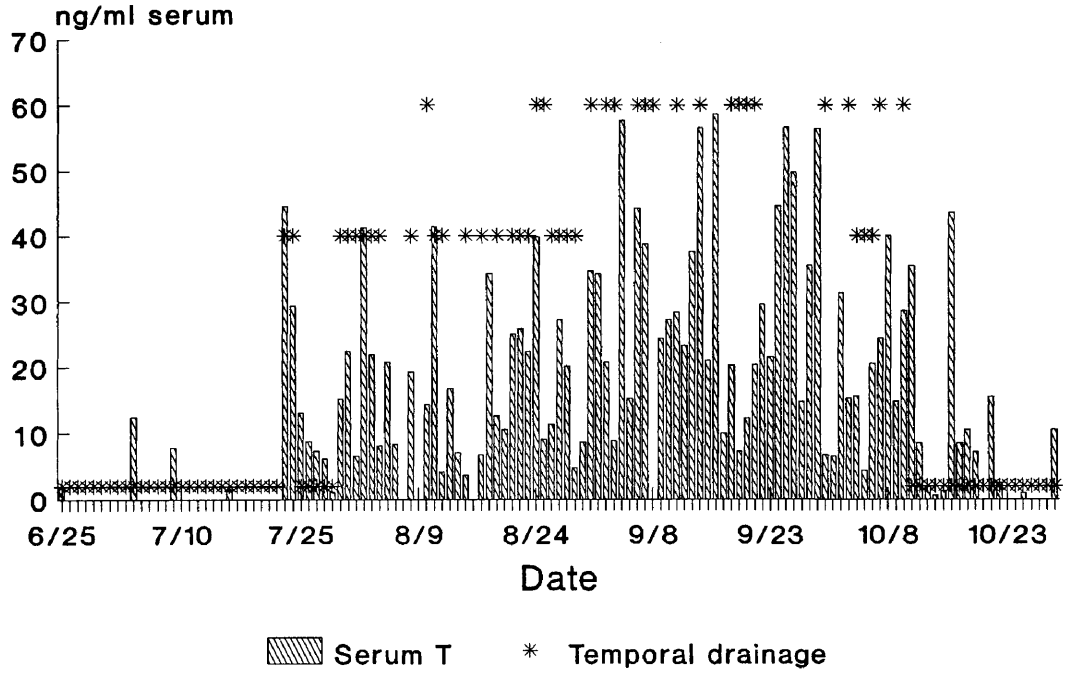

Figure 10. Serum concentrations of testosterone and degree of temporal gland secretion for animal "Onyx" during musth. Serum samples were collected daily over a 126 day period. Animal's age 24 years. Missing bars represent a day in which serum was not collected. Each individual asterisck indicates a daily observation and missing asterisks represent a day in which behavioral observation was not recorded. The asterisks closest to the X-axis indicate an observation of "no temporal gland secretion", the asterisks in the middle of the profile indicate an observation of "moderate temporal gland secretion" and the asterisks at the top of the profile indicate an observation of "heavy temporal gland secretion". Note that higher concentrations of testosterone are associated with a greater degree of temporal gland secretion.

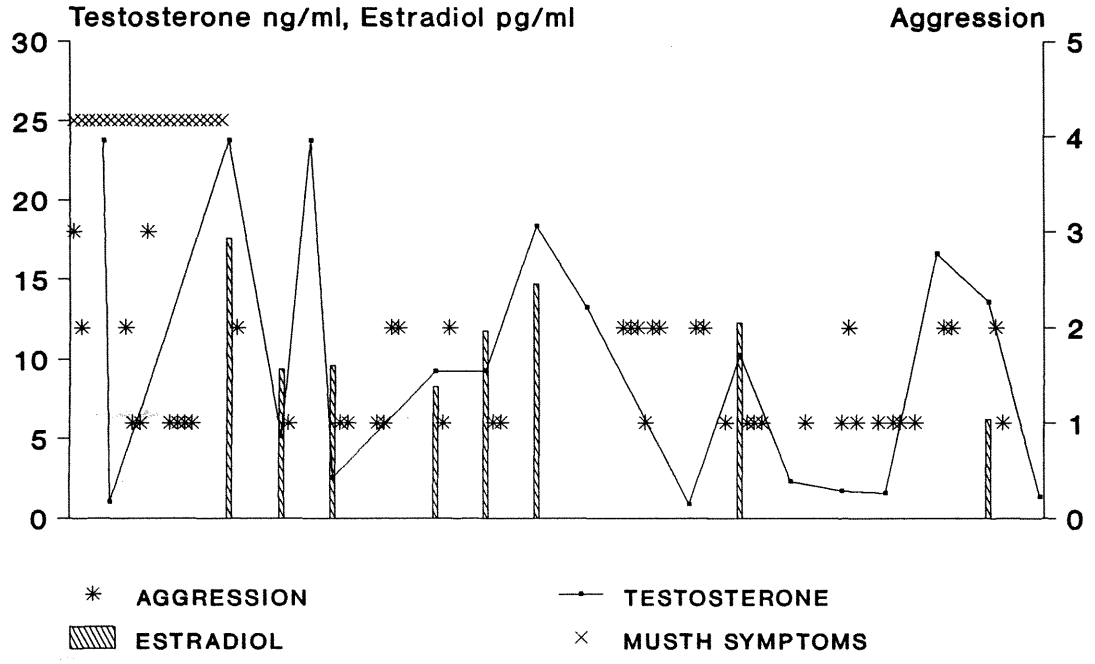

Figure 11. Serum concentrations of testosterone and estradiol and degree of aggression for animal "Indy". Degree of aggression is plotted on the right axis, 1=lowest, 4=highest. Each individual asterisk represents the day and degree of aggression noted for this bull. Each individual X indicates a day of temporal gland secretion. All serum samples available for this time period were used for the testosterone data. All serum samples with adequate volume for this time period were used for the estradiol data. A score of 3, which occurred during behavioral musth, is the highest intensity of aggression recorded for this bull during this time period.

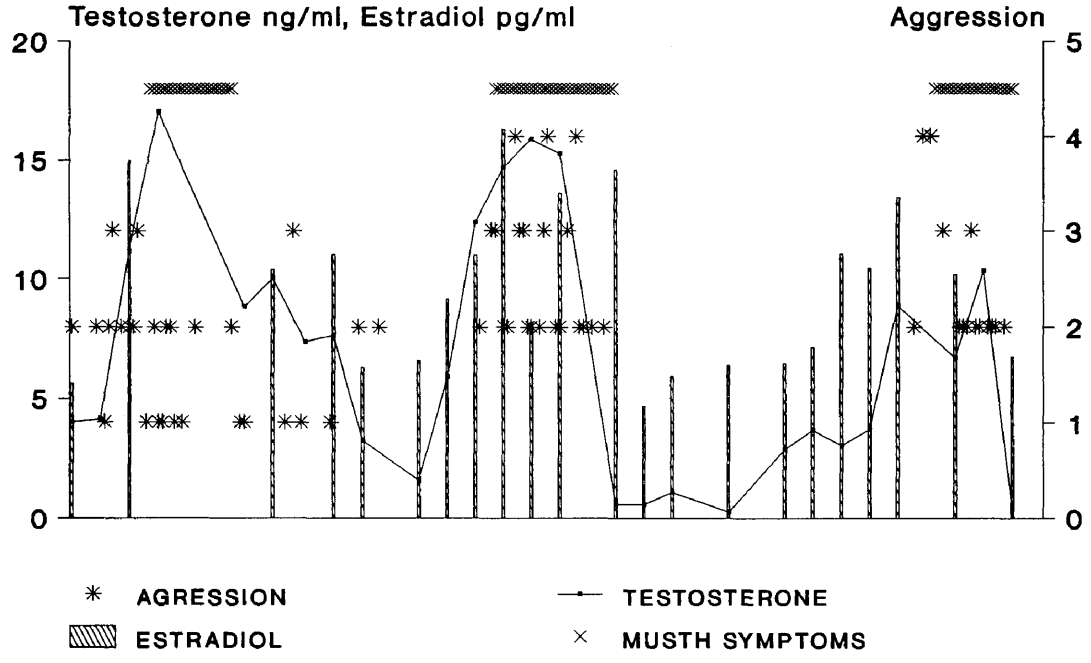

Figure 12. Serum concentrations of testosterone and estradiol and degree of aggression for animal "Sneezy". Degree of aggression is plotted on the right axis, 1=lowest, 4=highest. Each individual asterisk represents the day and degree of aggression noted for this bull. Each individual X indicates a day of temporal gland secretion. All serum samples available for this time period were used for the testosterone data. All serum samples with adequate volume for this time period were used for the estradiol data. An aggression score of 4 occured only during musth when both testosterone and estradiol levels were elevated.

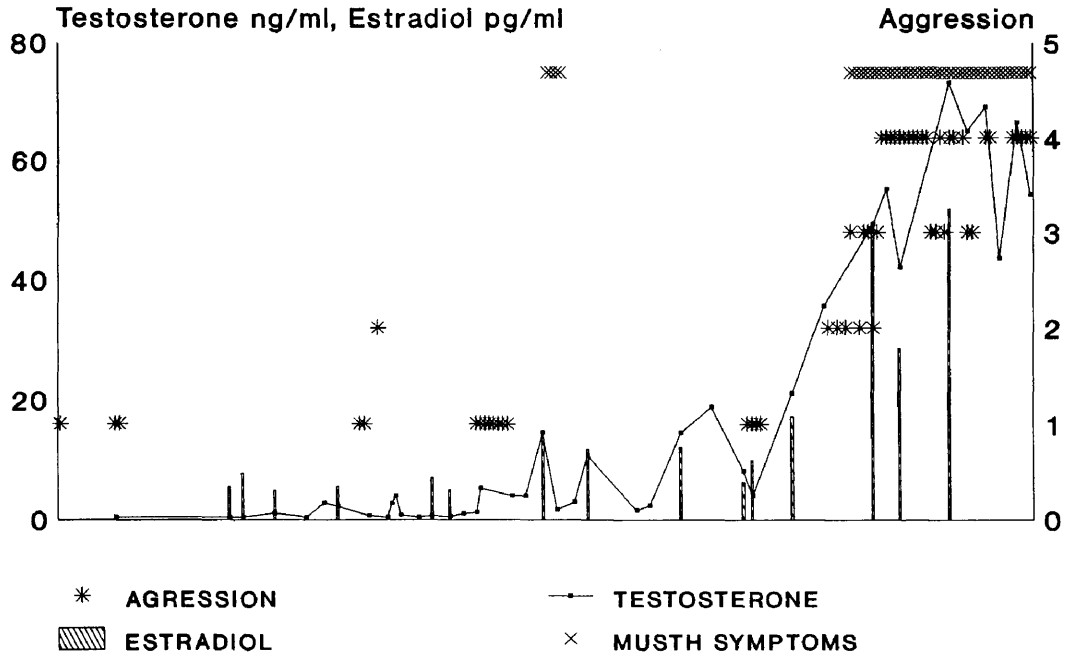

Figure 13. Serum concentrations of testosterone and estradiol and degree of aggression for animal "Dalip". Degree of aggression is plotted on the right axis, 1=lowest, 4=highest. Each individual asterisk represents the day and degree of aggression noted for this bull. Each individual X indicates a day of temporal gland secretion. All serum samples available for this time period were used for the testosterone data. All serum samples with adequate volume for this time period were used for the estradiol data. A clear relationship between musth, a higher aggression score, elevated testosterone and elevated estradiol can be observed.

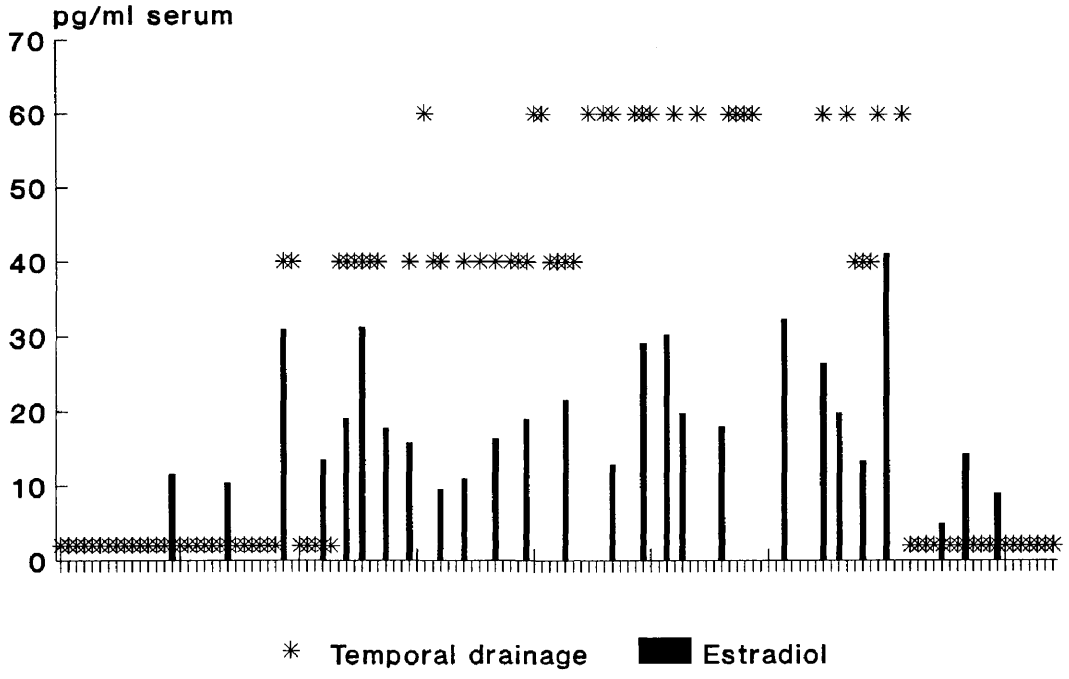

Figure 14. Serum concentrations of estradiol and degree of temporal gland secretion during musth in animal "Onyx. The data represent the same time period as in figure 10 with the estradiol data replacing testosterone data. Each individual asterisk indicates a daily observation and missing asterisks represent a day in which behavioral observation was not recorded. The asterisks closest to the X-axis indicate an observation of "no temporal gland secretion", the asterisks in the middle of the profile indicate an observation of "moderate temporal gland secretion" and the asterisks at the top of the profile indicate an observation of "heavy temporal gland secretion". A clear pattern of increasing estradiol with degree of temporal gland secretion is not apparant.

Pituitary luteinizing hormone stimulation of testosterone secretion and musth

Serum testosterone and LH concentrations for the group, analyzed by simple linear regression produced a significant linear trend with a Pearson's correlation coefficient of .39. However, when data from individual animals were analyzed separately, a much stronger correlation between serum testosterone and LH was demonstrated. Only two animals (Maverick and Spike) did not produce a significant linear relationship. The correlation coefficients for the individual's producing a significant linear trend were; Kuhn-chorn 0.39, Bandara 0.69, Sneezy 0.60, Indy 0.61, Dalip 0.74 and Onyx 0.95. The daily serum samples collected during an entire musth period for Onyx produced a correlation coefficient of 0.48.

The combined data representing the transition period into musth for 10 musth periods are depicted in figure 15. Circulating concentrations of LH and testosterone, measured in weekly samples, begin increasing 4 weeks before the onset of musth (based on temporal gland secretion). Furthermore, the circulating concentrations of LH began to decrease immediately after the onset of musth symptoms with a similar trend in testosterone levels seen lagging by about 1 week.

Concentrations of LH for the group ranged from less than 0.15 ng/ml to 11.26 ng/ml. Time series graphs of serum LH, and testosterone, representing animals of different age and musth status are depicted in figures 16-18. The daily musth hormone profile for the animal "Onyx" (figure 19) demonstrates the variation in LH concentrations that can be detected during musth with concentrations ranging from less than 0.15 ng/ml to 10.62 ng/ml.

Noninvasive methods to monitor musth

There were no significant differences between creatinine concentrations during musth and non-musth. Creatinine concentrations during musth were 0.48 $\pm$ 0.41 mg/ml and during non-musth concentrations were 0.399 $\pm$ 0.27 mg/ml. Concentrations of

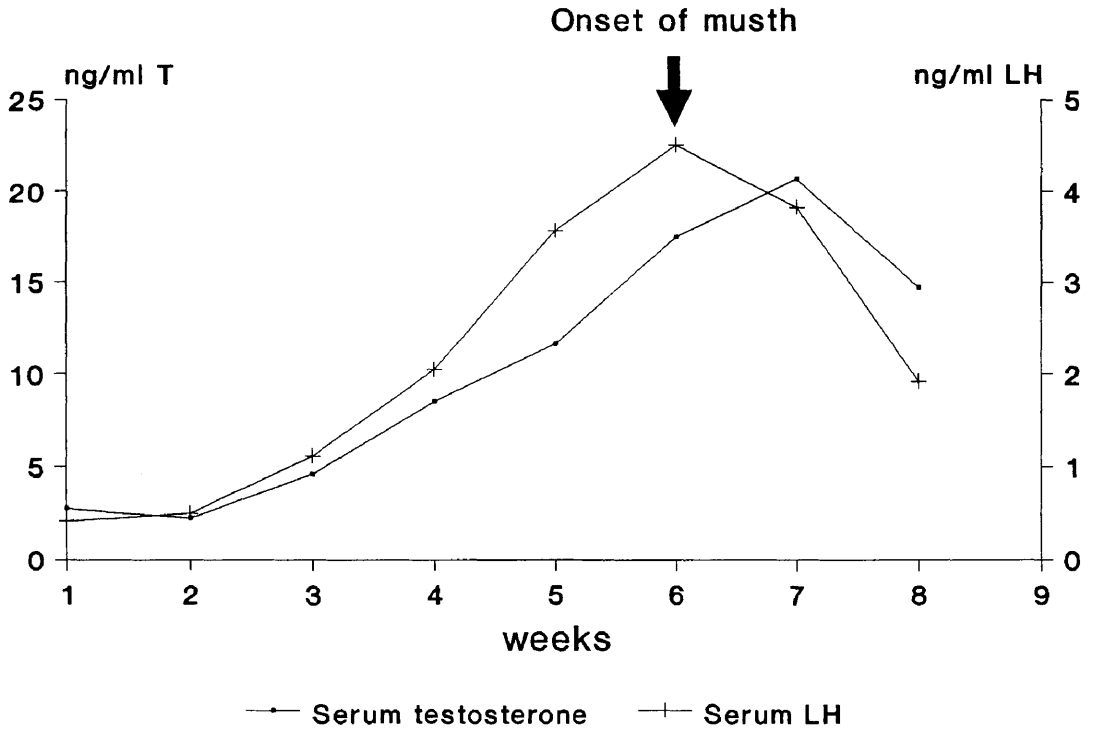

Figure 15. Serum concentrations of LH and testosterone before onset of musth symptoms. The weekly LH and testosterone level preceding musth symptoms (temporal gland secretion) were averaged for 10 musth periods. Note that LH and testosterone concentrations begin to rise 4 weeks before onset of musth symptoms.

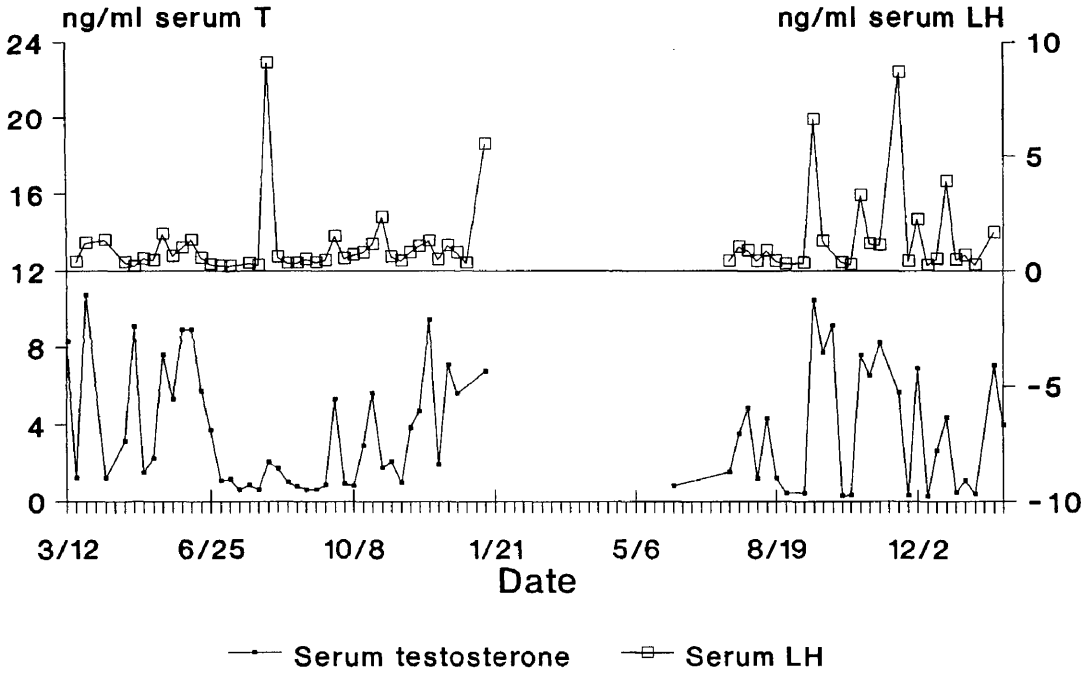

Figure 16. Weekly concentrations of serum LH and testosterone for the animal "Kuhn-chorn. This animal is 11-13 years of age. The lower graph represents testosterone and the upper graph represents LH. No musth symptoms were observed.

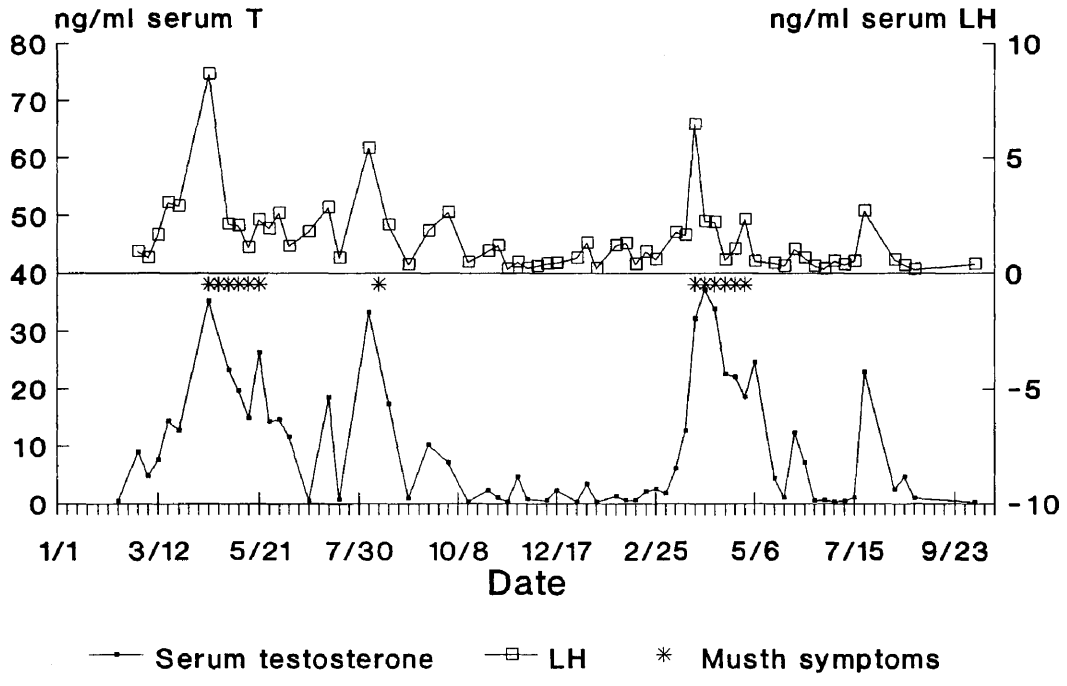

Figure 17. Weekly concentrations of serum LH and testosterone for the animal "Bandara". This animal is 15-16 years of age. The lower graph represents testosterone and the upper graph represents LH. Note that LH and testosterone levels begin to rise before musth symptoms are observed.

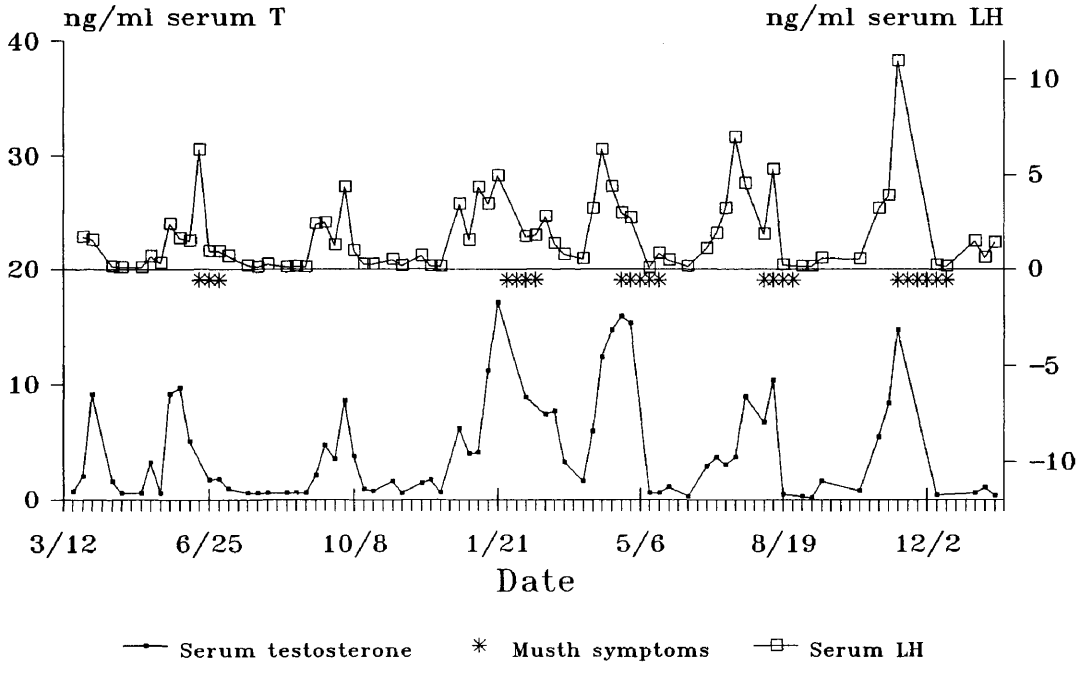

Figure 18. Weekly concentrations of serum LH and testosterone for the animal "Sneezy". This animal is 17-19 years of age. The lower graph represents testosterone and the upper graph represents LH. Note the consistent pattern of increasing LH and testosterone before the onset of musth symptoms. Levels of LH and testosterone are also seen to decrease before musth symptoms have ended.

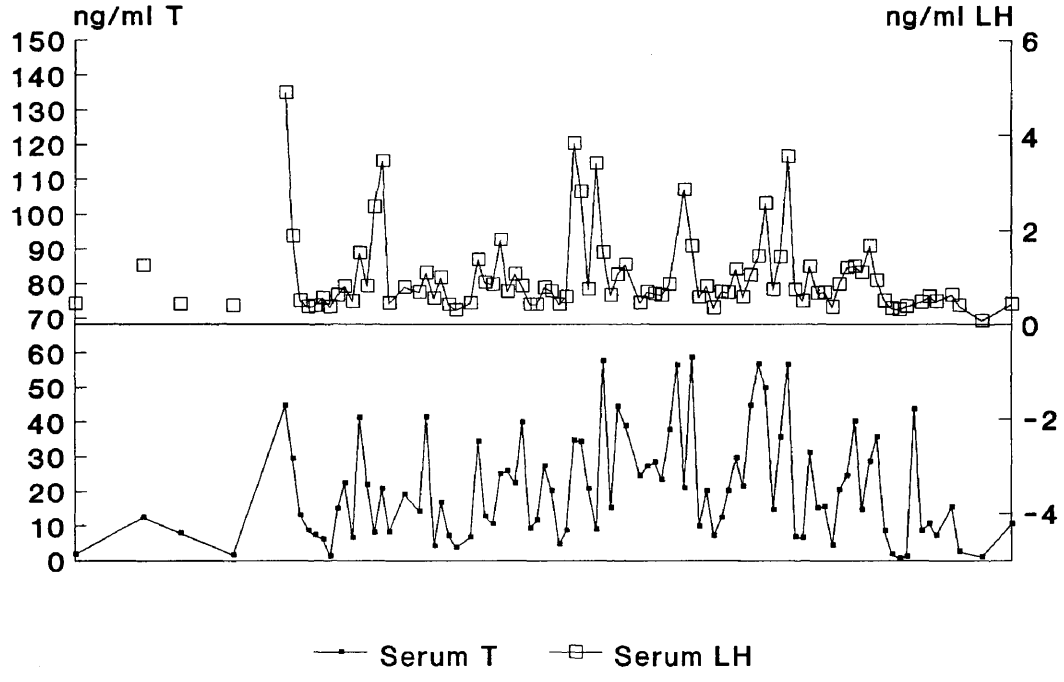

Figure 19. Daily concentrations of LH and testosterone during musth for the animal "Onyx". Serum concentrations are depicted for a 126 day musth period. Note the daily variation in LH concentrations.

creatinine for the Fort Worth zoo animals, analyzed separately were 0.064 $\pm$ 0.061 mg/ml. Radioimmunoassay of a pooled elephant urine sample after 24 hour treatment at 5^{o} C, 37^{o} C and hydrolysis at 37^{o} C, revealed that the majority of immunoreactive testosterone in the urine was conjugated. Concentrations were 1450 $\pm$ 75 pg/ml, 1813 $\pm$ 263 pg/ml and 121250 $\pm$ pg/ml, respectively. The efficiency of enzyme hydrolysis was 52 $\pm$ 7%.

A significant difference in mean concentrations of urinary immunoreactive testosterone was demonstrated for musth and non-musth samples analyzed for the group. Concentrations were 335 $\pm$ 270 ng/mg Cr and 733 $\pm$ 674 ng/mg Cr for non-musth and musth samples, respectively. These concentrations reflect values that were not corrected for hydrolysis or extraction recovery. Significant differences during musth and non-musth were also detected in the exceptionally dilute urine samples collected from the Fort Worth Zoo. These concentrations, however, were not comparable to the levels obtained from the remaining experimental group. Non-musth and musth values, respectively, were 875 $\pm$ 854 ng/mg Cr and 1631 $\pm$ 1152 ng/mg Cr. The simple linear regression analysis between serum and urinary testosterone for the group revealed a significant positive linear relationship with a correlation coefficient of 0.37. The Spearman's rank correlation coefficient for the group was 0.32. The relationship between serum and urinary testosterone is demonstrated for two animals in figures 20 and 21.

The first fecal experiments provided evidence that considerable amounts of immunoreactive testosterone was present in the feces of male Asian elephants. The first extraction procedure (experiment 1) produced a significant positive linear relationship between serum and fecal immunoreactive testosterone with a correlation coefficient of 0.52. The results of the sephadex co-chromatography (experiment 2) revealed that the majority of immunoreactivity co-eluted with standard ^{3}H testosterone. This is illustrated graphically in figure 22.

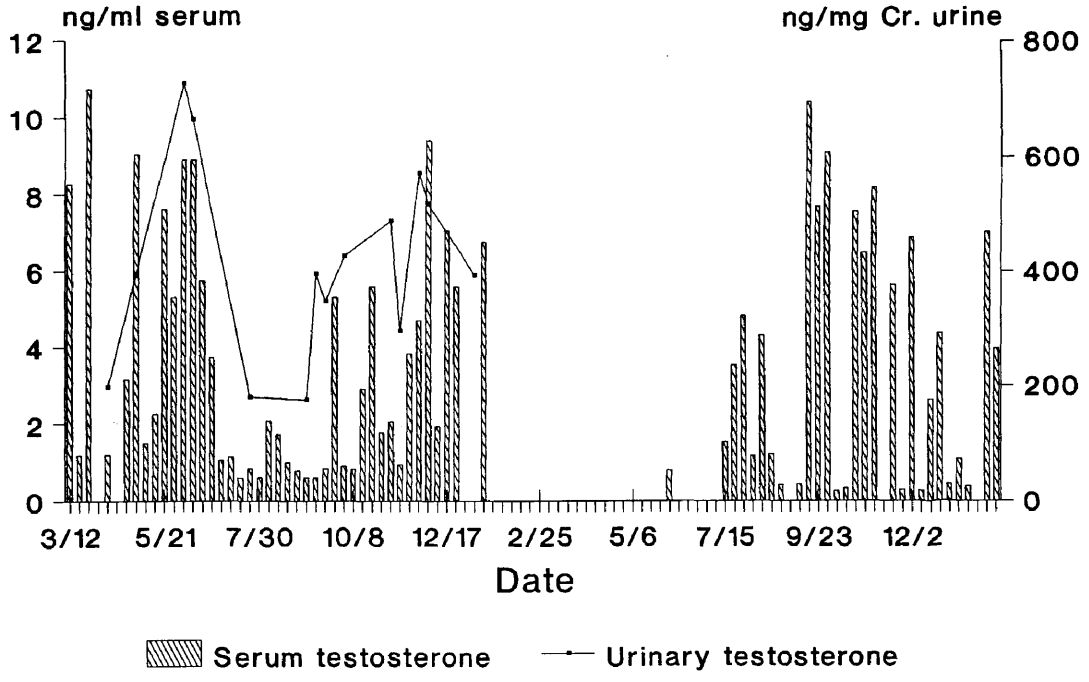

Figure 20. Serum and urinary testosterone profile comparison for animal "Kuhnchorn". A similar pattern of hormone secretion is observed.

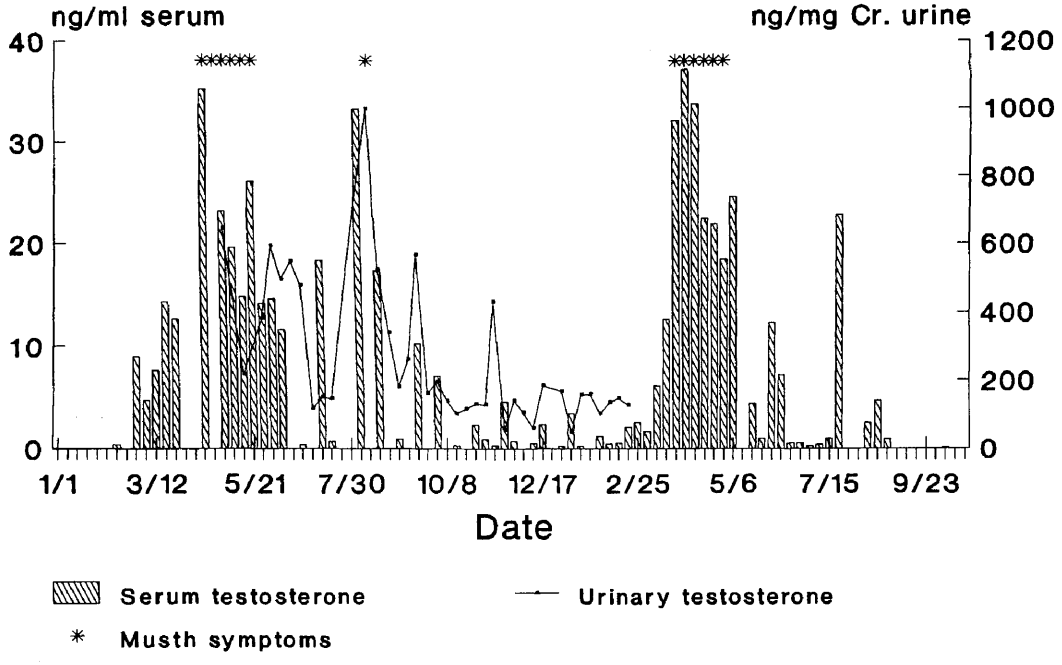

Figure 21. Serum and urinary testosterone profile comparison for animal "Bandara". Serum and urinary testosterone exhibit a similar pattern of hormone secretion.

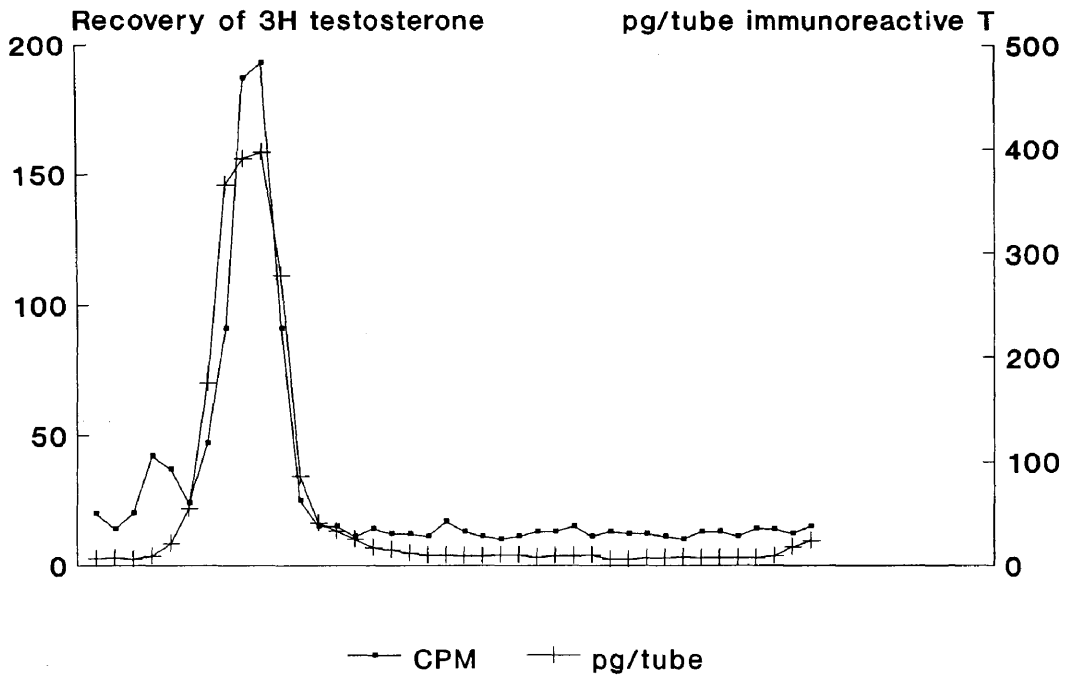

Figure 22. Sephadex co-chromatography of fecal extract and 3H testosterone. Each (.) symbol represents a fraction collected from the sephadex column and the amount of 3H testosterone measured in a portion of the fraction. Each (+) symbol represents a different portion of the same corresponding fraction measured for immunoreactive testosterone. Both radioactive standard testosterone and immunoreactive testosterone peaked in the same fractions.

The solubilization experiments determined that ethanol would be the best solution for recovery of testosterone from the feces. The percent recovery of ^{3}H testosterone and the efficiency of counting were as follows; Tris-gel buffer, 11% at 65% efficiency; sodium citrate pH 5, 13% at 68% efficiency; sodium citrate pH 4, 13% at 72 % efficiency; absolute ethanol, 72% at 62% efficiency and methanol, 49% at 41% efficiency (n=4 replicates).

Using this new solubilization procedure, a significant and stronger positive linear relationship with a correlation coefficient of 0.67 was demonstrated. Also, the recovery of ^{3}H testosterone monitored in each sample revealed adequate repeatability. The mean and coefficient of variation for recovery after measurement of the ethanol extract directly and after evaporating and reconstituting in buffer were 61% and 13%, and 54% and 12%, respectively (n=50). When the residual feces, collected after solubilization, was dried and the corresponding testosterone concentrations corrected for the fecal dry weight, no improvement was found in the strength of the correlation (r=0.62).

The lyophilization of fecal samples did not appear to improve the strength of the correlation between serum and fecal testosterone. The same fecal samples assayed wet and producing a correlation coefficient of 0.67, produced a correlation coefficient of 0.52 after lyophilization. The significant positive correlation between duplicate wet and dry samples was 0.72 and a time series plot of the data revealed nearly identical profiles.

Fecal concentrations of immunoreactive testosterone for the group averaged 10 $\pm$ 5 ng/gram wet feces during non-musth and 20 $\pm$ 7.8 ng/gram wet feces during musth. These concentrations were significantly different. The simple linear regression analysis did reveal a significant positive relationship between serum and fecal immunoreactive testosterone for the group with a Pearson's correlation coefficient of 0.49 and a Spearman's rank correlation coefficient of 0.48. When individual animals were examined for a linear relationship between serum and fecal testosterone, 3 animals did not produce a significant linear relationship and 2 animals did. The animals which did produce a

significant relationship produced a correlation coefficient of 0.63 (Sneezy) and 0.68 (Onyx). The relationship between serum and fecal testosterone is depicted in figures 23-26. The fecal testosterone data were extremely valuable for replacing serum data in 2 of the animals because blood collection was not possible.

A significant positive linear relationship was observed between fecal and urinary immunoreactive testosterone for the group and for the Fort Worth Zoo animals. The Pearson's correlation coefficient for the group was 0.49 and the Spearman's rank correlation coefficient was 0.47. The Pearson's and Spearman's rank correlation coefficients for the Fort Worth zoo animals were 0.49 and 0.55, respectively. This relationship is presented graphically for three animals in figures 27-29. Because blood collection was not possible from two of the animals (figures 27 and 28), without non-invasive analysis, endocrine data would not have been obtained.

The slopes of the regression lines for serial diluted extracted elephant feces, hydrolyzed extracted urine and standard testosterone were non-different (Urine, $F=0.072$; Feces, $F=0.83$, alpha$=0.05$, $df_1=1$, $df_2=4$). However, HPLC co-chromatography did reveal the presence of cross-reacting substances in both the urine and feces. Using the isocratic HPLC system for co-chromatography of a fecal extract, the majority of immunoreactivity co-eluted with standard ^3H testosterone. A second immunoreactive peak eluted after testosterone (figure 30). Using the gradient HPLC system, it appeared that the second immunoreactive peak detected with the isocratic system was actually 4 smaller immunoreactive peaks (figure 31). Co-chromatography of a urine pool extract determined that the majority of testosterone immunoreactivity co-eluted with standard ^3H testosterone but two small immunoreactive peaks eluted after testosterone (figure 32).

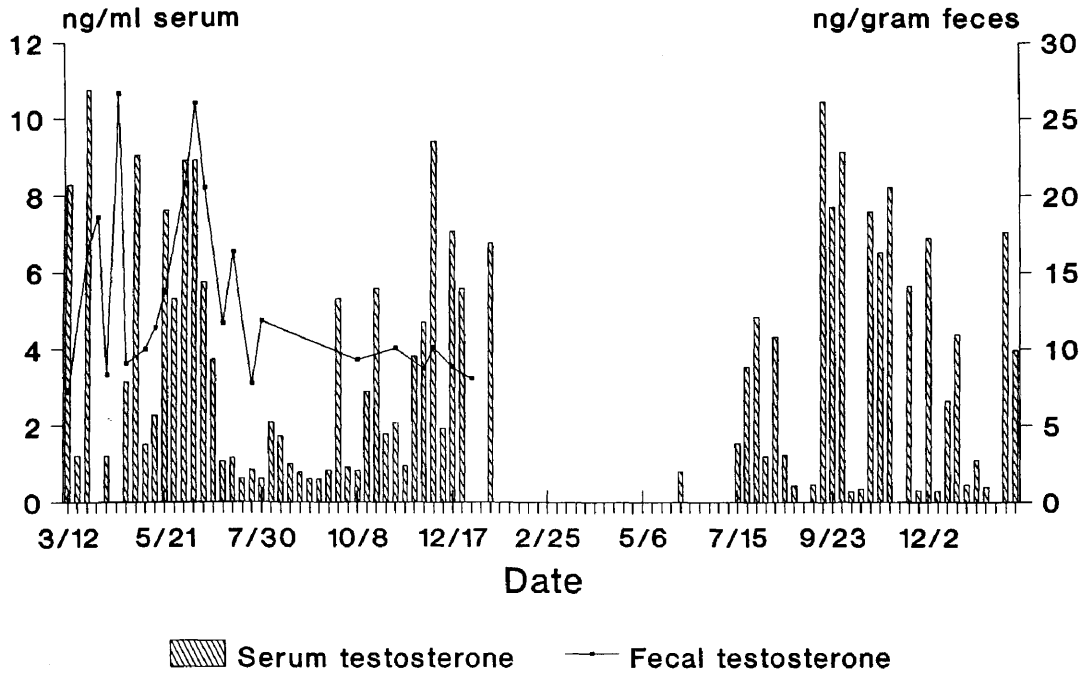

Figure 23. Serum and fecal testosterone profile comparison for animal "Kuhn-chorn". These data produced a correlation coefficient of 0.45 but a significant linear relationship between serum and fecal testosterone was not obtained for this individual animal using a simple linear regression model.

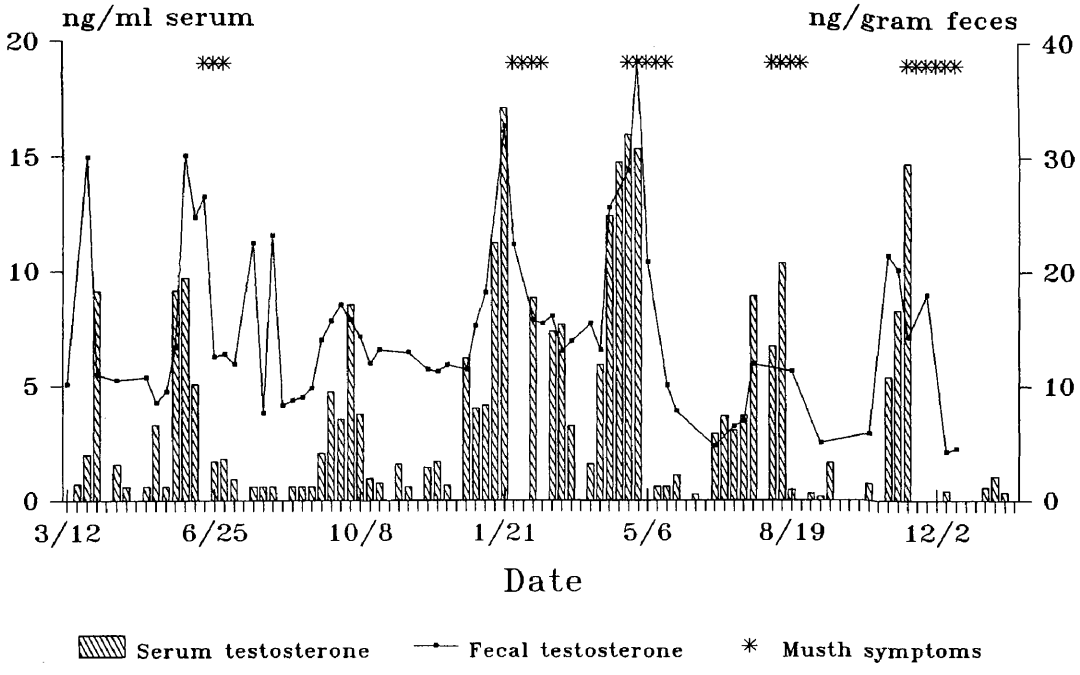

Figure 24. Serum and fecal testosterone profile comparison for animal "Sneezy". These data produced a significant linear relationship and a correlation coefficient of 0.64. Note the similar hormonal profiles produced.

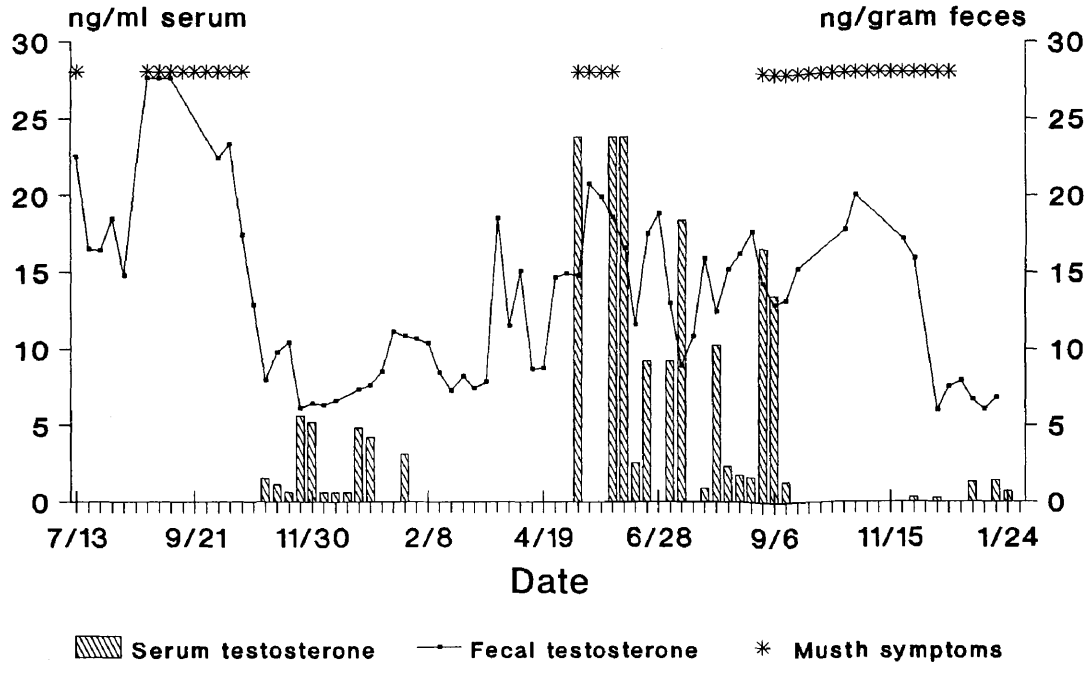

Figure 25. Serum and fecal testosterone profile comparison for animal "Indy". These data produced a correlation coefficient of 0.27 but a significant linear relationship between serum and fecal testosterone was not obtained. Serum samples could only be obtained periodically. This profile, however, demostrates increased fecal testosterone during temporal gland secretion.

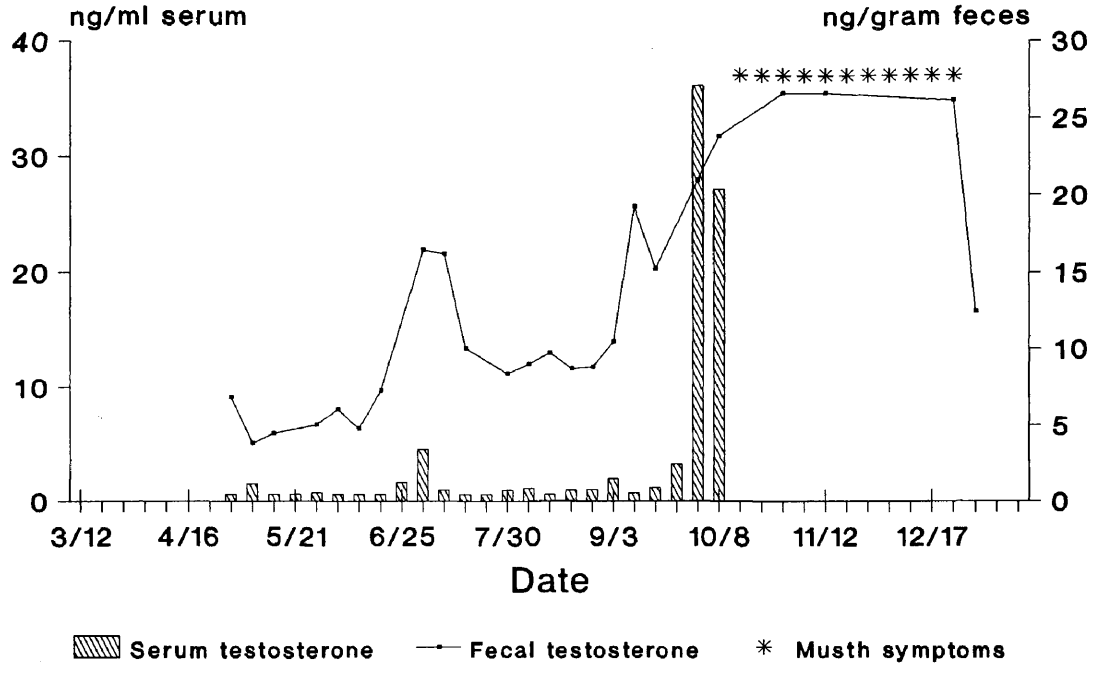

Figure 26. Serum and fecal testosterone profile comparison for animal "Onyx". These data produced a significant linear relationship with a correlation coefficient of 0.68. Serum collection was not possible once the animal began experiencing musth. Fecal testosterone increases dramatically during the period of temporal gland secretion.

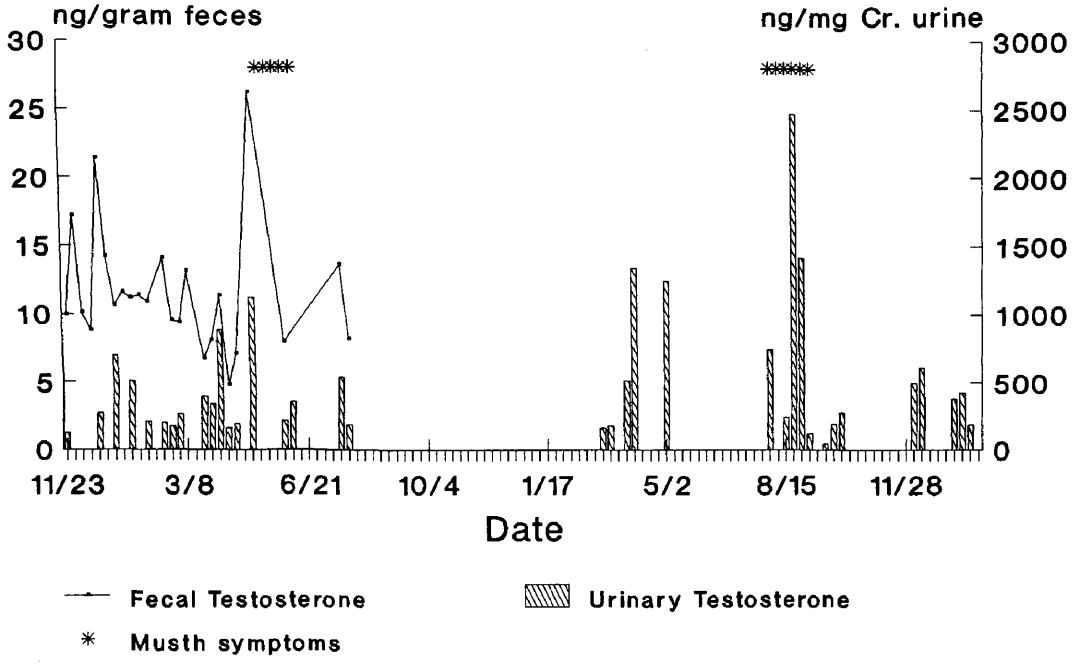

Figure 27. Fecal and urinary testosterone profile comparison for animal "Thong-trii". Samples were collected weekly over two 50 week periods. Animal's age 10-12 years. Missing data represent dates in which samples were not collected. Each individual asterisk indicates a week in which temporal gland secretion was observed. Blood collection was not possible from this animal.

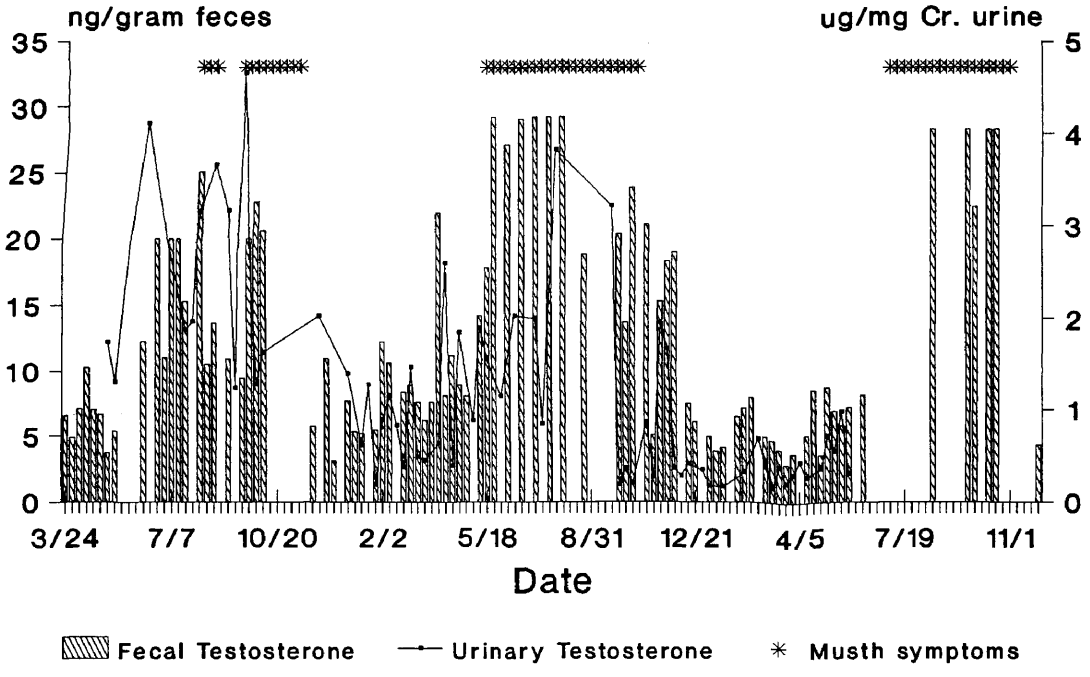

Figure 28. Fecal and urinary testosterone profile comparison for animal "Groucho". Samples were collected weekly over a 139 week period. Animal's age 19-21 years. Missing data represent dates in which samples were not collected. Each individual asterisk indicates a week in which temporal gland secretion was observed. Blood collection was not possible from this animal. Note the similar trends in testosterone excretion.

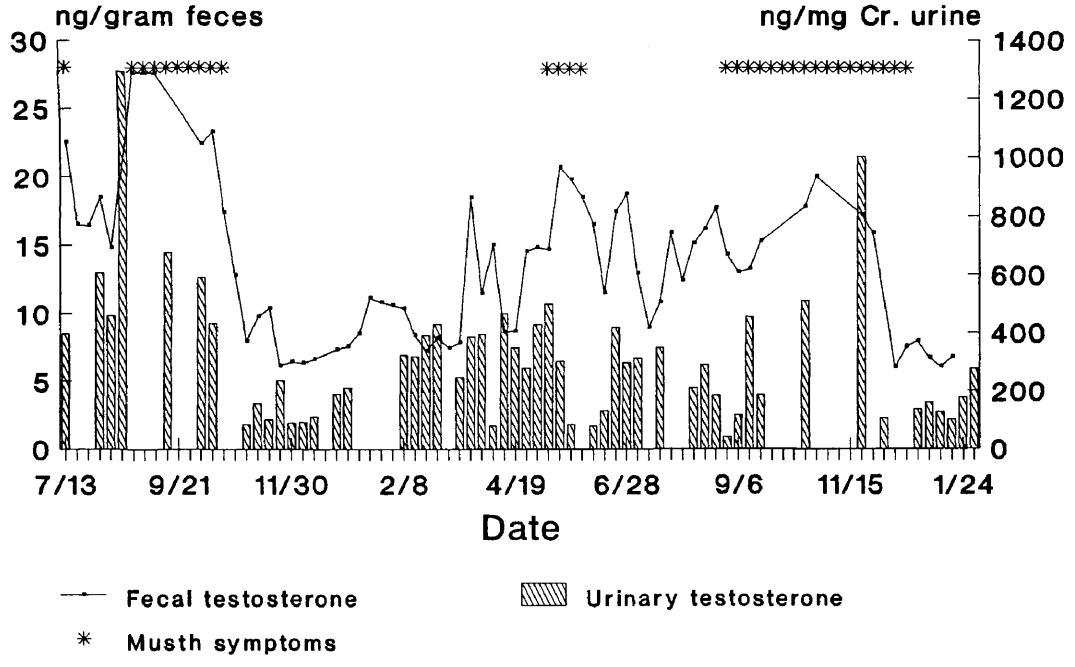

Figure 29. Fecal and urinary testosterone profile comparison for animal "Indy". Samples were collected weekly over an 80 week period. Animal's age 18-20 years. Missing data represent dates in which samples were not collected. Each individual asterisk indicates a week in which temporal gland secretion was observed. A correlation between fecal and urinary testosterone is not clearly demonstrated from this animal.

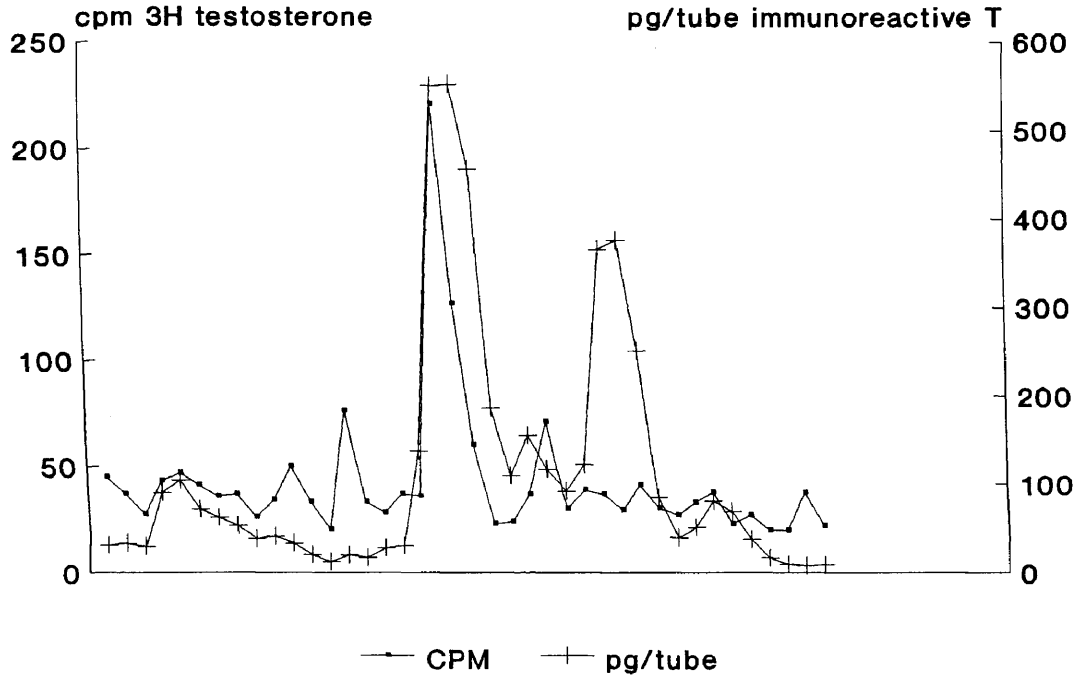

Figure 30. HPLC co-chromatography of a fecal extract and 3H testosterone using an isocratic system. Each (.) symbol represents a fraction collected from the HPLC and the amount of 3H testosterone measured in a portion of the fraction. Each (+) symbol represents a different portion of the same corresponding fraction measured for immunoreactive testosterone. Note the second immunoreactive testosterone peak.

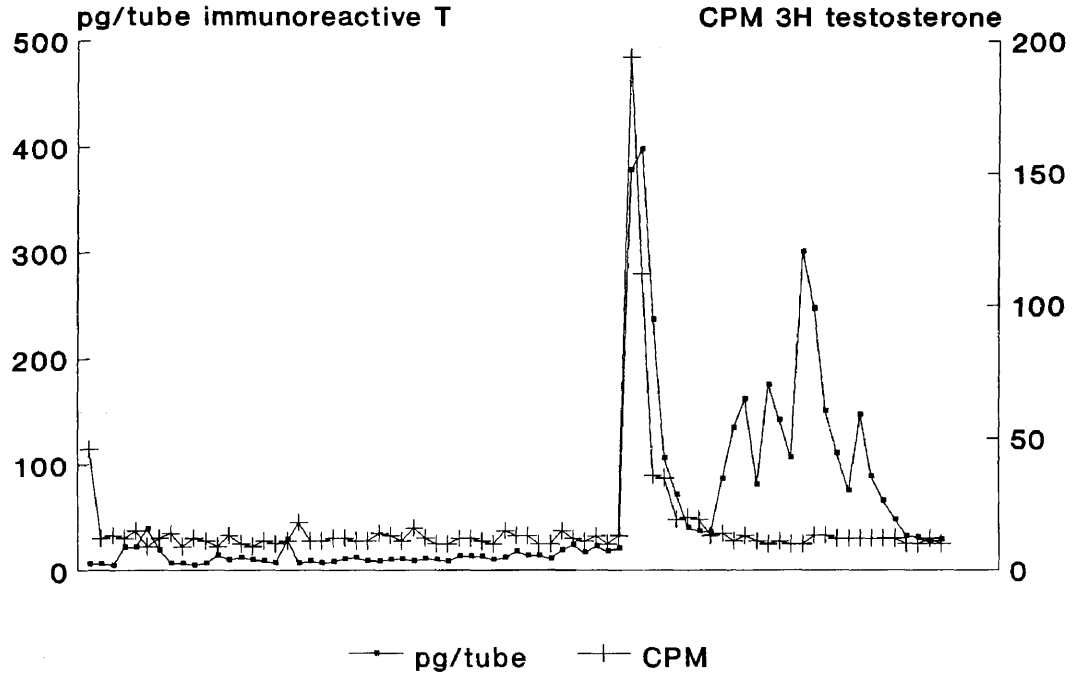

Figure 31. HPLC co-chromatography of a fecal extract and 3H testosterone using a gradient system. Each (+) symbol represents a fraction collected from the gradient system and the amount of 3H testosterone measured in a portion of the fraction. Each (.) symbol represents a different portion of the same corresponding fraction measured for immunoreactive testosterone. Note that now, the immunoreactivity eluting after testosterone is actually 4 peaks.

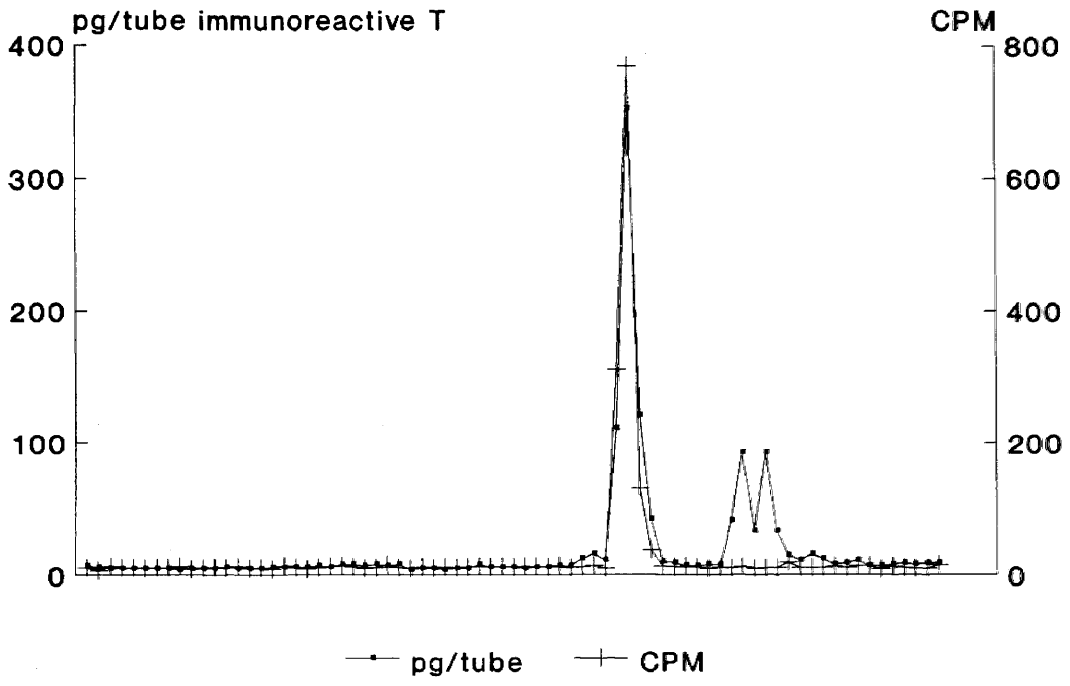

Figure 32. HPLC co-chromatography of a hydrolyzed urine extract with 3H testosterone using a gradient system. Each (+) symbol represents a fraction collected from the gradient system and the amount of 3H testosterone measured in a portion of the fraction. Each (.) symbol represents a different portion of the same corresponding fraction measured for immunoreactive testosterone. Note that the majority of immunoreactive testosterone does coelute with the radioactive standard.

Of the total 233uCi ^{14}C testosterone injected, 148 uCi (64%) were recovered during the collection period; 57% in the feces and 7% in the urine. When calculated as a percentage of recovered radioactivity, 89% of the activity was found in feces, 11% in the urine. A considerable amount of quench correction was required to estimate the amount of radioactivity present in ethanol fecal extracts and raw urine. Counting efficiency ranged from 40-93% for raw urine and 24-86% for fecal extracts. When two consecutive ethanol solubilization procedures were carried out on the same fecal extracts, (n=20) the first solubilization recovered 73.8 $\pm$ 5.74% of the two solubilizations combined.

The kinetics of excretion in the urine and in the feces differed greatly. In the urine, The greatest amount of activity, 8.55 uCi, was found in the first sample collection 10 hours after injection. The amount of activity collected over the next 10 hours (collected twenty hours after injection) decreased to 5.85 uCi and during the subsequent 14 hours (collected 34 hours post-injection) the activity decreased to less than 1 uCi. The activity remained low until 94 hours postinjection when levels over background could not be detected. The amount of activity totaled 2.35 uCi during this period. During the first 10 hours, the elephant had detected the modified drain system and removed the PVC pipes. It was suspected that a considerable amount of urine was lost through the drain during this time and an estimated volume of urine was used for the calculations. Figure 33 depicts the kinetics of ^{14}C testosterone excretion in the urine.

The peak of radioactivity in the feces occurred during the period 48-58 hours post-injection. However, the distribution of activity excretion was bell shaped resulting in a prolonged, increasing, then decreasing pattern. The kinetics of fecal excretion of ^{14}C testosterone is depicted in figure 34.

In the urine, 70% of the radioactivity present was in the conjugated form. These conjugates appeared to be quite resistant to enzyme hydrolysis but were adequately

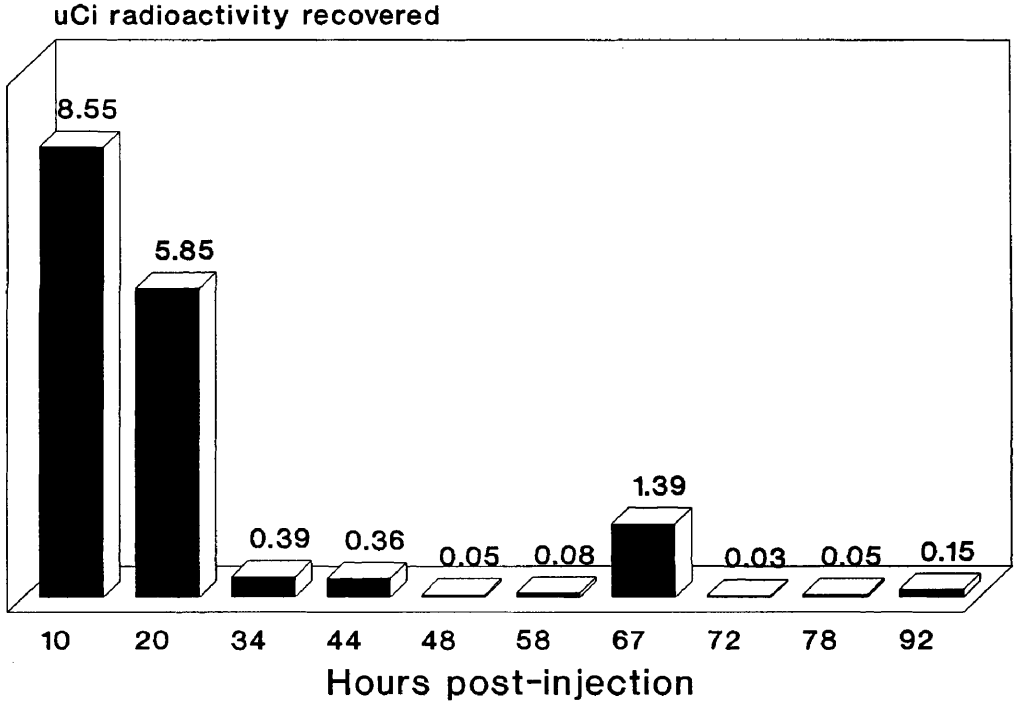

Figure 33. Kinetics of 14C testosterone excretion in the urine. Each bar represents the amount of radioactivity measured in the urine collected since the preceding collection period. The peak of activity occured with the first 24 hours.

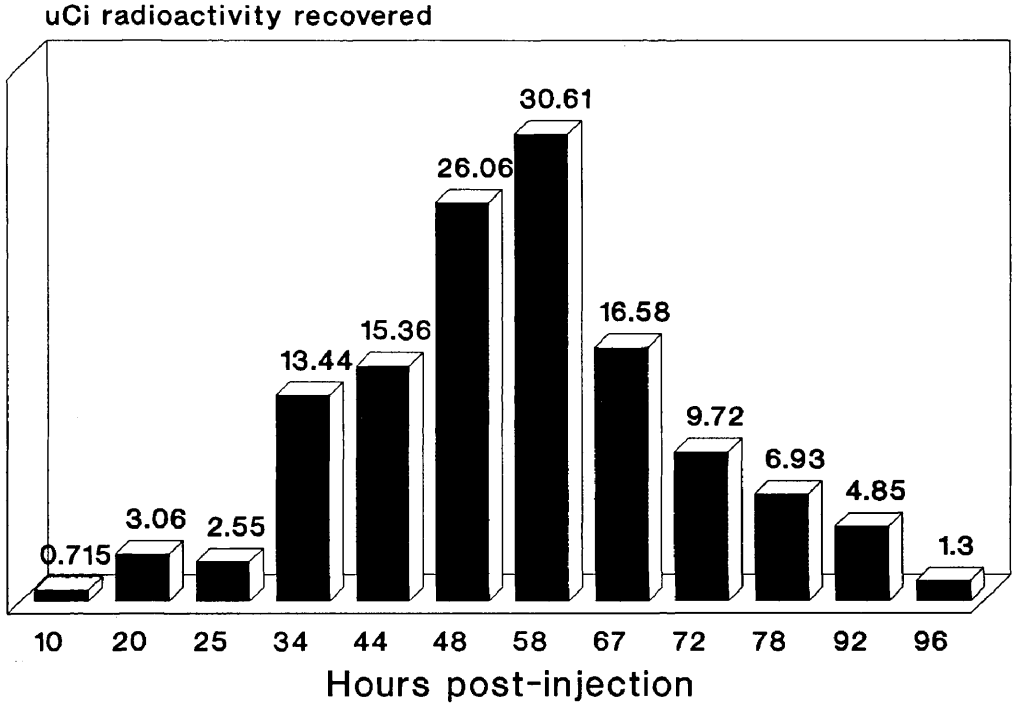

Figure 34. Kinetics of 14C testosterone excretion in the feces. Each bar represents the amount of radioactivity measured in the feces collected since the preceding collection period. Note that the pattern of excreted radioactivity in the feces is bell shaped, peaking at 58 hours post-injection.

hydrolyzed by solvolysis. Forty percent of the radioactivity was still present in the aqueous phase after enzyme hydrolysis and ether extraction. Solvolysis, on the other hand, was successful in liberating 97% of the radioactivity present. Nearly 100% of the radioactivity recovered in the feces was unconjugated. After the samples containing radioactivity were extracted with ether, the aqueous phase (representing conjugated steroids) for each sample was counted (n=20). These data combined contained a total of 0.0001 uCi of activity. Although this radioactivity present was nearly non-detectable, it was significantly different from background levels based on Student's t-test at a p-value of 0.048.

The HPLC analysis of radioactivity in fecal and urine extracts revealed significant in-vivo metabolism of ^{14}C testosterone. When dual standard peaks were detected, chromatograph error was suspected and the analysis was discarded (n=2). The HPLC profiles for hydrolyzed and non-hydrolyzed urine were nearly identical with perhaps a qualitative change in the size of the peaks evident. The standard ^{3}H testosterone peaked in fraction 50-51. Five ^{14}C radioactive peaks were consistently detected in all of the urine HPLC profiles. The fecal ^{14}C radioactive peaks were more difficult to detect over background because of problems with adequately concentrating and cleaning up the sample before injection into the HPLC system. Fecal ^{14}C peaks were similar to the peaks detected in urine. Only one peak detected in urine was not detected in the feces (fraction 46-48). Representative urine and fecal HPLC profiles are depicted in figures 35 and 36 and table 4 summarizes data for the entire analysis.

Table 4. Summary data for HPLC separation of fecal and urine radioactivity. The number represents the fraction where peak activity was measured.

Analysis	3H peaks	peak 1	peak 2	peak 3	peak 4	peak 5	peak 6
urine	51		47	51	57	60	63
urine	50		46	50	56	59	62
urine	51		48	51	57	61	63
urine		12	47	50	56	59	63
feces	50	10		50	56		64
feces	50				56		63
feces	50					61	

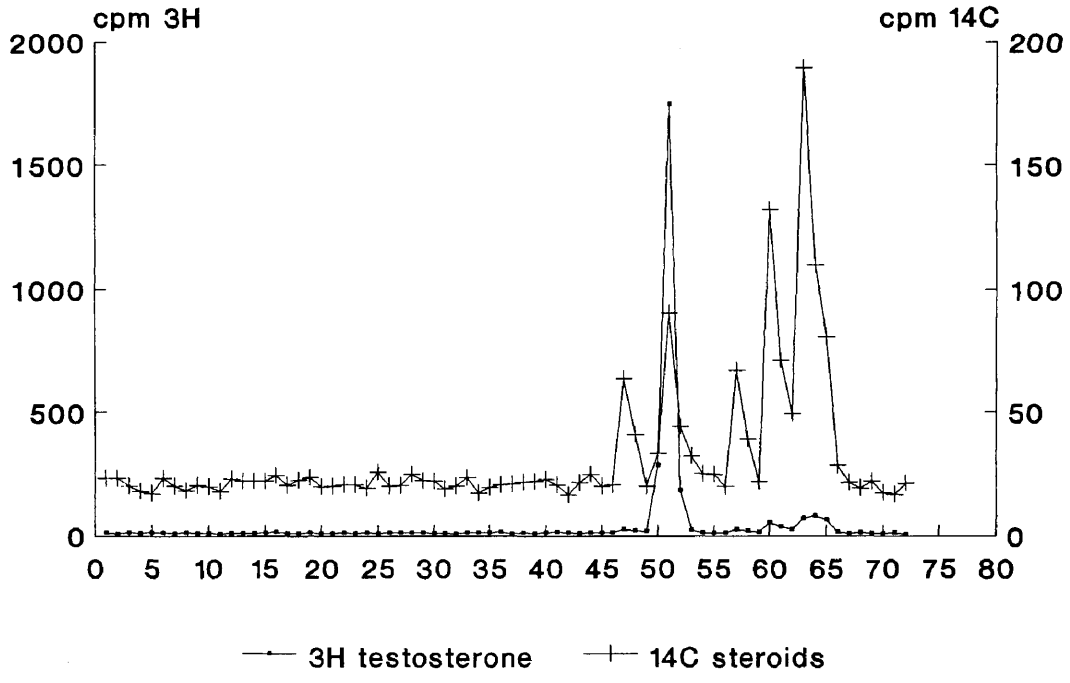

Figure 35. HPLC separation of urinary 14C testosterone after in-vivo metabolism. Each (.) symbol represents a fraction collected from the HPLC and the amount of 3H testosterone measured in a portion of the fraction using channel 1. Each (+) symbol represents a different portion of the same corresponding fraction measured for 14C steroids using channel 2. Note that metabolism results in at least 4 peaks in addition to testosterone.

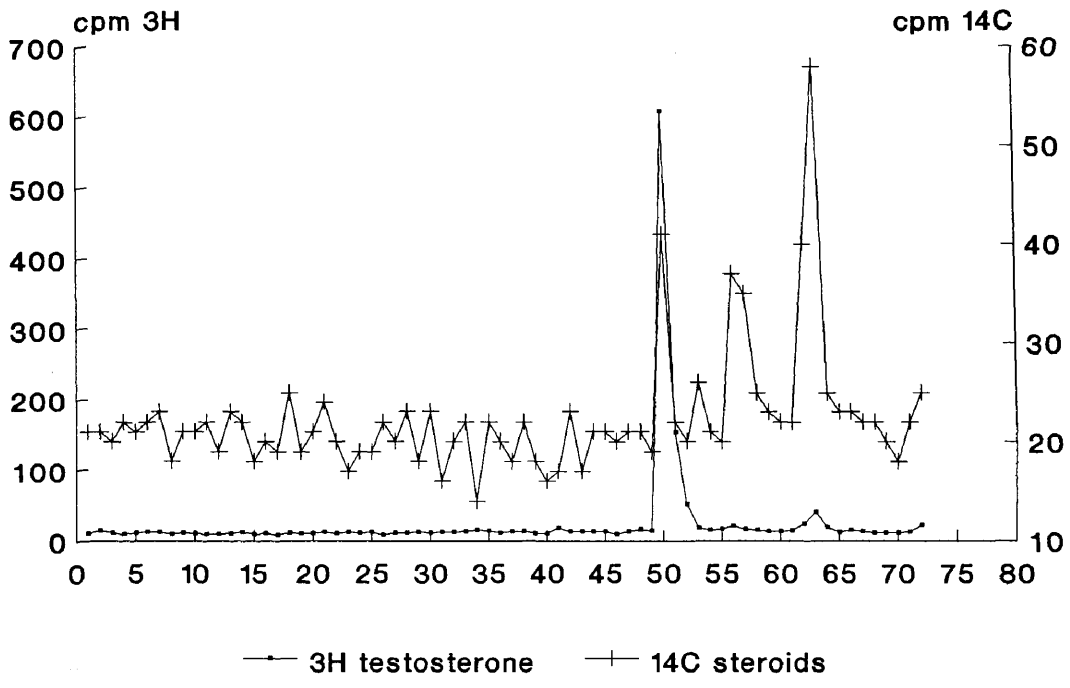

Figure 36. HPLC separation of fecal 14C testosterone after in-vivo metabolism. Each (.) symbol represents a fraction collected from the HPLC and the amount of 3H testosterone measured in a portion of the fraction using channel 1. Each (+) symbol represents a different portion of the same corresponding fraction measured for 14C steroids using channel 2. This profile indicates that fecal metabolites are similar to what is excreted in the urine.

DISCUSSION

Temporal patterns of musth--age and environmental factors

Musth, based on the incidence of temporal gland secretion, occurred in an animal as young as 11 years old. In addition, musth occurred in all animals 15 years and older. When compared to the literature, these results provide additional evidence that musth occurs at an earlier age in North American captive situations.

These results also demonstrate that older animals (21-30 years) as well as younger animals (15-20 years) display a sporadic incidence of musth. Although one animal (Groucho, 19-22 years) displayed a more cyclic incidence of musth on an annual basis. These observations tend to refute the hypothesis that musth patterns are sporadic and short in younger animals, more cyclic and/or prolonged in older animals. However, older animals did remain in musth a greater percentage of the duration of the study than did younger animals. Concentrations of serum testosterone in older musth animals were significantly greater than concentrations of testosterone during musth in younger animals, furthermore, peak concentrations of testosterone demonstrated a general trend towards greater values as animals increased in age. This does appear to represent a continuum of physiological development. Information related to the intensity of musth between animals in this study was difficult to analyze, therefore conclusions based on the intensity of musth and animal age can not be made.

All of the animals in the present study would still be considered young if they were compared to the free-ranging African elephants utilized by Poole (1987a). The author demonstrated that only animals older than 24 experienced musth and after close examination of the data, all of these animals younger than 35 years of age experienced shorter and more sporadic musth periods. Therefore based on this information, the incidence of sporadic musth activity in even the oldest bull in this study is not surprising. However, Jainudeen et. al (1972a) indicated that 90% of domesticated Asian working

bulls experienced musth in a cyclic pattern with irregularities most often observed in animals 15-20 years old. Also, one animal in this study, and two captive Asian males in other studies (Cooper et al., 1990; Niemuller and Liptrap, 1991), all younger than 23 years of age, have exhibited a cyclic pattern of musth. Based on all of this information, the conclusions that can be made are that musth occurs at a younger age in captivity and duration of musth is related to age. Furthermore, based on the information from the oldest bull (Onyx), captive bulls may experience an increased incidence of musth. Finally, a considerable amount of individual variability occurs with no clear predictor of when, or for how long, musth will occur.

In the present study, musth was defined as the incidence of temporal gland secretion. Although this is the most obvious physical sign of musth for the Asian elephant, it may be argued that temporal gland secretion and musth may not be strictly analogous. In several of the animals studied, bouts of temporal gland secretion were separated by small periods of time when temporal gland secretion was not noted (30-45 days). During many of these periods it is not known if testosterone levels remained elevated because samples were not obtained. It must be pointed out however, that for the oldest animal in the present study (Onyx), if these short intermusth periods had not occurred, he would have experienced musth for up to 10 months in length. Temporal gland secretion in the African elephant, unlike the Asian elephant, can occur at times other than during musth. Therefore, Poole (1987a) found that urine dribbling was the most reliable means of defining musth in free-ranging African elephants. Even then, urine dribbling was observed to cease for short periods of time, while temporal gland activity was evident, then subsequently resume. The profile of testosterone concentrations graphed against the degree of temporal gland secretion (figure 10) does support that temporal gland secretion is a good indicator of musth in the Asian elephant. Nevertheless, more stringent methods may be required to define musth, such as, the incidence of elevated testosterone in combination with one or two physical symptoms.

There are a number of other factors for which there were no controls in the present study. These include; the degree of exposure to social primers such as pheromones from estrous females, stress due to management policies used by the individual institutions, photoperiod and climatic conditions. All of these factors could have contributed to the interanimal musth variability demonstrated in this study.

Circulating levels of estradiol--musth and aggression

This study is the first to report circulating concentrations of estradiol in male Asiatic elephants. Plotka et al., (1975) reported that concentrations of estradiol in female Asian elephants range from 9-37 pg/ml serum, however, more recent publications indicate that levels of estradiol are less than 20 pg/ml (Hess et al., 1983; Taya et al., 1991). Based on this information, it appears that serum levels of estradiol in the male Asian elephants during non-musth are similar, but concentrations during musth exceed those reported for female Asian elephants. The non-musth concentrations of estradiol obtained during this study are comparable to the concentrations reported for normal men (Coffey, 1985). The musth concentrations, however, resemble the concentrations reported by Allrich et al., (1982) for the developing boar. The source of the elevated estradiol levels in these male elephants is not known but presumably could come from testicular production and/or aromatization of androgens (Horton, 1976; MacDonald, 1976).

A very strong correlation was demonstrated between testosterone and estradiol concentrations, and elevated concentrations of estradiol, like testosterone, were significantly elevated during musth. From the graphs of testosterone, estradiol and intensity of aggression, there does appear to be a relationship between elevated concentrations of these hormones and increased aggression. The elevated hormone concentrations and increased aggression are also seen to be associated with musth, defined by the occurrence of temporal gland secretion. Based on this information and the information reported by Rasmussen et al., (1990) we now know that all three steroids implicated for a role in mediating male sexual behavior and aggression; testosterone,

dihydrotestosterone and estradiol, are elevated during musth. The role each of these steroids plays at the level of the CNS for mediating desirable male behavior (mating) and undesirable male behavior (aggression) in the Asian elephant awaits further investigation. A recent study published by Crichton et at., (1991), reporting on manipulation of hormone levels in castrated male sheep, demonstrated that dihydrotestosterone alone has little effect on the CNS, estradiol alone has a reduced effect but dihydrotestosterone appears to potentiate the effect of estradiol on the CNS, equal to the level of male sexual behavior obtained from administration of testosterone. Perhaps similar studies on castrated male elephants (if available) would provide useful information on the role of these steroids in controlling musth behavior.

It must also be mentioned here that serum binding proteins such as testosterone-estrogen-binding globulin (TeBG) may also play a significant role in the biological effect of these steroids. In the human male, less than 2% of the total testosterone in plasma circulates in the free form and it is generally accepted that only unbound steroids are biologically active (Coffey, 1988). Furthermore, changes in the hormone ratios can have a profound effect on the amount of the individual steroids circulating in bound/free form (Coffey, 1988). In the present study, hormones were measured in serum by specific RIA after ether extraction. This procedure eliminates the conjugated forms of the steroids but also liberates the steroid from the binding proteins, resulting in estimation of total unconjugated steroid. Further work is needed to determine if changes in bound/free ratios of these relevant steroids occur during the musth cycle.

Pituitary luteinizing hormone stimulation of testosterone secretion and musth

A strong positive correlation between serum testosterone and LH is evidence that the increased concentrations of immunoreactive testosterone are caused by LH stimulation of testicular steroidogenesis. This correlation occurred independent of the musth status. Since it was clear that serum LH levels begin to rise before musth and that during musth, levels of LH begin to decrease (figure 15), concentrations were not analyzed for

significant differences between musth and non-musth. Although more frequent sampling is necessary for fully describing the change in pituitary LH secretion, the increasing then decreasing circulatory level observed for the elephant in this study is comparable to what occurs in male fallow deer during the transition into the rut (Asher and Peterson, 1991). This provides additional evidence for the similarity between musth and rut.

These data also indicate that it is important to reinvestigate the report of Neimuller and Liptrap (1991) indicating no difference in the pulse frequency of LH during musth and non-musth. It may be necessary to delineate between the transition into musth as well as various stages as the animal progresses through the musth cycle.

Noninvasive methods to monitor musth

The results of the creatinine analysis conflict with the data presented by Brannian et al., (1989) for one male African elephant. No significant differences were detected in musth and non-musth creatinine concentrations in the present study. The concentrations of creatinine obtained for this study were intermediate in comparison to the musth and non-musth concentrations reported by Brannian et al., (1989) but exhibited a greater degree of variability (non-musth 0.399 $\pm$ 0.27, musth 0.48 $\pm$ 0.41 vs non-musth 0.24 $\pm$ 0.08, musth 1.47 $\pm$ 0.17 mg/ml, respectively). The dilute concentrations of creatinine obtained from the Fort Worth Zoo animals were presumably due to the design of the facility and the routine used by the keepers while watering the elephants.

Once it was demonstrated that the majority of testosterone immunoreactivity in the urine was conjugated, the methods reported by Poole et al., (1984) were employed. The urinary concentrations of immunoreactive testosterone obtained during musth in this study (733 $\pm$ 674 ng. mg Cr) were higher than the concentrations of testosterone (241 $\pm$ 78.4 ng/mg Cr) reported by Poole et. al., (1984) but very similar to the concentrations of urinary androstenedione (737 $\pm$ 134 ng/mg Cr) reported by Brannian et al., (1989). Both Poole et al., (1984) and Brannian et al., (1989) studied African elephants. Non-musth concentrations in the present study (335 $\pm$ 270 ng/mg Cr) were much higher than

non-musth concentrations reported by Poole et al., (1984) (146.7 $\pm$ 62.6 ng/mg Cr) and Brannian et al., (1989) (103 $\pm$ 8 ng/mg Cr). A greater degree of variation about the mean was also evident when comparing the results of the present study to those of the previous studies. The present study also utilized a much greater number of urine samples and employed serial sampling. The analysis of urine samples from the Fort Worth Zoo animals presented some interesting results. When measuring urinary hormones, many investigators exclude urine samples that are below 0.1 mg/ml creatinine (Kirkpatrick et al., 1991; Monfort et al., 1991). Practically all of the samples obtained from the Fort Worth zoo animals surpassed this threshold level. These samples produced hormone concentrations much higher than the remaining group after they were indexed for mg/ml creatinine but the profiles still produced useful trends in hormone secretion (see figure 23).

The results of the initial experiments to develop the fecal assay clearly demonstrate how each step of the assay was developed. When experimentation began for the fecal assay, very little information was available on the methods of analysis. Furthermore, there had been no reports of the use of fecal analysis to measure testosterone. Using the method of Mostl et al., (1984), it appeared that testosterone was present in the feces of male Asian elephants and experimentation was then performed to improve the analysis. Concentrations of immunoreactive testosterone per gram of feces is similar to the range of testosterone per ml serum. The fecal androgen concentrations obtained in this study were not comparable to the androgen concentrations reported by Flood et al., (1992), for intact adult male Muskoxen (461 $\pm$ 26 ng/gram). This however, is most likely due to the fact that Flood et al., (1992) reported concentrations per gram of dry weight.

There is no doubt that collection of urine or fecal samples from these animals is much easier than collecting serum. Ease of collection however, may not always justify the decision to investigate certain endocrinological events without obtaining a blood sample. The type of hormone (ie. steroid, protein), the mode of utilization (ie. telocrine,

paracrine), the rhythm of secretion (ie. infradian, ultradian) type of transport (ie. prehormone, bound/free) and mode of excretion, to name a few, must be carefully examined before making this decision. The present investigation was confined to a study of steroid secretion and specifically to the androgen testosterone. Testosterone, produced by the Leydig cells of the testes is released into the general circulation via the spermatic venous system and is then available to activate various cellular functions throughout the body (Griffin and Wilson, 1985). The general circulation therefore, reflects changes in this hormones secretion rate. If the metabolism and excretion rate for this hormone remains relatively constant or can be corrected for excretion rate, then urinary and/or fecal hormone concentrations should equally reflect the testosterone secretion rate. This investigation demonstrated a significant positive relationship between urine, fecal and serum testosterone but the strength of the correlation was not high. The fact that both urine and fecal testosterone concentrations were significantly elevated during musth, however, indicates that these assays are useful for monitoring musth. Evaluating the individual profiles provides additional evidence for the value of these assays. Although both assays appear useful, the fecal assay may be the method of choice with this species because of the simplicity of the assay and the convenience of sample collection

There may have been several factors which affected this correlation. Some of these factors can be controlled, thereby improving the strength of the correlation. Retrospectively, there were some inefficient aspects of the assays that could have contributed to variability. The ^{14}C testosterone experiments demonstrated that 70% of the activity in the urine was conjugated and a considerable amount of these conjugates were resistant to the enzyme hydrolysis methods used. Furthermore, the HPLC analysis of the in-vivo metabolized ^{14}C testosterone in the urine indicated that the largest peak of activity is not testosterone. A direct assay to the conjugated form of this hormone would limit the number of steps required and therefore limit the variability. For the fecal assay, although the ethanol solubilization procedure demonstrated adequate recovery (73.8%),

when the evaporated acetone-hormone mixtures were reconstituted in assay buffer for assay, recovery was reduced (51.3%). This indicates some adherence of hormone to the tubes which could be related to the abundant amount of green pigments extracted from the fecal sample during solubilization. Identifying a simple method of separating these pigments from the steroids may limit adherence of steroids to the assay tubes and thereby limit some of the variability.

Another factor which can affect the strength of the correlation is the matching of samples for the regression analysis. In this analysis, each serum sample was compared to a corresponding urine and/or fecal sample collected on the same day, at or about the same time. The results of the ^{14}C testosterone experiments indicate that this is not ideal. In the present study, it appears that circulating testosterone is excreted within 24 hours. Previous work indicates that the majority or urinary steroid excretion occurs within the first 2 hours in the elephant but continues for up to 16 hours (Czekala et al., 1992). In the feces the peak of excretion appears to be delayed for up to 58 hours. Based on this information, a much greater correlation would most likely be produced if serum samples were compared to urine samples collected 2-24 hours later and fecal samples collected 48-58 hours later.

Another concern when comparing serum levels of testosterone to testosterone levels in the urine and feces is the pattern and/or rhythm of secretion. In a number of species, including the elephant, the testicular Leydig cells respond to the pulsatile secretion of LH by releasing testosterone in a pulsatile manner (Sanford et al., 1976; Lincoln and Kay, 1979; Niemuller and Liptrap, 1991). Variations in testosterone concentration as great as threefold can be demonstrated during a 24 hour sampling period (Griffin and Wilson, 1980). The data on daily levels of testosterone during musth for the bull "Onyx" presented in this study add additional evidence that a considerable amount of daily variation in serum testosterone occurs during musth. Testosterone is also secreted diurnally in a variety of mammalian species (Tureck and vanCauter, 1988). The

concentration of testosterone measured in the serum reflects the concentration in circulation at that precise moment. On the other hand, the results of the ^{14}C testosterone experiments indicate that circulating testosterone is excreted over a 24 hour period in the urine and over a 40 hour period in the feces. Therefore, what is measured in the urine and/or feces at a given point, is an additive or general value of what was in the serum over an extended time period. The effect this has on the hormone profile is probably small for the urine assay but much more dramatic for the fecal assay. Fecal testosterone would most likely exhibit less ultradian variation. This in fact appears to be the case, since fecal testosterone produced less variation than serum testosterone obtained during this study. Because of this, the need to obtain a stronger correlation may not be necessary or even possible in some cases. In addition, fecal hormone analysis may actually have advantages over serum and urinary analysis for long-term endocrinological studies when samples are collected infrequently.

Obtaining parallelism between the standard curves and the serial diluted extracted feces and urine indicate that the assays used were quantitative. The results of the HPLC analysis, however, indicates that the antiserum used cross-reacted with other steroids in the urine and feces. The non-specific immunoreactive peaks observed for the urine extract were very small, therefore the hormone concentrations reported are a good estimate of testosterone. The non-specific immunoreactive peaks observed for the fecal assay are more significant, therefore, the hormone concentrations reported cannot be considered specifically testosterone. The identification of these non-specific immunoreactive peaks was not attempted because the assay was quantitative and the use of these procedures will not be confined to the use of this antiserum only. Different antisera would probably alter the number and affinity of cross-reacting substances. It should be mentioned, however, that several steroids were tested for cross-reactivity with this antiserum. Significant cross-reactivity was found for only one endogenous steroid, 50% for dihydrotestosterone. All other endogenous steroids were 1% or less. Some of

the non-specific immunoreactive peaks detected by HPLC could be this steroid and/or isoforms of this steroid. The remaining steroids tested which did not produce significant cross-reactivity can be eliminated as a possible identity of the HPLC peaks.

The 64% of radioactivity recovered after injection in the present study is comparable to what was reported by Hindle and Hodges (1990) for the white rhinocerous (61%). In the present study, however, it is believed that a considerable amount of urine may have been lost and would account for some of the remaining radioactivity. Considering this, these results still indicate that over half of the injected label was excreted in the feces. Urinary excretion of ^{14}C testosterone occurred within 24 hours. This is comparable to the results of Czekala et al., (1992) for urinary excretion of ^{3}H estradiol in a female Asian elephant. The authors reported that the peak of activity occurred during the first collection, 30 minutes post-injection and the activity had returned to background within 16 hours. Although, the labeled steroid was injected intramuscularly in the present study and would slow down excretion to some degree, more frequent collection would probably have demonstrated quicker excretion. Very little excretion was reported on the first day by Hindle and Hodges (1990) presumably due to administration of a sedative for injection of the label. Forty-five percent of the administered ^{14}C testosterone was recovered within 96 hours post-injection in gelded horses (Hougnton and Dumasia, 1979).

Fecal excretion of radioactivity peaked at 48-58 hours post-injection in the present study which is comparable to the fecal excretion of radioactivity reported by Hindle and Hodges (1990). No activity was detected when feces were collected for 24 hours after injection from a female Asian elephant (Czekala et al., 1992).

The demonstration of several radioactive peaks after HPLC separation of in-vivo metabolized ^{14}C testosterone is not surprising. The metabolism of testosterone, as demonstrated by Houghton and Dumasia, (1979) for the horse, is substantial. Identification of all of the radioactive peaks is beyond the scope of this project and

therefore is not reported here. This of course, does not mean that identification of the radioactive compounds would not have some significance for understanding musth. Perhaps these types of studies would be beneficial for determining if metabolic changes occur in animals of different age groups or musth status. For purposes of this project, we can conclude that circulating testosterone is excreted as testosterone in the urine and feces, mostly conjugated in the urine and non-conjugated in the feces. Testosterone, however, is not the major excreted metabolite of circulating testosterone. The major metabolite of circulating testosterone is less polar than testosterone. Finally, the same metabolites appear to be excreted in both the urine and the feces.

Possible methods of manipulating musth

Before we can even begin to select a suitable method of manipulating musth we must visualize the interaction of factors which are involved in the regulation of this complex phenomenon. Based on a model about control of the reproductive endocrine system in other mammals (Bronson, 1988) and what has been elucidated about musth in this and other studies, a model related to regulation of musth has been constructed (Figure 37). This model, an oversimplification, must be considered preliminary due to the lack of knowledge about musth. It will however, facilitate the selection and elimination of certain mechanisms which can be altered for manipulation of musth.

Musth is most probably mediated by an interaction of environmental influences as have been proposed by Bronson (1988) for seasonal regulation of breeding in mammals. The environmental stimuli depicted at the top of this model exert their effect indirectly or directly on the neuroendocrine pathways located in the higher regions of the brain. There is direct and anecdotal evidence to support some of these influences in the male elephant. Social primers include pheromones from estrus females and/or other male elephants Jainudeen et al., 1972a; Rasmussen et al., 1984). Emotional stress includes subordination to other musth males or could include subordination to human handlers in

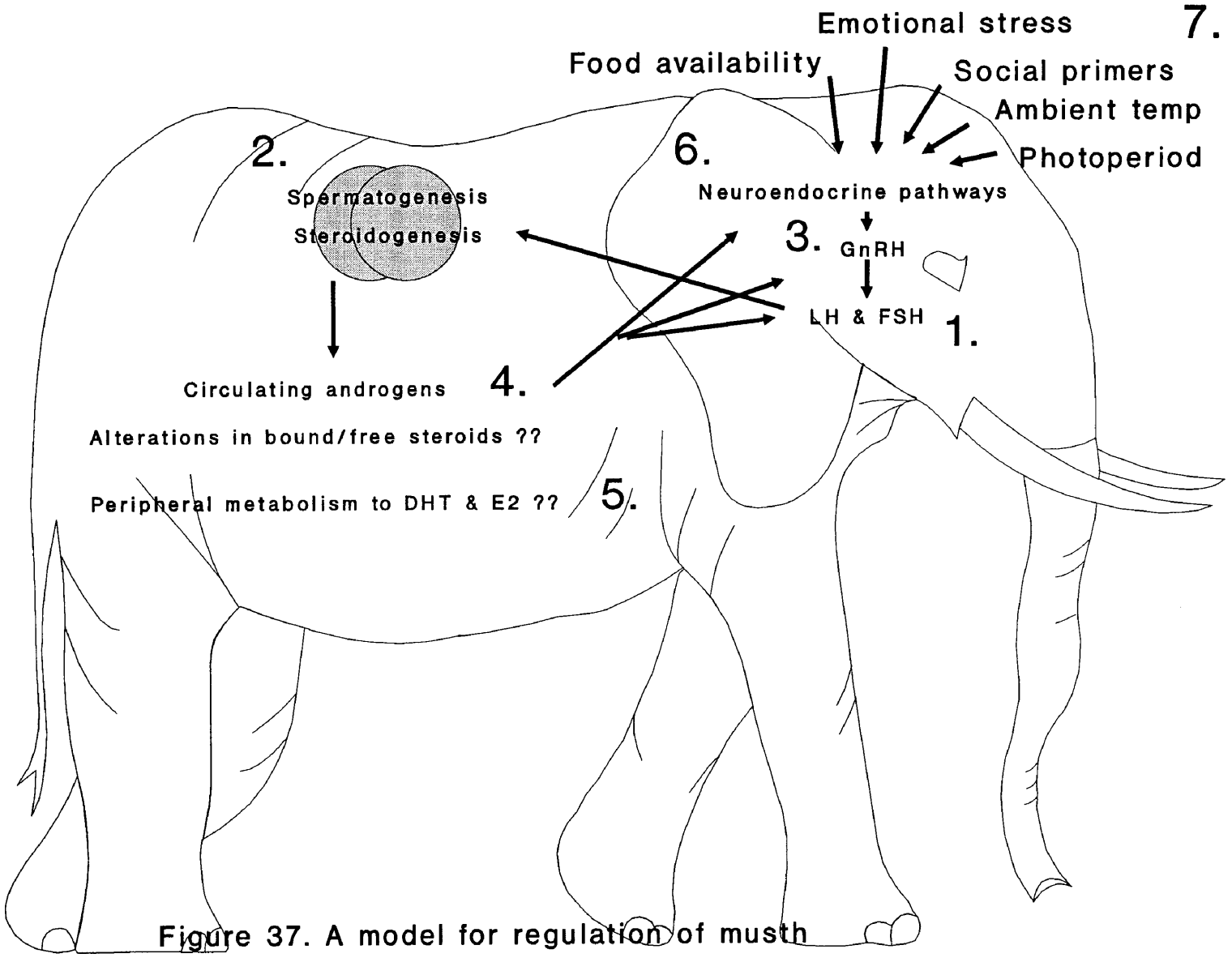

Figure 37. A model for regulation of musth

captivity. In fact, Bronson and Eleftheriou (1965) have presented evidence that habitual subordination in mice can result in depressed LH release just by the presence of the dominant individual. There is direct evidence for this suppressive effect on male elephants by other dominant males (Poole, 1989a). and anecdotal evidence supports the effect that dominant human handlers can have on male elephants (Jeff Glazier, personal communication; Ross Garcia, personal communication). Other emotional stress could include, confinement, transferring the male from one institution to another or changes in the structure of the captive herd. As reported by Jainudeen et al., (1972a), sickness and overwork also tend to suppress musth activity.

Nutrition, or food availability, have been reported to exert some effects on musth with the majority of information indicating a suppression (Jainudeen et al., 1972a; Poole 1987a; Cooper et al., 1990).

Neither photoperiod nor ambient temperature have been investigated as stimuli capable of altering musth but may also be important. In seasonal rutting animals, photoperiod plays a major role in regulating the LH and testosterone cycle. However, other environmental cues (nutrition, dominance, stress etc.) also play a part. It is feasible that musth is regulated much less, but to some extent, by photoperiod and is more strongly affected by the other environmental stimuli. Investigation of the influence of photoperiod and/or ambient temperature on musth would require rigorously controlled experiments. For now we must extrapolate from what is known about other species of mammals.

The neuroendocrine pathways mediated directly or indirectly by these environmental stimuli relay the appropriate stimulatory or inhibitory signals to the hypothalamus causing alterations in the pulsatile secretion of GnRH. Changes in GnRH secretion are then responsible for the pattern and amount of LH and FSH secretion from the pituitary. We also know from studies in sheep (Lincoln and Short, 1980) that the pituitary gonadotrophs and the testes change in respect to there responsiveness to GnRH and LH

respectively. The result of all this is an increase in testicular steroid secretion and increased circulating levels of these steroids. Increased circulating levels of androstenedione, testosterone, dihydrotestosterone (DHT) and estradiol (E2) are now known to increase in the male elephant during musth (this study; Rasmussen et al., 1984; Niemuller and Liptrap, 1991). It is not known if increases in DHT and/or E2 are due to testicular production or reductase and aromatase activity in the male elephant. Nor do we know if alterations in protein bound/free steroid ratios are important here. However, testosterone and estradiol are known to be important for feedback effects on the hypothalamopituitary axis in other mammals (Negro-Vilar et al., 1988). Also, there is evidence that DHT and E2, produced by the testes or by peripheral and/or neural metabolism, have an effect on the mammalian brain to control male sexual behavior and aggression (Callard et al., 1978; Brain, 1979; Crichton et al., 1991).

Along with this visual conceptualization, a numbering of areas that could be manipulated to possibly alter the mechanisms responsible for mediating musth or aggressive behavior is included. Important here is whether or not the particular individual is valuable for breeding purposes and if so, the animal's stage of physiological development. Since we are dealing with the reproductive endocrine system, if the animal is needed for breeding purposes, then our goal is to alter the magnitude of the chemical messages responsible for aggression without adversely affecting fertility or libido. This becomes even more critical when we are dealing with immature animals since alterations could irreversibly alter normal physiological development.

Pituitary gonadotropin secretion can be manipulated by the administration of potent GnRH agonists or antagonist (number 1, pituitary). Antagonist function by interfering with the binding of natural GnRH to the receptors on the pituitary gonadotrophs resulting in decreased LH and FSH secretion and subsequently testicular spermatogenesis and steroidogenesis. GnRH agonists have a more complex mechanism of action. These agonists bind to the pituitary GnRH receptors causing an initial increase in LH and

testosterone secretion followed shortly by a paradoxical inhibition of gonadotropin secretion and consequently testosterone secretion (Labrie et al., 1980). The mechanisms of action of agonists are multiple and include; reduction of pituitary gonadotropin responsiveness, a reduction of testicular gonadotropin receptors, direct effects on testicular steroidogenesis and inhibition of spermatogenesis including degeneration of the testes and the accessory organs (Labrie et al., 1980). Certain dosages of the GnRH analogs essentially shut down gonadotropin and testicular function. For this reason, use of these compounds for controlling musth should be approached with caution if the animal is needed for breeding purposes.

As discussed by Brain, (1979) castration-induced reductions in aggression are among the more consistent phenomena in the field of hormone/aggression correlation's. Therefore, castration (number 2, testes) would be an obvious choice for elephants not needed for breeding purposes. For the elephant, however, castration requires major surgery since the testes are located intra-abdominally. Although castration of the male Asian elephant is possible (Fowler et al., 1973) no scientific data have been published regarding post-castration sexual or aggressive behavior.

Interfering with the binding of androgens to target tissue in the CNS (number 3, CNS) is another possible method of controlling the aggressive behavior associated with musth. The administration of anti-androgens such as cyproterone acetate or flutamide interfere with androgen action by inhibiting binding at the receptor level and have altered sexual behavior to some extent in other mammals (Sachs and Meisel, 1988). However, since it is not known which steroids or combination of steroids are responsible for the aggressive behavior in the male elephant, anti-androgen experiments should probably be expanded to include anti-estrogens as well.

Manipulation of circulating hormone levels could be accomplished by passive or active immunization against the selected steroids and/or administration of selected steroids (number 4, circulating androgens). Immunization would reduce the amount of

steroid available to bind with target tissue, however, a number of factors would need to be considered here, including the effect of repeated treatment and the degree of reversibility. Administration of steroids may have an effect on gonadotropin secretion via the negative feedback system. Here again it would be important to know which steroids are responsible for mediating aggression and which steroids are most effective for negative feedback on the hypothalamopituitary axis in the male elephant.

Number 5 represents the use of aromatase or 5-alpha-reductase enzyme inhibitors. If it can be demonstrated in the elephant, that peripheral or neural conversion of testosterone to estradiol via the aromatase enzyme and/or conversion of testosterone to dihydrotestosterone via the 5-alpha-reductase enzyme, is responsible for mediating the aggressive manifestations of musth, then inhibiting this conversion would be a possible method by which to control aggression. In fact, experimentally blocking these enzymes may be useful in determining if metabolism of testosterone to these active metabolites are responsible for the aggressive behavior. To add support to this hypothesis, Brain (1979) has demonstrated that specific doses of anti-aromatases (4-androsten-3,6,17trione and androst-1,4,6-triene,3,17dione) will prevent testosterone induced fighting in castrated mice.

Another possible but somewhat more complicated method of suppressing musth is represented by number 6, the CRF/ACTH/adrenal axis. This method would include the administration of hormones that have been investigated for their role in the stress response. Stress induces an elevation of endogenous opiods, corticotrophin releasing hormone (CRF), adrenocorticotrophic hormone (ACTH) and corticosteroids (Hulse and Coleman, 1981). Endogenous and exogenous opiods have been demonstrated to have a suppressive effect on LH secretion in mammals (Gabriel et al., 1985; Gilbeau et al., 1985; Matteri and Moberg, 1985; Schanbacher, 1985). As have other areas of the CRF/ACTH/adrenal axis (Welsh and Johnson, 1981; Echterkamp, 1984; Rivier and Vale, 1984). Bronson, (1988) indicates that the CRF/ACTH/adrenal axis appears to act

at higher neuroendocrine elements as well as secondary elements downstream. Therefore, the decision of which hormone, the dosage and the timing of administration would require a great deal of experimentation. To complicate things further, all of this data is conflicting with data reviewed by Brain, (1979) demonstrating some aggression stimulating effects of ACTH and corticosteroids. Nevertheless, these areas warrant investigation for a role in mediating musth in the male elephant. Some of the old remedies used for controlling musth in Asian elephants include administration of doses of opium (McGaughey, 1963).

Finally, musth could probably be influenced by altering the environmental factors which are involved in the regulation of musth (number 7, environmental influences). Changing the nutritional availability, social domination and exposure to estrous female elephants would alter musth to some degree. Again, it is possible that photoperiod and/or ambient temperature could also be used as a tool in regulating musth. The work of Rasmussen et al., (1990) indicates that it is conceivable to isolate certain volatile substances involved in chemical communication between elephants. Specific substances could then be used for manipulating musth behavior.

SUMMARY

Serum, urine and fecal samples were collected along with behavioral data from 11 male Asian elephants ranging from 4-30 years of age for up to 4 years. Serum samples were measured for testosterone, estradiol and luteinizing hormone by radioimmunoassay following validation. Fecal and urinary testosterone assay procedures were developed and validated. All of the endocrine data were subsequently correlated with two common symptoms of musth; temporal gland secretion and aggressive behavior. The in-vivo metabolism and excretion of testosterone in one male Asiatic elephant was studied after injection of 233 uCi Carbon 14 testosterone using HPLC.

Serum testosterone concentrations analyzed by Newman-Kuels demonstrated that musth testosterone concentrations were significantly greater than non-musth testosterone concentrations. Also, musth values for the 21-30 year old group were significantly greater than musth values for the 15-20 year old group. A trend towards greater testosterone concentrations and increased incidence of musth with increasing age was demonstrated. Neither behavioral nor endocrine data revealed a clear predictor as to when or for how long musth will occur. Serum concentration of estradiol during musth were significantly greater than during non-musth and a significant positive linear relationship was detected between serum testosterone and estradiol. Time series plots illustrate that a higher intensity of aggression is shown to be associated with greater testosterone and estradiol concentrations. Positive linear relationships were observed between individual serum testosterone and LH concentrations. Combining LH data representing the transition period into musth for 10 musth periods revealed that LH levels begin to rise 4 weeks before, and begin decreasing immediately after, the onset of temporal gland secretion.

There were no significant differences between urinary creatinine concentrations during musth and non-musth samples. Analyzing samples consistently diluted with water

before collection and containing less than 0.01 mg/ml creatinine produced useful urinary testosterone profiles. Concentrations of urinary testosterone were significantly elevated during musth and a positive linear relationship was observed between serum and urinary testosterone values. A simplified RIA for fecal testosterone analysis was developed and utilizing this procedure fecal testosterone levels during musth were observed to be significantly greater than during non-musth. A positive linear relationship was also demonstrated between serum and fecal testosterone.

After injection of ^{14}C testosterone into one male Asiatic elephant during musth, 57% of the radioactivity was excreted in the feces, 7% in the urine and 36% was unrecovered. The majority of urinary testosterone was excreted within 24 hours in the urine whereas testosterone excretion in the feces was prolonged and peaked 48-58 hours post-injection. In the urine, 70% of the excreted steroids were conjugated. These conjugates were somewhat resistant to enzyme hydrolysis but were adequately liberated by acid solvolysis. Nearly 100% of steroids in the feces were unconjugated. In-vivo metabolism of testosterone was extensive as revealed by HPLC. Fecal and urinary profiles were similar and demonstrate five distinct peaks of radioactivity. One of these peaks co-eluted with testosterone standard, however, the largest peaks were less-polar metabolites.

LITERATURE CITED

Abraham, G.E., Swerdloff, R. Tulchinsky, D. & Odell, W.D. (1971) Radioimmunoassay of plasma progesterone. *J. clin. Endo. Metab.* **32,** 619-624.

Allrich, R.D., Christenson, R.K., Ford, J.J. & Zimmerman, D.R. (1982) Prepubertal development of the boar: Testosterone, estradiol-17B, cortisol and LH concentrations before and after castration at various ages. *J. Anim. Sci.* **55,** 1139-1142.

Asher, G.W., Peterson, A.J. & Bass, J.J. (1989) Seasonal pattern of LH and testosterone secretion in adult male fallow deer, (*Dama dama*) *J. Reprod. Fert.* **85,** 657-665.

Asher, G.W. & Peterson, A.J. (1991) Pattern of LH and testosterone secretion of adult male fallow deer (*Dama dama*) during the transition into the breeding season. *J. Reprod. Fert.* **91,** 649-654.

Bamberg, E., Choi, H.S., Mostl, E., Wurm, W., Lorin, D. & Arbeiter, K. (1984) Enzymatic determination of unconjugated oestrogens in faeces for pregnancy diagnosis in mares. *Equine vet. J.* **16,** 537-539.

Bamberg, E., Mostl, E., Patzl, M. & King, G.J. (1991) Pregnancy diagnosis by enzyme immunoassay of estrogens in feces from nondomestic species. *J. Zoo Wild. Med.* **22,**(1) 73-77.

Benedict, F.G. (1936) *The Physiology of the elephant.* Carnegie Institute, Washington.

Brain, P.F. (1979) Effects of endocrine manipulations on fighting and threat in vertebrates. In: *Hormones, Drugs and Aggression.* vol 3 pp. 50-75, Eden Press. St Albans, Vermont.

Brannian, J.D., Griffin, F. & Terranova, P.F. (1989) Urinary androstenedione and luteinizing hormone concentrations during musth in a mature African elephant (*Loxodonta africana*). *Zoo Biol.* **8,** 165-170.

Bronson, F.H. (1988) Seasonal regulation of reproduction in mammals In: *The Physiology of Reproduction.* pp. 1860-1863. Eds. E., Knobil & J.D., Neill. Raven Press. New York.

Bronson, F.H. & Eleftheriou, B.E. (1965) Adrenal response to fighting in mice: separation of physical and phsycological causes. *Science.* **147,** 627-628.

Brown, J.L., Citino, S.B., Bush, M., Lehnhardt, J. & Phillips, L.G. (1991) Cyclic patterns of luteinizing hormone, follicle-stimulating hormone, inhibin, and progesterone secretion in the Asian elephant (*Elephas maximus*) *J. Zoo Wild. Med.* **22**,1: 49-57.

Buiter, R.M., Schaftenaar, W. & Dieleman, S.J. (1992) Application of fecal steroid analysis to several zoo animals in the Netherlands and Belgium. *Proc. 1st Int. Sym. on Fecal Steroid Monitoring in Zoo Animals,* Rotterdam Zoo. pp 21-24.

Buss, I.O. & Johnson, O.W. (1967) Relationships of leydig cell characteristics and intratesticular testosterone levels to sexual activity in the African elephant (*Loxodonta africana). Anat. Rec.* **157,** 191-196.

Callard, G.V., Zoltan, P., & Ryan, K.J. (1978) Conversion of androgen to estrogen and other steriods in the vertebrate brain. *Amer. Zool.* **18,** 511-523.

Chappel, S.C. & Schmidt, M.J. (1979) Cyclic release of luteinizing hormone and the effects of luteinizing hormone-releasing hormone injection in Asiatic elephants. *Am. J. Vet. Res.* **40,** 3: 451-453.

Coffey, D.S. (1985) Biochemistry and physiology of the prostate and seminal vesicles. In: *Campbell's Urology.* 5th ed. Eds. P.C., Welsh et al. W.B. Saunders. Philidelphia.

Coffey, D.S. (1988) Androgen action and the sex accessory tissues. In: *Textbook of Endocrinology.* pp. 1086-1090. 7th ed. Eds. J.D., Wilson & D.W., Foster. W.B. Saunders. Philadelphia.

Cooper, K.A., Harder, J.D., Clawson, D.H., Fredrick, D.L., Lodge, G.A., Peachey, H.C., Spellmire, T.J. & Winstel, D.P. (1990) Serum testosterone and musth in captive male African and Asian elephants. *Zoo Biol.* **9,** 297-306.

Crichton, J.S., Lishman, A.W., Hundley, M. & Amies, A. (1991) Role of dihydrotestosterone in the control of sexual behaviour on castrated male sheep. *J. Reprod. Fert.* **93**, 9-17.

Czekala, N.M., Roocroft, A., Bates, M., & Lasley, B.L. (1992) Estrogen metabolism in the Asian elephant (*Elephas maximus*). *Biol. Reprod.* **11,** 75-80.

Darwin, C. (1871) *The decent of man and selection in relation to sex.* 2nd ed. A.L. Burt. New York.

Echterkamp, S.E. (1984) Relationship between LH and cortisol in the acutely-stressed beef cow. *Theriogenology.* **22,** 305-311.

Eisenberg, J.F., McKay, G.M. & Jainudeen, M.R. (1971) Reproductive behavior of the Asiatic elephant (*Elephas maximus*). *Behavior* **38,** 193-225.

Espmark, Y. (1964) Rutting behavior in reindeer (*Rangifer tarandus L.*) *Anim. Behav.* **12,** 159-163.

Fennessy, P.F., Suttie, J.M.,, Crosbie, S.F. Corson, I.D., Elgar, H.J. & Lapwood, K.R. (1988) Plasma LH and testosterone responses to gonadotropin-releasing hormone in adult red deer (*Cervus elaphus*) stags during the annual antler cycle. *J. Endocr.* **117,** 35-41.

Flood, P.F., Thrush, B.J., Clark, G.D., Rawlings, N.C. & Cook, S. (1992) Androgens in the feces of male muskoxen. *Proc. 1st Int. Sym. on Fecal Steroid Monitoring in Zoo Animals,* Rotterdam Zoo. pp. 84-89.

Forrest, D.W., Fleeger, J.C., Long, C.R., Sorenson Jr., A.M. and Harms, P.G. (1980) Effect of exogenous prolactin on peripheral luteinizing hormone levels in ovarectomized cows. *Biol. Reprod.* **22,** 197-201.

Fowler, M.E. & Hart, R. (1973) Castration of an Asian elephant, using etorphine anesthesia. *J. Am. Med. Ass.* **163,** 539-543.

Gabriel, S.M., Simpkins, J.W., Kalra, S.P. & Kalra, P.S. (1985) Chronic morphine treatment induces hypersensitivity of testosterone-negative feedback in castrated male rats. *Neuroendocrinology.* **40,** 39-44.

Ganong, W.F. (1987) Renal function and micturition. In: *Review of medical physiology.* pp 581-606. 13th ed. Appleton & Lange. California.

Gilbeau, P.H., Almirez, R.G., Holaday, J.W. & Smith, C.G. (1985) Opiod effects on plasma concentrations of LH and prolactin in the adult rhesus monkey. *J. Clin. Endo. Metab.* **60,** 299-305.

Griffen, J.E. & Wilson. J.D. (1985) Disorders of the testes and male reproductive tract. In: *Textbook of Endocrinology.* pp. 269-270. 7th ed. Eds. J.D., Wilson & D.W., Foster. W.B. Saunders. Philadelphia.

Griffen, J.E. & Wilson. J.D. (1980) The testis. In: *Metabolic control and Disease.* 8th ed. Eds. P.K., Bondy. & L.E., Rosenberg. W.B. Saunders, Philadelphia.

Gross, T.S. (1992) Development and use of fecal steroid analysis in several carnivore species. *Proc. 1st Int. Sym. on Fecal Steroid Monitoring in Zoo Animals,* Rotterdam Zoo. pp.55-61.

Halasz, B., Kiss, J. & Molnar, J. (1989) Regulation of the gonadotropin-releasing hormone (GnRH) neuronal system: Morphological aspects. *J. Steroid Biochem.* **33,** 663-668.

Hall, P.F. (1988) Testicular steroid synthesis: Organizaztion and regulation. In: *The Physiology of Reproduction.* pp. 975-994. Eds. E., Knobil & J.D., Neill. Raven Press. New York.

Hall-Martin, A.J. (1987) Role of musth in the reproductive stategy of the African elephant *(Loxodonta africana). S. Afr. J. Sci.* **83,** 616-620.

Hall-Martin, A.J. & Van Der Walt, L.A. (1984) Plasma testosterone levels in relation to musth in the male African elephant, *(Loxodonta africana) Koedoe* **27,** 147-149.

Hess, D.L., Schmidt, A.M. & Schmidt, M.J. (1983) Reproductive cycle of the Asian elephant (*Elephas maximus*) in captivity. *Biol. Reprod.* **28,** 767-773.

Hindle, J.E. & Hodges, J.K. (1990) Metabolism of oestradiol-17B and progesterone in the white rhinoceros (*Ceratotherium simum simum*). *J. Reprod. Fert.* **90,** 571-580.

Hodges, J.K., Tari, S. & Heistermann, M. (1992) Fecal steroid excretion during the ovarian cycle in marmosets and tamarins. *Proc. 1st Int. Sym. on Fecal Steroid Monitoring in Zoo Animals,* Rotterdam Zoo. pp. 47-48.

Holtz, W. (1992) Pregnancy diagnosis through fecal estrogens in goats. *Proc. 1st Int. Sym. on Fecal Steroid Monitoring in Zoo Animals,* Rotterdam Zoo. pp. 62-67.

Hoppen, H.O., Diaz de Aguirre, L., Hagenbeck, D., Boer, M. & Schwarzenberger, F. (1992) Progesterone metabolites in elephant feces. *Proc. 1st Int. Sym. on Fecal Steroid Monitoring in Zoo Animals,* Rotterdam Zoo. pp. 51-55.

Horton, R.J. (1976) Benign prostatic hyperplasia. *Proc. of a workshop sponsored by the Kidney Disease and Urology Program of NIAMDD.* pp. 183-190. U.S. Gov. printing office, Washington, D.C.

Houghton, E. & Dumasia, M.C. (1979) Studies related to the metabolism of anabolic steroids in the horse: testosterone. *Xenobiotica.* **9,** 269-279.

Howard, J.G., Bush, M., deVos, V. & Wildt, D.E. (1984) Electroejaculation, semen characteristics and serum testosterone concentrations of free-ranging African elephants (*Loxodonta africana*). *J. Reprod. Fert.* **72,** 187-195.

Hulse, G.K. & Coleman, G.J. (1981) The role of endogenous opiods in the blockade of reproductive function in the rat following exposure to acute stress. *Pharm. Biochem. Behav.* **19,** 795-799.

Ilhus, A.W., Haynes, N.B., Lamming, G.E., Howles, C.M., Fairall, N. & Millar, R.P. (1983) Evaluation of LH-RH stimulation of testosterone as an index of reproductive status in rams and its application in wild antelope. *J. Reprod. Fert.* **68,** 105-112.

Jacobsohn, G.M. & Lieberman, S. (1962) Studies on the chemical clevage of the urinary glucuronosides of the 17-Ketosteroids. *J. Biol. Chem.* **227,** 1469-1475.

Jainudeen, M.R., Eisenberg, J.F. & Jayasinghe, J.B. (1971) Semen of the Ceylon elephant, (*Elephas maximus*) *J. Reprod. Fert.* **24,** 213-217.

Jainudeen, M.R., Katongole, C.B. & Short, R.V. (1972a) Plasma testosterone levels in relation to musth and sexual activity in the male Asiatic elephant (*Elephas maximus*) *J. Reprod. Fert.* **29,** 99-104.

Jainudeen, M.R., McKay, G.M. & Eisenberg, J.F. (1972b) Observations on musth in the domesticated Asiatic elephant (*Elephas maximus*). *Mammalia* **36,** 247-261.

Johnson, O.W. & Buss, I.O. (1967) The testis of the African elephant (*Loxodonta africana*). *J. Reprod. Fert.* **13,** 23-30.

Kirkpatrick, J.F., Kincy, V., Bancroft, K., Shideler, S.E. & Lasley, B.L. (1991a) Estrous cycle of the North American bison (*Bison bison*) characterized by urinary pregnanediol-3-glucuronide. *J. Reprod. Fert.* **93,** 541-547.

Kirkpatrick. J.F., Shideler, S.E., Lasley, B.L., Turner, J.W. (1991b) Pregnancy determination in uncaptured feral horses by means of fecal steroid conjugates. *Theriogenology.* **35,** 753-759.

Kirkpatrick, J.F., Shideler, S.E., Lasley, B.L., Turner, J.W. & Czekala, N.M. (1992) Field applications of fecal steroid technology to free-ranging ungulate species. *Proc. 1st Int. Sym. on Fecal Steroid Monitoring in Zoo Animals,* Rotterdam Zoo. pp. 25-34.

Knobil, E., Plant, T.M., Wildt, L., Belchetz, P.E. & Marshall, G. (1980) Control of the rhesus monkey menstrual cycle: permissive role of the hypothalamic gonadotropin-releasing hormone. *Science.* **207,** 1371-1373.

Knox, W.M., Miller, K.V., Collins, D.C., Bush, P.B., Kiser, T.E. & Marchinton, R.L. (1992) Serum and urinary levels of reproductive hormones associated with the estrous cycle in white-tailed deer (*Odocoileus virginianus*) *Zoo. Biol.* **11,** 121-131.

Labrie, F., Bellanger, A., Cusan, L., Seguin, C., Pelletier, G., Kelly, P., Reeves, L., Lefebvre, F., Lemay, A., Gourdeau, Y. & Raynaud, J. (1980) Antifertility effects of LHRH agonists in the male. *J. Androl.* **1,** 209-215.

Lasley, B.L. (1985) Methods for evaluating reproductive function in exotic species. *Adv. Vet. Sci. Comp. Med.* **30,** 209-228.

Lasley, B.L. & Kirkpatrick, J.F. (1991) Monitoring ovarian function in captive and free-ranging wildlife by means of urinary and fecal steroids. *J. Zoo Wildl. Med.* **22,** 23-31.

Lasley, B.L., Troedssoni, M., Bravo, W. & Haggerty, M.A. (1989) Estrogen conjugate measurements to monitor ovarian activity. *Theriogenology.* **31,** 1:127-139.

Lent, P.C. (1965) Rutting behavior in a barren-ground caribou population. *Anim. Behav.* **13,** 259-264.

Levine, J.E., Pau. K.F., Ramirez, V.D. & Jackson, G.L. (1982) Simultaneous measurement of luteinizing hormone-releasing hormone and luteinizing hormone release in unanesthetized overiectomized sheep. *Endocrinology.* **111,** 1449-1455.

Lincoln, G.A. & Davidson, W. (1977) The relationship between sexual and aggressive behavior, and pituitary and testicular activity during the seasonal sexual cycle of rams, and influence of photoperiod. *J. Reprod. Fert.* **49,** 267-272.

Lincoln, G.A. & Kay, R.N.B. (1979) Effects of season on the secretion of LH and testosterone in intact and castrated red deer stags (*Cervus elaphus*). *J. Reprod. Fert.* **55,** 75-80.

Lincoln, G.A. & Short, R.V. (1980) Seasonal breeding: Nature's contraceptive. *Recent Prog. Horm. Res.* **36,** 1-52.

Loskutoff, N.M., Kasman, L.H., Raphael, B.L., Ott-Joslin, J.E. & Lasley, B.L. (1987) Urinary steroid evaluations to monitor ovarian function in exotic ungulates: IV Estrogen metabolism in the Okapi (*Okapia johnstoni*). *Zoo Biol.* **6,** 213-218.

MacDonald, P.G. (1976) Benign Prostatic Hyperplasia. *Proc. of a workshop sponsored by the Kidney Disease and Urology Program of NIAMDD.* pp. 191-193. U.S. Gov. printing office. Washington D.C.

Matteri, R.L. & Moberg, G.P. (1985) The effect of opioid peptides on Ovine pituitary gonadotropin secretion *In-Vitro*. *Peptides.* **6,** 957-963.

McGaughey, C.A. (1963) Musth. *Ceylon Vet. J.* **11,** 105-107.

Monfort, S.L., Martinet, C. & Wildt, D.E. (1991) Urinary steroid metabolite profiles in female Pere David's deer (*Elaphurus davidianus*) *J. Zoo Wildl. Med.* **22,** 78-85.

Moss, C.J. (1983) Estrous behavior and female choice in the African elephant. *Behavior.* **86,** 167-196.

Mostl, E. (1992) Measuring steroids in feces of mammals to monitor the reproductive status, success and disappointments. *Proc. 1st Int. Sym. on Fecal Steroid Monitoring in Zoo Animals,* Rotterdam Zoo. pp. 5-9.

Mostl, E., Choi, H.S., Ismail, N. & Bamberg, E. (1984) Pregnancy diagnosis in cows and heifers by determination of oestradiol-17alpha in faeces. *Br. vet. J.* **140,** 287-291.

Musey, P.I. Wright, K. Preedy, J.R.K. & Collins D.C. (1979) Formation and metabolism of steroid conjugates: effect of conjugation on excretion and tissue distribution. *Steroid Biochem.* CRC Press. pp. 82-130.

Negro-Vilar, A., Ching, M., Culler, M., Johnston, C. & Valenca, M. (1988) New concepts in the physiology of LHRH and its role in pulsatile gonadotropin secretion. *Andrology and Human Reproduction.* pp. 85-96. Raven Press. New York.

Niemuller, C.A. & Liptrap, R.M. (1991) Altered androstenedione to testosterone ratios and LH concentrations during musth in the captive male Asian elephant (*Elephas maximus*). *J. Reprod. Fert.* **91,** 139-146.

Niemuller, C.A., Gentry, P.A. & Liptrap, R.M. (1990) Longitudinal study of haematological and biochemical constituents in blood of the Asian elephant (*Elephas maximus*). *Comp. Biochem. Physiol.* **96,** 131-134.

Nowak, R.M. (1991) *Walker's Mammals of the World.* 5th edition. pp. xvi. John Hopkins University Press. Baltimore.

Ott, L. (1988) Introduction to the Analysis of Variance. In: *An Introduction to statistical methods and data analysis.* pp. 325-352. 3rd ed. PWS-Kent. Boston.

Plotka, E.D., Seal, U.S., Schobert, E.E. & Schmoller, G.C. (1975) Serum progesterone and estrogens in elephants. *Endocrinology.* **97,** 485-487.

Poole, J.H. (1987a) Rutting behavior in African elephants: The phenomenon of musth. *Behaviour.* **102,** 283-316.

Poole, J.H. (1987b) Elephants in musth, lust. *Nat. Hist.* **96,** 46-53.

Poole, J.H. (1989a) Announcing intent: the aggressive state of musth in African elephants. *Anim. Behav.* **37,** 140-152.

Poole, J.H. (1989b) Mate guarding, reproductive success and female choice in African elephants. *Anim. Behav.* **37,** 842-849.

Poole, J.H. & Moss, C.J. (1981) Musth in the African elephant (*Loxodonta africana*). *Nature Lond.* **292.** 830-831.

Poole J.H., Kasman, L.H., Ramsay, E.C. & Lasley, B.L. (1984) Musth and urinary testosterone concentrations in the African elephant (*Loxodonta africana*). *J. Reprod. Fert.* **70,** 255-260.

Pryce, C.R. & Dobeli, M. (1992) Monitoring the excretion of sex steroids in neotropical monkeys: coping with different metabolic strategies. *Proc. 1st Int. Sym. on Fecal Steroid Monitoring in Zoo Animals, Rotterdam Zoo.* pp. 35-45.

Rasmussen, L.E. (1988) Chemosensory responses in two species of elephants to constituents of temporal gland secretion and musth urine. *J. Chem. Ecol.* **14,** 1687-1711.

Rasmussen, L.E., Buss, I.O., Hess, D.L. & Schmidt, M.J. (1984) Testosterone and dihydrotestosterone concentrations in elephant serum and temporal gland secretions. *Biol. Reprod.* **30,** 352-362.

Rasmussen, L.E., Hess, D.L. & Haight, J.D. (1990) Chemical analysis of temporal gland secretions collected from an Asian bull elephant during a four-month musth episode. *J. Chem. Ecol.* **16,** 2167-2181.

Reiter, E.O. & Grumbach, M.M. (1982) Neuroendocrine control mechanisms and the onset of puberty. *Ann. Rev. Physiol.* **44,** 595-613.

Rissman, E.F. (1991) Evidence that neural aromatization of androgen regulates the expression of sexual behaviour in female musk shrews. *J. Neuroendocrinology.* **3,** 441-448.

Rivier, C. & Vale, W. (1984) Influence of corticotropin releasing factor on reproductive function in the rat. *Endocrinology.* **114,** 914-920.

Sachs, B.C. & Meisel, R.L. (1988) The physiology of male sexual behavior. In: *The Physiology of Reproduction.* pp. 1422-1430 eds. E., Knobil & J.D., Neill. Raven Press. New York.

Safar-Hermann, N., Ismail M.N., Choi, H.S., Mostl, E. & Bamberg, E. (1987) Pregnancy diagnosis in zoo animals by estrogen determination in feces. *Zoo Biol.* **6,** 189-193.

Sanderson, G.P. (1876) *Thirteen Years Amoung the Wild Beasts of India.* pp. 59-60 W.H. Allen. London.

Sanford, L.M., Swierstra, E.E., Palmer, W.M. & Howland, B.E. (1976) The profile of luteinizing hormone and testosterone secretion in the boar. *Proc. 8th Int. Cong. Anim. Reprod. & A.I., Krakow* **3,** 96-99.

Schanbacher, B.D. (1985) Endogenous opiates and the hypothalamic-pituitary-gonadal axis in male sheep. *Dom. Anim. Endocr.* **2,** 67-75.

Scheurmann, E. & Jainudeen, M.R. (1972) Musth in Asiatic elephants (*Elephas maximus*). *Zool. Gart.* **42,** 132-136.

Schmidt, M.J. (1988) Breeding strategies for domesticated elephants. *Proc. of the January 1988 meeting of the Asian elephant specialist group of the I.U.C.N.* Thialand.

Shaw, H.J., Green, D.I., Sainsbury, A.W. & Holt, W.V. (1992) Monitoring ovarian function in scimitar-horned oryx (*Oryx dammah*) by fecal steroid analysis. *Proc. 1st Int. Sym. on Fecal Steroid Monitoring in Zoo Animals,* Rotterdam Zoo. pp. 68-70.

Shille, V.M., Haggerty, M.A., Shackleton, C. & Lasley, B.L. (1990) Metabolites of estradiol in serum, bile, intestine and feces of the domestic cat. (*Felis catus*) *Theriogenology.* **34,** 779-794.

Siegel, R.K. (1984) LSD-induced effects in elephants: Comparisons with musth behavior. *Bulletin of the Psychonomic Society.* **22,** 53-56.

Sitarz, N.E., Erb, R.E., Martin, T.G. & Malven, P.V. (1977) Effect of repeated sampling on concentrations of testosterone, LH and prolactin in blood of yearling angus bulls. *Theriogenology.* **7,** 151-158.

Strabo. (63 B.C.-21 A.D.). *The Geography of Stabo,* Book VII (Geography 15.1.43). Translated by H.L. Jones (1930). The Loeb Classical Library Harvard University Press. Boston. p. 73.

Sunde, A. & Lundmo, P.I. (1982) Separation of 5-alpha-reduced androgens by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **242,** 381-384.

Suttie, J.M., Lincoln, G.A. & Kay, R.N.B. (1984) Endocrine control of antler growth in red deer stags. *J. Reprod. Fert.* **71,** 7-15.

Taussky, H.H. (1954) A microcolorimetric determination of creatinine in urine by the Jaffe reaction. *J. Biol. Chem.* **208,** 853-861.

Taya, K., Komura, H., Kondoh, M., Ogawa, Y., Nakada, K., Watanabe, G., Sasamoto, S., Tanabe, K., Saito, K., Tajima, H. & Narushima, E. (1991) Concentrations of progesterone, testosterone and estradiol-17B in the serum during the estrous cycle of Asian elephants (*Elephas maximus*). *Zoo Biol.* **10,** 299-307.

Tureck, F.W. & van Cauter, E. (1988) Rhythms in reproduction. In: *Textbook of Endocrinology.* pp.1789-1823. 7th ed. Eds. J.D., Wilson & D.W., Foster. W.B. Saunders. Philadelphia.

Tuttle, D. (1991) Elephant management: Meeting the challenge. *Proc. of the 12th International Elephant Workshop.* Burnet Park Zoo, Syracuse, N.Y. pp. 1-5.

van der Hoeven, T., (1984) Assay of hepatic microsomal testosterone hydroxylases by high performance liquid chromatography. *Anal. Biochem.***138,** 57-65.

Wasser, S.K., Thomas, R.I., Nair, P.P., Monfort, S.L. & Wildt, D.E. (1992) Effect of diet on fecal steroid measurements. *Proc. 1st Int. Sym. on Fecal Steroid Monitoring in Zoo Animals,* Rotterdam Zoo. pp. 10-19.

Welsh, T.H. & Johnson, B.H. (1981) Stress-induced alterations in secretion of corticosteroids, progesterone, luteinizing hormone, and testosterone in bulls. *Endocrinology.* **109,** 185-190.

West, L.J., Pierce, C.M. & Thomas, W.D. (1962) Lysergic acid diethylamide: Its effects on a male Asiatic elephant. *Science* **138,** 1100-1103.

Yallow R.S. (1985) Radioimmunoassay of hormones. In: *Textbook of endocrinology.* pp. 123-132. 7th ed. Eds. J.D., Wilson & D.W., Foster. W.B. Saunders. Philadelphia.