ความสัมพันธ์ระหว่างฮอร์โมนโปรเจสเตอโรนจากเลือดกับอุจจาระในช้างเอเชีย ที่มีวงรอบการเป็นสัดปกติและอุ้มท้อง

นายพุทธิพงศ์ ขาวนวล

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RELATIONSHIP BETWEEN SERUM PROGESTERONE AND FAECAL PROGESTINS IN THE PREGNANT AND NON-PREGNANT ASIAN ELEPHANT (*Elephas maximus*)

Mr. Puttipong Khawnual

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
Faculty of Veterinary Science
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Academic Year 2006

Thesis Title	RELATIOSHIP BETWEEN SERUM PROGESTERONE AND FAECAL PROGESTINS IN THE PREGNANT AND NON-PREGNANT ASIAN ELEPHANT (*Elephas maximus*)
By	Puttipong Khawnual
Field of study	Theriogenology
Thesis Advisor	Associate Professor Chainarong Lohachit, Dr.Vet. Med.
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พุทธิพงศ์ ขาวนวล : ความสัมพันธ์ระหว่างฮอร์โมนโปรเจสเตอโรนจากเลือดกับอุจจาระในช้างเอเชียที่มีวงรอบการเป็นสัดปกติและท้อง. RELATIONSHIP BETWEEN SERUM PROGESTERONE AND FAECAL PROGESTINS IN THE PREGNANT AND NON-PREGNANT ASIAN ELEPHANT (*Elephas maximus*)
อาจารย์ที่ปรึกษา : รศ.น.สพ.ดร.ชัยณรงค์ โลหชิต, อาจารย์ที่ปรึกษาร่วม : ผศ.น.สพ.ดร.เผด็จ ธรรมรักษ์, ดร. เจนีน แอล บราวน์ 46 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาระดับฮอร์โมนโปรเจสเตอโรน (P_4) ในเลือดและโปรเจสตินในอุจจาระช้างเอเชียเพศเมียที่มีวงรอบการเป็นสัดปกติและอุ้มท้องในระยะแรก และทำการศึกษา หาความสัมพันธ์ระหว่างระดับฮอร์โมนทั้งสองดังกล่าวในช้างเอเชียที่มีวงรอบการเป็นสัดปกติจำนวน 6 เชือก และช้างอุ้มท้องจำนวน 5 เชือก ตัวอย่างเลือดและอุจจาระของช้างถูกเก็บทุกสัปดาห์ เริ่มตั้งแต่ตรวจพบการเป็นสัด โดยการศึกษาใช้ระยะเวลาประมาณ 1 ปี นำอุจจาระมาสกัดเพื่อแยกฮอร์โมนโปรเจสติน และนำมาตรวจด้วยวิธี enzyme-immunoassay (EIA) ซีรั่มนำมาตรวจฮอร์โมน P_4 โดยชุดตรวจ ^{125}I-radioimmunoassay (RIA) ทำการวิเคราะห์ข้อมูลทางสถิติด้วยวิธี Analysis of variance และ Spearman's correlation ผลการทดลองพบว่าช้างเอเชียที่ทำการศึกษามีวงจรการเป็นสัดเฉลี่ย 14.1±2.4 สัปดาห์ ระดับฮอร์โมน P_4 โดยเฉลี่ยในเลือดช้างที่เป็นสัดปกติกับอุ้มท้องมีความแตกต่างกันอย่างมีนัยสำคัญ (0.56 กับ 0.08 นาโนกรัม/มิลลิลิตร, P<0.001) โดยระดับฮอร์โมน P_4 ในช้างอุ้มท้องสูงกว่าช้างที่เป็นสัดปกติหลังจากผสมพันธุ์ 13 สัปดาห์ (0.89 กับ 0.03 นาโนกรัม/มิลลิลิตร, P=0.05) ระดับฮอร์โมน P_4 ในเลือดและโปรเจสตินในอุจจาระช้างเอเชียมีความสัมพันธ์กันอย่างมีนัยสำคัญ ทั้งในช้างที่มีวงรอบการเป็นสัดปกติ (r=0.32, P=0.004) และช้างที่อุ้มท้อง (r=0.33, P<0.001) การศึกษาระดับฮอร์โมน P_4 ในเลือดและโปรเจสตินในอุจจาระช้างเอเชียเป็นรายตัว พบว่าระดับฮอร์โมนมีความสัมพันธ์กันเป็นบางตัว โดยมีค่าสัมประสิทธิ์ความสัมพันธ์ระหว่าง r=-0.086 ถึง r=0.88 โดยระดับโปรเจสตินในอุจจาระช้างเอเชียมีความแปรปรวนสูง ต้องใช้จำนวนตัวอย่างและระยะเวลาในการเก็บข้อมูลเพิ่มมากขึ้น การตรวจฮอร์โมนโปรเจสเตอโรนในเลือดมีความน่าเชื่อถือกว่าการตรวจโปรเจสตินในอุจจาระ และสามารถประยุกต์ใช้ในการตรวจการตั้งท้องระยะต้นในช้างได้

ภาควิชาสูติศาสตร์เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต.....
สาขาวิชาวิทยาการสืบพันธุ์สัตว์ ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD: Asian elephant/ Serum progesterone/ Faecal progestins/ RIA/ EIA

PUTTIPONG KHAWNUAL: RELATIONSHIP BETWEEN SERUM PROGESTERONE AND FAECAL PROGESTINS IN THE PREGNANT AND NON-PREGNANT ASIAN ELEPHANT (*Elephas maximus*)

THESIS ADVISOR : ASSOC. PROF. CHAINARONG LOHACHIT, Dr. Med. Vet.,

THESIS CO ADVISOR : ASST. PROF. PADET TUMMARAK, Ph.D.,

DR. JANINE L. BROWN, Ph.D 46 pp.

The objectives of the present study were to measure the concentrations of serum progesterone and faecal progestins in non-pregnant and early pregnant female Asian elephants and to study the relationship between both hormones. The experiment included 6 non-pregnant and 5 pregnant elephants. Blood and faecal samples were collected weekly for 1 year starting from the first week of the behavioral estrous. The faecal progestins were extracted and analyzed using enzyme immunoassay (EIA). Serum P_4 were analyzed using ^{125}I-radioimmunoassay (RIA). The statistical analyses were performed using Analysis of variance and Spearman' correlation. This study found that the oestrus cycle was 14.1±2.4 weeks (mean±SD). Mean serum P_4 concentration differed significantly between pregnant and non-pregnant elephants (0.56 versus 0.08 ng/ml, P<0.001) and the serum P_4 in pregnants were significantly higher than non-pregnant elephants after the 13th week post mating (0.89 versus 0.03 ng/ml, P=0.05). The concentrations of serum P_4 and faecal progestins were significantly correlated both in non-pregnant (r=0.32, P=0.004) and pregnant Asian (r=0.33, P<0.001). However, the individual's correlation coefficient varied between animals from r=-0.086 to r=0.88. A high variation among individual elephants in the faecal progestin data suggest more individuals and times need to be examined. The serum P_4 was more accurate than the faecal progestins. The serum P_4 could be used to predict early pregnancy in female Asian elephant.

Department of Obstretrics Gynaecology and Reproduction Student's signature.....

Field of study Theriogenology Advisor's signature.....

Academic year 2006 Co-advisor's signature.....

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ABBREVIATIONS

anLH	anovulatory Lutinizing Hormone
ºC	degree celcius
µg	microgram
17α OHP	17α-hydroxyprogesterone
20-OXO-P	20-oxo-progestagens
5α –DHP	5α-Pregnane-3,20-dione
5α –P-3-OH	5α-Pregnane-3-ol-20-one
CBG	corticosteroid-binding globulin
CL	corpus luteum
CNS	central nervous system
CV	coefficient of variation
DHT	5α-reduced dihydrotestosterone
E_2	estradiol-17β
EIA	enzyme immunoassay
FSH	follicle stimulating hormone
g	gram
h	hour
Kg	kilogram
LH	lutenizing hormone
min	minute
ml	milliliter
ng	nanogram
ovLH	ovulatory Lutinizing Hormone
P_4	progesterone
pg	picogram
RIA	radio immunoassay
rpm	round per minute
SHBG	sex hormone-binding globulin
Yr	year

CHAPTER 1

INTRODUCTION

The Asian elephant (*Elephas maximus*) is the largest land mammal in Asia and is an important cultural icon in many Asian countries, including Thailand. The Asian elephant has been registered in CITES Appendix 1 (F) and classified as an endangered species. The number of wild elephants in the world is estimated to range between 37,530 and 48,180, while the numbers of domestic Asian elephants is 16,785 (Table 1).

Table 1.1 The estimated numbers of wild and domestic Asian elephants in 17 countries (source: Santiapillai et al., 1990; Lair, 1997, 1998)

Country	Wild elephant	Domestic elephant
India	23,500 - 27,500	3,000
Myanma	4,000 - 6,000	6,400
Indonesia	3,500 – 5,000	570
Sri Lanka	2,000 – 3,000	500
Malaysia	1,700 – 2,300	20
Thailand	1,200 – 1,500	3,800
Cambodia	500 – 1,000	500
China	330 – 370	-
Vietnam	300 – 600	225
Lao PDR	200 – 500	1,200
Bangladesh	200 – 250	500
Bhutan	50 – 100	-
Nepal	50 – 60	70
Total	37,530 - 48,180	16,785

In Thailand, elephants are recognized as an important part of the ancient history. They have played a role in Thai culture, religion, lifestyle, and war, as well as being an important working animal. During ancient times, wild elephants were captured from the

forest, tamed and used for many purposes. From 1965 to 1994, there was a dramatic decrease in the number of Thai domestics elephants, from 11,192 to 2,502 individuals. Furthermore, it has been estimated that the population of Thai domestic elephants has decreased 3-5 % annually from 1979-1999 (Mahasawangkul, 1999). The reduction in number of Asian elephants in Thailand is due to many factors, including illegal logging, poaching, loss of habitat, agriculture conversion of land, deforestation and overall poor management. The lack of public and government awareness of these problems exacerbates their precarious position in Thailand.

The proper management of domestic elephants in Thailand has not been clearly established yet. In general, elephant handlers use management strategies passed down by their ancestors. Unfortunately, many elephants are not kept under ideal conditions and suffer from poor husbandry practices, such as housing animals individually and not meeting social needs, feeding diets poor in nutrition and variety, not allowing access to salt licks and providing poor veterinary care. These practices have resulted in an unsustainable population of domestic elephants in Thailand and other range countries.

In part, because of poor husbandry and lack of interest, reproductive performance of the domesticated elephant is low. The gestation period of the female is approximately 22 months, the longest among mammals. Under ideal conditions, the calving interval of female elephants generally is 4 to 5 years. Knowledge of the reproductive status of individual elephants is needed to improve captive breeding success and preserve this valuable elephant population. Obtaining this information can be difficult, however, due to a lack of suitable facilities and equipment for handling the animals and limited cooperation between the owner and veterinarians. Not all elephants are trained for blood collection, which is necessary for longitudinal serum progesterone (P_4) analyses. But with the advent of noninvasive faecal hormone monitoring techniques, it may be possible to assess reproductive activity in elephants without the need for handling or restraint. This study compared the efficacy of a faecal progestin enzymeimmunoassay (EIA) with a standard P_4 radioimmunoassay (RIA) for assessing ovarian activity and pregnancy in a group of captive Thai elephants.

CHAPTER 2

LITERATURE REVIEW

2.1 Reproductive organs of the female elephant

In general, the reproductive organs of the female elephant are similar to other mammals, except for the enormous the size and weight (Fig 2.1) (Hildebrandt et al., 2000). The reproductive organs of the female elephants consist of vestibule, clitoris, vagina, cervix, body of uterus, uterine horn and ovaries (Fig. 2.1). The vestibule is a flat tube about 100-140 cm in length, which can accomodate the s-shape of the male genital organ during natural mating. The vestibule is located vertically away from the rectum. The clitoris is located at the anterior distal end of the vestibule and is 6 – 8 cm in length. The vagina is a cranial sac 20-40 cm in length and located at the anterior dorsal part of the vestibule with the opening of the urethra. The vagina consists of folds for natural semen deposition. During pregnancy, a vaginal mucus plug is secreted and released 2-3 days before parturition. The cervix consists of mucosal folds about 8 – 15 cm thick.

The uterus of the elephant is a bicornuate type, 8-15 cm in length with a short uterine body (convoluted). The structure is similar to the horse uterus (Fig. 2.2). The uterine horns are located cranio-ventral about 5-7 cm from the bifurcation. During pregnancy, the elephant embryo is implanted in the uterine horn lateral to the ovulated follicle.

The ovaries of the elephants are covered by an infundibulum and are 7x5x2.5 cm (lengthxwidexthickness). The ovaries produced ovum during oestrus cycle.

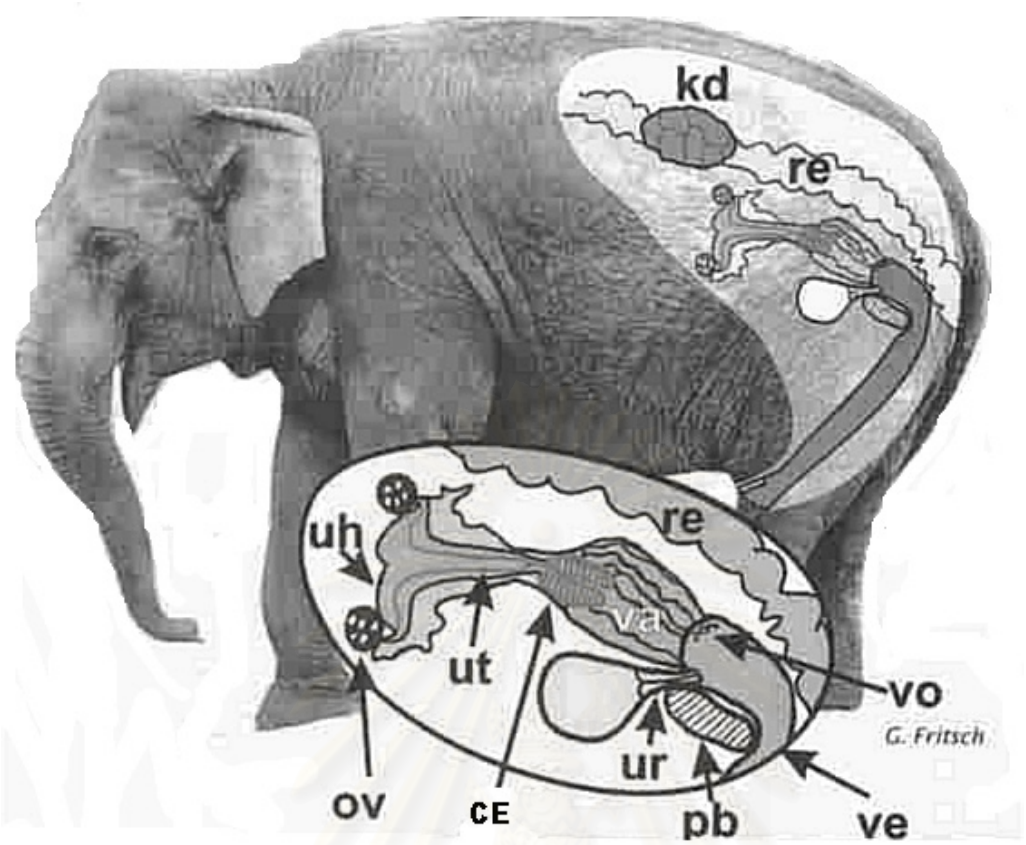

Fig 2.1 Reproductive organs of the female Asian elephant. ce=cervix, kd=kidney, ov=ovary, pb=pubic bone, re=rectum, uh=uterine horn, ur=urinary bladder, ut=uterus, va=vagina, ve=vestibule, vo=vagina opening (source: Hildebrandt et al., 2000)

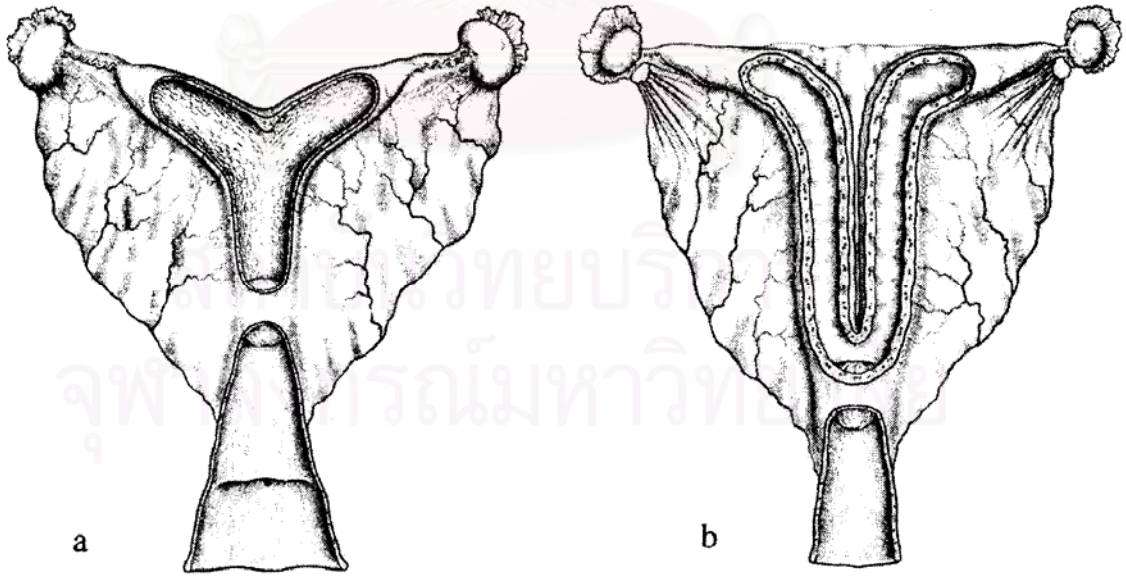

Fig 2.2 Comparison between the female's reproductive tracts of elephant (a) and horse (b) (source: Allen et al., 2003)

2.2 Oestrus cycle of the elephant

The female elephant has oestrus cycle about 13-16 weeks in duration. The oestrus interval consists of 2 phases, the follicular and the luteal phases (Hess et al., 1983; Plotka et al., 1988; Mainka et al., 1990; Brown et al., 1991, 1995a, 1999, 2004a; Taya et al., 1991; Niemuller et al., 1993, Olsen et al., 1994; Kapustin et al., 1996, Schwarzenberger et al., 1997, Dehnhard et al., 2001). The follicular phase is 4-6 weeks in duration, during which two LH surges 3 weeks apart occur (Brown et al., 1991, 1995b). The anovulatory LH (anLH) surge occurs after the formation of several small follicles, whereas the ovulatory LH (ovLH) surge occurs in conjunction with selection of a single large follicle that ovulates (Hermes et al., 2000; Brown, 2000).

In elephants, circulating pulses of pituitary gonadotropins are related to the the decrease in luteal P_4. After the decrease in P_4, estradiol-17β (E_2) concentrations increase and reach a threshold concentration that simulates a large, pre-ovulatory surge of leutinizing hormone (LH). Serum follicle stimulating hormone (FSH) concentrations are highest at the beginning of the non-luteal phase, decline to nadir concentrations within 4 days of the ovLH surge, remain low until the ovLH surge occurs and then increase during the luteal phase (Brown et al., 1999, 2004b) (Fig 2.3). A cyclic pattern of FSH in Asian elephants is prolonged for 12–14 weeks, with low levels during the late follicular-early luteal phase followed by a relatively long period of elevated levels (at least 7–8 weeks) during the late luteal and early follicular phases (Brown et al., 2004a). E_2 rises (12–15 pg/ml) prior to each of the two inter-luteal phase LH peaks, and peaks coincide with increase mounting behavior by male (Ramsay el al., 1980). In Asian elephants, serum prolactin concentrations are stable throughout the oestrus cycle, which is different from African elephants where prolactin increases during the nonluteal phase (Brown et al., 2004b) (Fig 2.4).

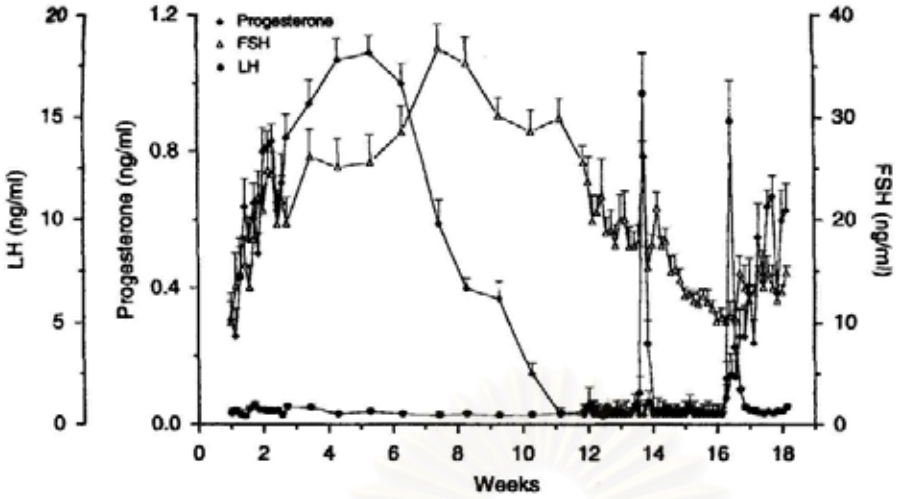

Fig 2.3 Circulating concentrations of serum P_4, FSH, LH during oestrus cycle in female Asian elephant (source: Brown et al., 1999)

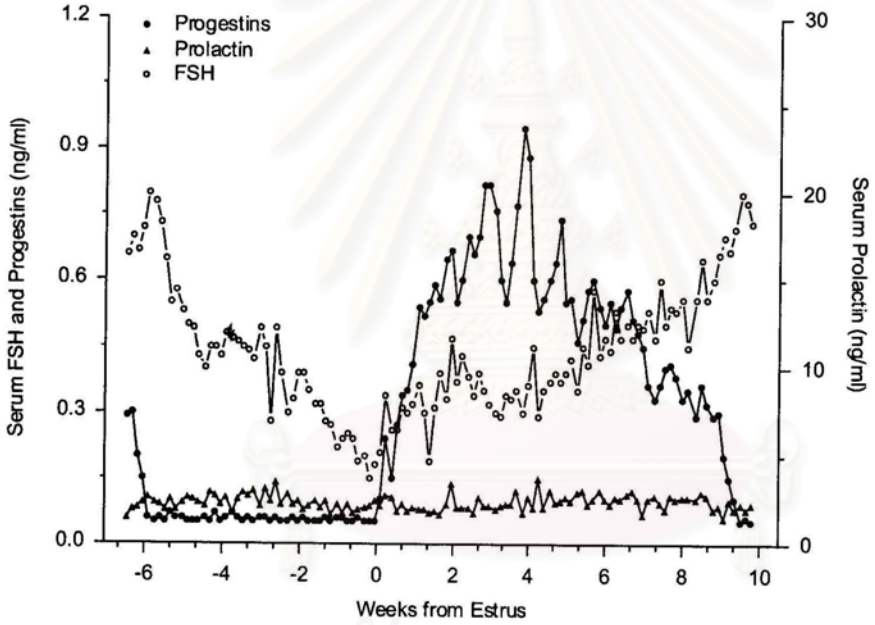

Fig 2.4 Circulating concentrations of serum progestins, FSH, prolactin during oestrus cycle in female Asian elephant (source: Brown et al., 2004b)

2.3 Oestrus behavior and oestrus detection

The onset of oestrus in female elephants is associated with behavioral changes, such as a sudden decrease in responsiveness to mahout's commands and a rearward presentation to bulls. In addition, a sticky translucent vaginal discharge, often from the genital organs, though not always, is observed. A frequent attempt to smell the reproductive organs of other elephants also has been observed. In general, the bull will sniff the vagina of a particular female more often than usual and the bull's genital organ

protrudes while sniffing (Fig 2.5). When the bull approaches, the female displays an increased tolerance of and attentiveness to his interest, turns to present rearward, the responds with hind legs that widen slightly during the act of sniffing (Fig 2.5) (Poole and Moss, 1989, Khawnual and Clark, 1998, Slade et al., 2003).

The vemeronasal organ is a special organ in the roof of the mouth in the elephant (Ester, 1972, Rasmussen ,1998, 2003). Female elephants release the pheromone (Z)-7-dodecenyl acetate in their urine during the pre-ovulatory period (Ramussen et al., 1996, 1997). This pheromone production correlates with positive responses in the male sniff test, which include heightened interest, genital erection, penis protrusion and increased sniffing of female urine (Fig 2.5) (Hall et al., 1987, Poole et al., 1989, Slade et al., 2003).

Fig 2.5 Oestrus detection in the elephant: courtship behavior (a), sniff test (b), male genital organ protrusion and mounting (c), natural mating (d) (source: Ayutthaya elephant's camp, Thailand)

2.4 Pregnancy and parturition

The gestation period of the female elephants is approximately 22 months (Mainka et al., 1990). The serum P_4 and prolactin profile during gestation are demonstrated in Fig. 2.6. It has been shown that circulating P_4 is at baseline values during the week preceding parturition. Cortisol in both plasma and urine are increased 1–2 days before and markedly on the day of and shortly after parturition (Brown et al., 2004a).

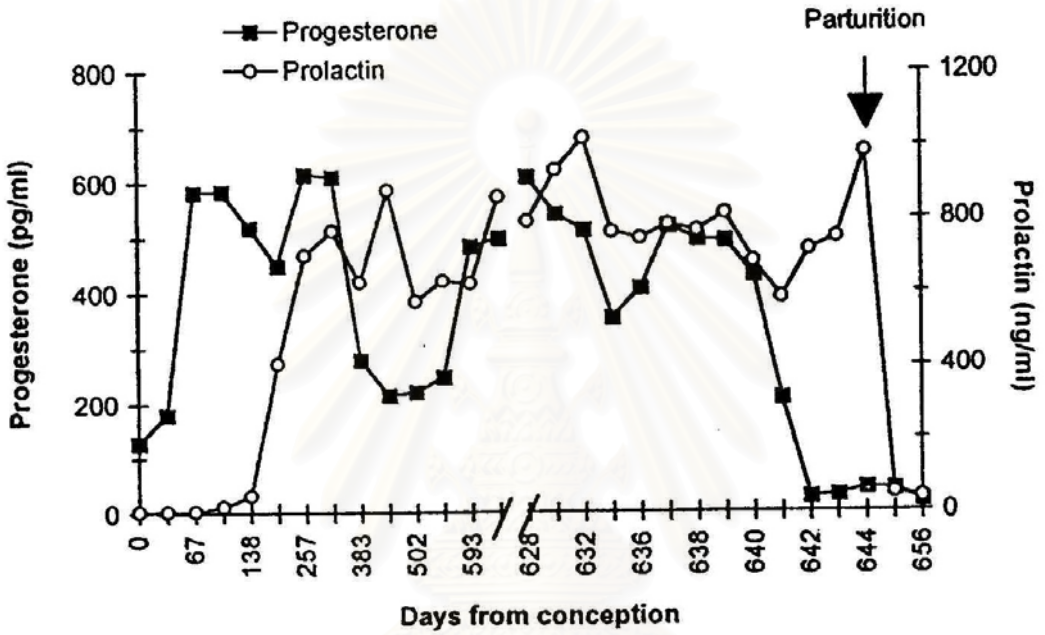

Fig 2.6 Serum P_4 and prolactin profile during gestation in an Asian elephant (source: Carden et al., 1998)

One to three days before parturition, elephants exhibit moderate changes in behavior including decreased response to mahout commands, decreased appetite and increased anxiety. A clear to yellowish vaginal discharge is release from the mucous plug of the cervix (Mainka et al., 1990, Khawnual and Clark, 1998, Brown et al, 2004a).

During delivery, emergence of the fetus from the cervix is indicated by distension at the posterior part of the vagina. The fetus is discharged, preceded by loud vocalizations, extreme agitation, and an erected tail. The birth is normally in the dorsal longitudinal posterior presentation position. As the fetus emerges, it curls into the ventral position. Some elephants prod the infant with their trunk or forelegs. Elephants may

attack the infant to release the infant from the amniotic sac and stimulate the circulatory and respiratory system of the infant. The infant can stand up by itself within 10-60 minutes after birth (Eisenberg et al., 1971, Khawnual and Clark, 1998).

Placental expulsion stage occurs within 24 hours of the end of stage two. If the expulsion of the placenta was not occur until the 96-hour, retained placenta might be suspected (Khawnual and Clark, 1998).

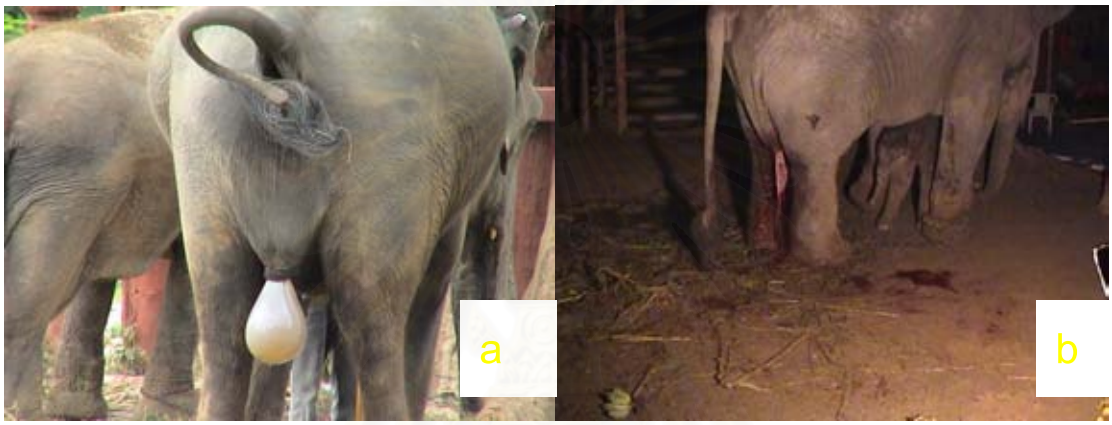

Fig 2.7 Parturition process in the female elephant: protrusion of the amniotic sac (a), the 1 day old calve (b) (source: Ayutthaya elephant's camp, Thailand)

2.5 Ovarian function

The female elephant has a single ovulation (Laws, 1969) but multiple corpus luteum (CL) (range between 2-42) are observed during the luteal phase and pregnancy (Smith and Buss, 1975; de Villiers et al., 1989; Hildebrandt et al., 2000). Ovulation and male interest occur as P_4 increases at the end of the follicular phase, and as a result of the ovLH surge (Carden et al., 1998; Brown, 2000).

After ovulation, formed CL are functional for approximately 10 weeks, during the luteal phase (Brannian et al., 1998). Earlier studies demonstrated that exogenous prostaglandins-$F_{2\alpha}$, had a luteolytic effect in elephants (Gross et al., 1991). An examination of the ovaries of both pregnant and non-pregnant African elephants have shown that multiple CL exist, up to 6 to 8 CL per animal (Smith and Buss, 1975). About 30–40% of these CL had a visible ovulation point or stigma (Hildebrandt et al., 1997).

Ovarian cycles are associated with the formation of multiple CL. These CL undoubtedly are responsible for the rise in P_4 during the luteal phase, but non-ovulatory CL also are formed during the oestrus cycle and their function is unclear. The rapid increase in P_4 observed at the end of the follicular period and preceding ovulation suggests the presence of pre-formed structures that respond more quickly to a second LH stimulus (Brown et al., 2004b). Therefore, multiple CL could reflect accumulation of structures between cycles (Hanks and Short, 1972, Short, 1996). The P_4 levels are not correlated with the number of CL and the multiple CL are formed within each cycle may persist until subsequent cycles (Hodges, 1998).

Luteal tissue in the elephant is steroidogenically active (Ogle et al., 1973; Smith and Buss, 1975); however, it has been found that P_4 is of minor importance and that the most abundant progestins are 5α-reduced compounds, like 5α-dihydro-progesterone (5αDHP) and 5α-pregnan-3-ol-20-one (Hodges et al., 1994, 1998; Schwarzenberger et al., 1997). Despite the major progestins being pregnanes, however, many P_4 assays crossreact with circulating metabolites and thus can be used for routine monitoring of luteal activity (Hodges et al., 1994; Brown, 2000). For this reason, although P_4 assays are used, we refer to luteal steroids in circulation as 'progestins' or 'P_4 metabolites'.

Recently, it was found that 5αDHP had a high affinity binding to the elephant endometrial P_4 receptor (Greyling et al., 1997; Meyer et al., 1997). Therefore, 5αDHP may be the biological active progestin in elephant.

2.6 Steroid hormone synthesis and metabolisms

Steroids are lipophilic with a low-molecular weight and are derived from cholesterol. They are synthesized by the ovary and feto-placental unit and released into the blood circulation. They act both on peripheral target tissues and the central nervous system (CNS). An important function of the steroid hormones is to coordinate physiological and behavioral responses for specific biological purposes. Gonadal steroids influence the sexual differentiation, secondary sexual characteristics during development, sexual maturation, maintenance of their functional state and control sexual

behavior. It has been shown that the CNS is also able to synthesize a number of biologically active steroids directly from cholesterol (Feder, 1981). Steroid metabolism is important for the production of these hormones and for the regulation of their cellular and physiological actions.

Cholesterol is made up of three hexagonal carbon rings and a pentagonal carbon ring to which a side-chain (carbons 20-27) is attached (at position 17 of the polycyclic hydrocarbon). Removal of part of the side-chain gives rise to C21-compounds of the *pregnane* series (progestins and corticosteroids). Total removal produces C19-steroids of the *androstane* series, whereas loss of the 19-methyl group yields the *estrane* series, to which estrogens belong. Individual compounds are characterised by the presence or absence of specific functional groups at certain positions of the carbon skeleton. The adrenals produce both androgens and corticosteroids. The ovaries can secrete estrogens and progestins (Fig. 2.8). However, the biochemical pathways involved are strikingly similar in all tissues, the difference in secretory capacity mostly due to the presence or absence of specific enzymes.

In general, steroids are released into the blood circulation as soon as they are formed. Secretion rates are related to the biosynthetic activity of the gland and the blood flow rate. In fluid, steroids are usually found in a conjugated form e.g., sulfates and glucuronides. In plasma, unconjugated steroids are mostly bound to carrier proteins (Johnson et al., 1980). The binding of steroids to plasma albumin is rather unspecific, whereas binding to either corticosteroid-binding globulin (CBG) or the sex hormone-binding globulin (SHBG) are more stringent. The free fraction (1-10% of total concentration in plasma) represent the active fraction. There is a recent evidence that, in some cases, the specific binding proteins may facilitate steroid to entry into the target tissues. The roles of plasma binding proteins act as a buffer for active hormones. The non-covalent nature of the protein-bound steroids are released into the plasma in free form when the free concentration decrease.

Steroids are lipophilic and diffuse through the cell membranes. In the target tissues, steroids are concentrated by an uptake mechanism which relies on their binding to intracellular proteins. High concentration of steroids is also found in adipose tissue, although this is not a target for hormone action. Most of the peripheral metabolism occurs in the liver and to some extent in the kidneys, which are the major sites of hormone inactivation and elimination, or catabolism (Norman et al., 1987). The biological activity of a steroid molecule depends on its ability to interact with a specific binding site on the corresponding receptor. In most cases, biological activity correlated with binding affinity. The affinity of a steroid for its specific receptor is dependent upon the presence or absence of particular functional groups and the overall three-dimensional structure of the molecule. Target tissue metabolism is not limited to the local production of active metabolites. Inactivation occurs within the target cell, and this mechanism contribute to the regulation of the intracellular concentration of biologically active molecules (Norman et al., 1987).

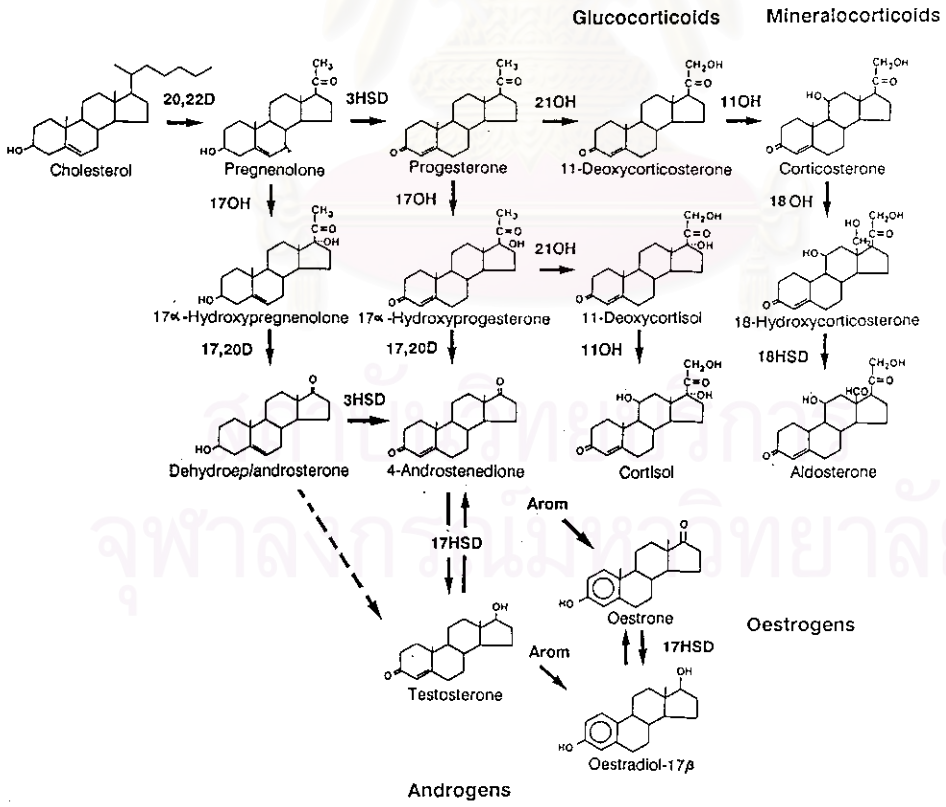

Fig 2.8 Steroid biosynthesis pathways (source: Griffin et al., 1988)

Cholesterol side-chain removal is blocked specifically by aminoglutethimide, a steroid biosynthesis inhibitor. From pregnenolone, steroid biosynthesis can proceed either through the delta-5 pathway (17a-hydroxypregnenolone, dehydroepiandrosterone, testosterone), or through the delta-4 pathway. P_4 is the starting point for mineralocorticoid synthesis, whereas glucocorticoids are derived from its metabolite, 17a-hydroxyprogesterone. Estrogens are formed from androgens. Most reactions are irreversible (Fig. 2.8). Reversible reactions depend on cofactor availability.

2.7 Non-invasive monitoring of steroid hormone

Non-invasive techniques to monitor luteal steroid hormone activity is based on the measurement of progestin metabolites in urine and faeces (Schwarzenberger et al., 1996). Pregnanetriol, derived from circulating 17αOHP is identified as a urinary progestagen in the Asian elephant, while circulating PROGESTINS are virtually undetectable. Pregnanetriol measurements for monitoring ovarian cycles of exotic animals has been widely used among European zoos (Schwarzenberger et al., 1996). Group specific measurement of 5α-20-oxo-pregnanes (a combination of 5αDHP and 5α-pregnan-3-ol-20-one) enable monitoring of the major circulating and excreted progestins in elephants. Circulating E_2 is not practical for monitoring ovarian function due to low levels and unclear profiles (Taya et al., 1991). Studies describing urinary E_2 profiles are few and limited, especially in the Asian elephant. Faecal E_2 monitoring is not appropriate because most of those metabolites are excreted in urine, not feces (Wasser et al., 1996).

Faecal steroid hormone monitoring, such as E_2 and P_4 metabolites, has been used to study female reproductive cycles and indicate various reproductive states such as pregnancy, abortion, puberty and abnormalities of the ovaries in a variety of species (Lasley et al., 1991; Schwarzenberger et al., 1997). Recently, a urinary estradiol-3-glucuronide assay characterized two waves of steroidogenic activity in elephants, in agreement with previously published ultrasound data (Czekala et al., 2000; Hermes et al., 2000). In the African elephant, multiple (2-4) small follicles developed during the first wave and stimulated an LH surge. About 1 to 5 days before the LH surge, accessory CL

are visible. These may be remnant structures of the non-ovulatory wave and the source of elevated progestins observed 2-3 days before the LH surge. During the second wave only one large antral follicle develops beginning 5-7 days before the LH surge and becomes dominant (Niemuller et al., 1993; Wasser et al., 1996; Heistermann et al., 1997). Approximately 55% of progestin radioactivity is found in feces, making this valid approach for noninvasive monitoring of luteal activity. There is an interesting species difference in the excretion of 5α-pregnanetriol, with the 17α-OHP metabolite being abundant in the urine of the Asian elephant (Niemuller et al., 1993).

2.8 Hormonal profile during the oestrus cycle

Previously, studies based on observation of oestrus behavior and vaginal cytology (Jainudeen et al., 1971), serum LH peak analysis (Chappel and Schmidt, 1979, Schmidt et al., 1981) and urinary E_2 immunoactivity (Ramsay et al., 1980) suggested that the oestrus cycle of the Asian elephant was about 18-27 days. Subsequently, more recent studies have proved that those findings are false. The development of radioimmunoassays (RIA) for measuring P_4 metabolites in the elephant indicates that the oestrus cycle is actually 16.3 ± 0.4 weeks, with a luteal phase of 10.5 ± 0.3 weeks and a follicular phase of 5.1 ± 0.4 weeks (Hess et al., 1983; Plotka et al., 1988; Mainka et al., 1990; Brown et al., 1991, 1999, Taya et al., 1991; Niemuller et al., 1993) (Table 2).

Table 2.1 The oestrus cycle and the levels of serum P_4 during the normal oestrus cycle in the female Asian elephant measured by radioimmunoassay technique (RIA)

Parameters measured	Oestrus cycle (week)	N	P_4 (ng/ml)	References
Serum P_4	16.3 (5.8:10.5)*	15	0.6-0.8	Hess et al., 1983
Serum P_4 & LH	14.7 (4.2:10.6)*	10	0.4-0.8	Plotka et al., 1988
Serum P_4	16.1	NA	0.15-0.4	Mainka et al., 1990
Serum P_4	13.2 (3.4:9.8)*	14	0.3-0.43	Brown et al., 1991
Serum P_4 & E_2	16.8	14	0.5-1.7	Taya et al., 1991
Plasma 17-hydroxy P_4	15.2	15	0.11-2.4	Niemuller et al., 1993

* number in the bracket means follicular phase: luteal phase; E_2= estradiol-17β, FSH=follicle stimulating hormone, LH=luteinizing hormone, NA=data not available, N=number of animal

Serum P_4 analysis is now used exclusively to monitor the reproductive cycle of female elephants (Brown et al., 1991; Taya et al., 1991; Olsen et al., 1994; Kapustin et al., 1996). The elephant is unique in exhibiting two LH peaks that occur 3 weeks apart during the follicular phase (Brown et al., 1991, 1995b; Kapustin et al., 1996). The first surge occurs approximately 12-21 days after the fall in P_4 to baseline and does not cause ovulation. E_2 levels increase after P_4 levels decrease to baseline level indicative of follicular development during the non-luteal phase (Fig. 2.9)

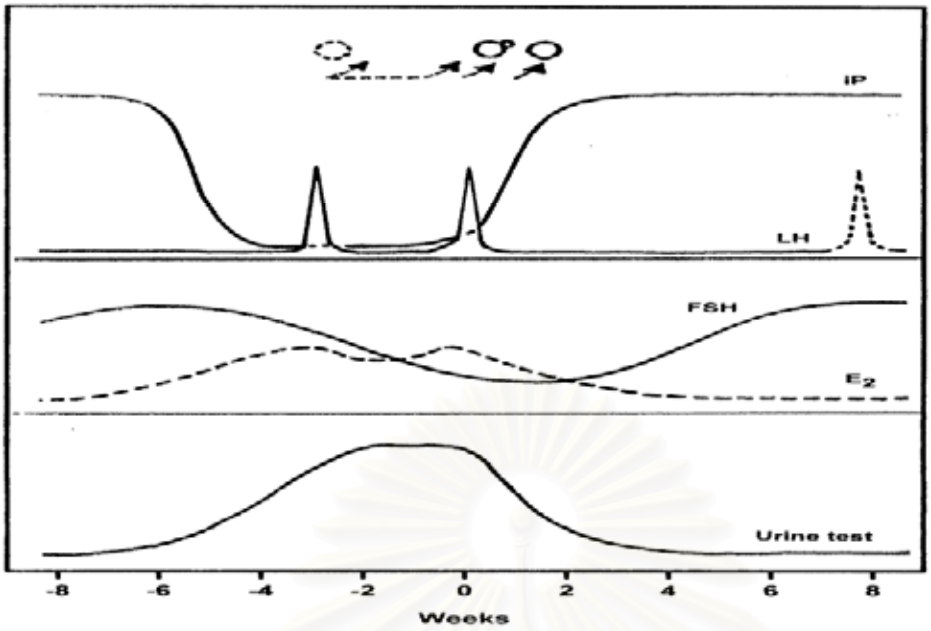

Fig 2.9 Model of elephant oestrus cycle, E_2=estrogen, FSH=follicle stimulating hormone, iP=immunoreactive progesterone, LH=leutinizing hormone (source: Hodges, 1998)

The ovaries of the elephants produce testosterone during the luteal phase (Taya et al., 1991). After conception, P_4 concentrations increase and sometime exceed the levels observed during the luteal phase (Mainka et al., 1990, Brown, 2000). In general, pregnancy diagnosis based on single samples analyzed for P_4 is not accurate because of high variability among and within animals (Schwarzenberger et al., 1997; Brown, 2000). Elephants are known to develop several corpora lutea during pregnancy (Smith et al., 1969; Hanks et al., 1972). P_4 decline to baseline 2-5 days before parturition, giving the managers an adequate time to prepare for birth (Brown et al., 2004a) (Fig 2.6).

The female elephant shows few overt signs of behavioral oestrus; however, bulls can detect fertility status by smelling pheromones excreted in the female's urine (Rasmussen et al., 1998, Bagley et al., 2006). The female elephants advertise a forthcoming ovulation by releasing (Z)-7-dodecen-1-yl acetate in their urine during the pre-ovulatory period (Rasmussen et al., 1998). Urine sniff testing usually is followed by mounting and mating behaviors. This has been shown to be correlated with an increase in the level of P_4 at the beginning of the luteal phase (Fig. 2.10) (Hess et al., 1983;

Carden et al., 1998). Male interest in the female also corresponds with the peak in total E_2 production during the late follicular phase (Mainka et al., 1990)

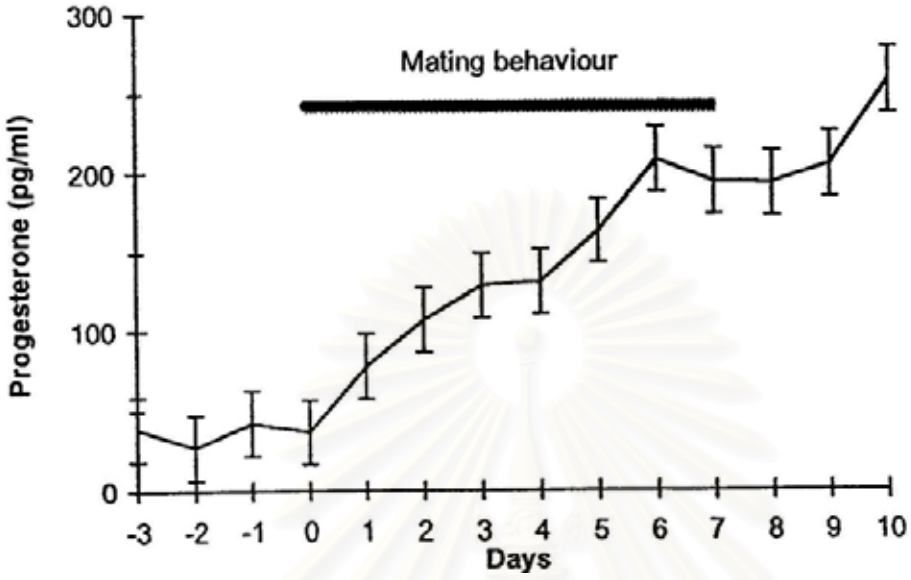

Fig 2.10 Serum P_4 in relation to mating behavior (source: Carden et al., 1998)

2.9 Hypothesis

Concentrations of serum P_4 correlate with faecal progestins in pregnant and non-pregnant Asian elephants.

2.10 Objectives of the study

The objectives of the present study are to:

1. Measure the concentrations of serum P_4 and faecal progestins in early pregnant and non-pregnant Asian elephants

2. Study the relationship between serum P_4 and faecal progestins in pregnant and non-pregnant Asian elephants

CHAPTER 3

MATERIALS AND METHODS

The present experiment is consisted of 4 parts:

Part I: Study the serum P_4 profiles in pregnant and non-pregnant Asian elephants

Part II: Study the relationship between serum P_4 and faecal progestins during normal oestrus cycle in the Asian elephant

Part III: Study the relationship between serum P_4 and faecal progestins in pregnant Asian elephants

Part IV: Study the individual variations in serum P_4 and faecal progestin concentrations

3.1 Animals

The study was performed from August 2005 to July 2006 and involved 11 female Asian elephants from the Ayutthaya elephant camp, Thailand (Fig 3.1). The elephant camp produced 32 calves during a 7 years period from 2000-2006 by natural mating. The age of the elephants was 26.1±7.6 years (18-40 years) and the body weight was 2559.6±341.9 kg (1890-3100 kg) (Table 3.1). All of the elephants had previously calved at least once (parity 1-2) within the past 5 years. The elephants were kept outdoors and were fed with commercial pellet, variety of fruit, pineapple tree, corn tree, Napia glass, variety of vitamins and minerals (Fig 3.2). Water was provided to ad-libitum. The feed were provided to the animals during the day until midnight and the commercial pellet of minerals were provided once a day. The health of the elephants was supervised by the camp's veterinarian. In general, the elephants in the camp worked for tourist riding for a period of 2-3 weeks and rested for 1-2 weeks in each month. The working period was 10-12 hours per day.

Fig 3.1 Elephant calves at Ayutthaya elephant's camp (a,b) (source: Ayutthaya elephant's camp, Thailand)

Fig 3.2 The elephant feed at Ayuttaya elephant's camp including pine apple tree (a), corn (b), commercial pellets (c), vitamin and mineral (d) (source: Ayutthaya elephant's camp, Thailand)

In part I, 8 female Asian elephants were studied. The elephants were divided into 2 groups: group I (n=4, P2-P4 and P5) were naturally mated after standing oestrus; group II (n=4, NP1-NP4) were not mated after the standing oestrus.

In part II, 13 oestrus cycles from 6 non-pregnant female Asian elephants were evaluated (NP1-NP6). Weekly blood and faecal samples from each animal were collected 24 h apart, respectively. The correlation between serum and faecal progestins in these elephants was analyzed.

In part III, 3 pregnant elephants were evaluated (P1-P3) (Table 3.1). Serum and faecal samples were collected weekly from mating until approximately 35 weeks of gestation.

In part IV, individual data from 8 of 11 elephants elephants are graphically presented.

Table 3.1 General information of the 11 female Asian elephants utilized in this study

Female's name (ID)	Group	Age (year)	Weight (kg)	No. of calves
Jumpee (NP1)	NP	32	2894	1
Jamjuree (NP2)	NP	20	1890	2
Namphet (NP3)	NP	18	2300	2
Karawek (NP4)	NP	23	2450	1
Doksano (NP5)	NP	20	2550	1
Sopha (NP6)	NP	40	3100	2
Mayuree (P1)	P	22	2436	1
Chommanat (P2)	P	25	2660	1
Kamlaiphet (P3)	P	33	2646	2
Namphung (P4)	P	35	2930	1
Wandek (P5)	P	19	2300	1

NP: non pregnant group, P: pregnant group

Table 3.2 General information of the 4 bulls Asian elephants utilized in this study

Bull's name (ID)	Age (year)	Weight (kg)	No. of calves
Nga-thong (M1)	26	3300	13
Kotcharat (M2)	19	3000	2
Sud-lor (M3)	35	3600	5
Kong-ka (M4)	32	3500	2

Table 3.3 Information of the bull sniff test in each individual female elephant

Female's ID	Oestrus cycle	Oestrus/mating date	Bull's ID	Standing response in relation to serum P_4 profile
NP1	1	15/10/2005	M1	Early follicular phase
	2	21/2/2006	M1	Late follicular phase
	3	27/6/2006	M1,M2	Late follicular phase
NP2	1	22/11/2005	M1,M2	Early follicular phase
	2	21/3/2006	M1	Late follicular phase
	3	11/7/2006	M1	Late follicular phase
NP3	1	15/10/2005	M2,M3	Early follicular phase
	2	7/2/2006	M1	Late follicular phase
	3	30/5/2006	M1	Early follicular phase
NP4	1	24/10/2005	M1	Early follicular phase
	2	7/2/2006	M1,M2	Late follicular phase
	3	25/4/2006	M1	Late follicular phase
NP5	1	24/1/2006	M1	Early follicular phase
	2	17/5/2006	M1	Late follicular phase
NP6	1	27/12/2005	M1,M4	Early follicular phase
	2	25/4/2006	M1	Early follicular phase
P1	1	29/11/2005	M1,M2	Early follicular phase
	2	28/3/2006	M1	Late follicular phase
P2	1	15/11/2005	M1	Early follicular phase

3.2 Oestrus detection and mating

A total of 4 bull elephants (M1-M4) were used for sniff testing in all females elephants for 2-3 times per week (Table 3.2). Information of the bull sniff testing in each individual female elephant is presented in Table 3.3. Briefly, the bull used his trunk to determine the responsiveness of the females. Firstly, the male approached the lateral side of the female and then used the trunk tip to hover the female body. The sniffing often resulted in a loud (humbling) vocalization by the female. Secondly, the male placed his trunk tip around the female body and to search for the female genital organs. Finally, the male used his trunk to approach the female vulva lips and check for her readiness (Flehmen's reaction) by inserting his trunk tip into the vemeronasal organ at the end of the hard palate (Altmann et al., 1974; Schulte and Rasmussen, 1999). After the oestrus was observed, natural mating was arranged within 2-3 days using a proven fertile bull elephant. In case that the first bull did not natural mate the female within 1-2

days, additional attempts would be arranged using other bulls. The elephants' mahouts supervised all aspects of sniff tests, courtship and natural mating activities. In the non-pregnant group (Group II), elephants were separated from the bull after oestrus behavior was detected. The bull sniff test was used to check for a return to oestrus in Group I and II elephants. In general, the sniff test was performed at least once a week after mating throughout the study.

3.3 Blood and faecal collection

Blood samples (5-10 ml) were collected weekly from the ear veins of all elephants for about 43 weeks. Blood was allowed to clot at room temperature for 30 minutes and centrifuged for 10 min at 1500 rpm. Serum was collected and stored at –20°C until hormonal analyses.

Faecal samples were collected from each elephant at the ground within 24 hrs after the blood collection. Each faecal sample consisted of inner and outer bolus aliquots, and was placed in a 16x125 mm plastic tube. Samples were kept in an ice box during transportation to the laboratory and then stored at –20°C.

3.4 Faecal extraction

All faecal samples were dried in a hot air oven (Memmert model 600 D06062, Schwabach) at 75-80 ºC for 15-20 h and were kept in plastic tubes at room temperature until an extraction similar to that previously described (Wasser et al, 1996). Dried feces 0.1 g was added to 5 ml of 90% ethanol (ETOH) and vortexed briefly. The samples were boiled in a water bath (96 ºC) for 20 minutes with additional ETOH being continually added to prevent samples from drying out. The final extract was brought up with ETOH to 5 ml and centrifuged at 2500 rpm for 20 minutes. The supernatant was poured off into a second set of identically labeled glass tubes. Another 5 ml of 90% ETOH was added to the remaining faecal pellets and samples were vortexed for 30 seconds and centrifuged at 2500 rpm for 15 minutes. The supernatants was poured off into the second set of tubes containing the first extract. Extracted samples were dried (96 ºC) under air and reconstituted in 3 ml of absolute ETOH, vortexed and dried completely.

This extract was reconstituted in 1 ml MeOH, vortexed and sonicated for 15 min and dried again. The final dried extract was reconstituted in 1 ml phosphate-buffered saline dilution buffer and vortexed. The extracted sample was stored at –20°c until analysis.

3.5 Serum progestin analysis

Serum samples were thawed at room temperature for 3-4 hrs and progestins measured using a solid-phase ^{125}I-P_4 radioimmunoassay (RIA) (Coat-a-Count®, Diagnostic Products, Los Angeles, CA). Briefly, the kit provides tracer steroid and tubes coated with P_4 antibody. A 100 µl sample and 1.0 ml iodinated P_4 tracer was pipetted into tubes in duplicate and vortexed. After 3 h of incubation at room temperature, the liquid was decanted and tubes counted for 1 min in a gamma scintillation counter (Mini instruments scaler ratemeter type 6-90, Burnham on Crouch, England). Assay sensitivity was 0.03 ng/ml. The intra assay coefficient of variation (CV) for low and high concentration P_4 were 7.74 % and 8.20 %, and the inter assay CV for low and high concentration P_4 were 1.42 % and 2.74 %, respectively.

3.6 Faecal progestin analysis

Samples were thawed at room temperature for 3-4 hrs and the extracts analyzed for progestins using an EIA with a P_4 monoclonal antibody (CL425, Munro and Stabenfeldt, 1984) that cross reacts with relevant metabolites (Brown et al., 2005). Briefly, the antibody dilution (1:10,000) was prepared by adding 25 µl antibody stock (1:100) to 5 ml coating buffer. The P_4 standards were prepared to concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.12, and 0.78 pg /well by serial dilution of the standard working stock (4 ng/ml or 200 pg/well) using 200 µl stock plus 200 µl EIA buffer. Serum samples and controls were diluted in dilution buffer. The P_4 horseradish peroxidase (HRP) enzyme tracer working dilution (1:40,000) was prepared by adding 30 µl of HRP to 6 ml EIA buffer. For the assay, each well on the plate (NUNC, Maxisorb plates), except for nonspecific binding (NSB) wells, were coated with 50 µl of antibody solution and incubated overnight (12 h) at 4°C. Plates were washed 5 times with washing buffer and the plate blotted on a towel to remove excess washing solution. 50 µl of standard, control and sample per well were pipetted using a plate map. Afterward 50 µl of diluted

P_4 HRP were added to all wells. The samples and standards were pipetted into the wells within 10 min and incubated at room temperature for 2 h. 100 µl of ABTS substrate (40 µl 0.5 M H_2O_2, 125 µl 40 mM ABTS and 12.5 ml substrate buffer) was added to all wells and incubated at room temperature with shaking. Plates were read at 405 nm (ELISA reader, Labsystem Multiskan MS type 352 serial 352002098, Finland). Optical density (OD) for 0 wells is > 0.7 to < 1 OD. Assay sensitivity at 90% binding was 0.016 ng/well. The intra assay CV for low and high controls were 2.61% and 2.3 % and inter assay CV for low and high controls were 20.5 % and 10.2 %, respectively.

3.7 Statistical analysis

The statistical analyses were performed using SAS (SAS Inst. Version 9.0, Cary, NC., USA). Descriptive data were presented as mean±standard deviation (SD). The length of the oestrus cycle, follicular phase and the luteal phases were calculated. The follicular and luteal phase lengths were calculated based on serum P_4 profiles of each animal. Increases in serum P_4 were considered indicative of a luteal phase if concentrations exceeded >0.05 ng/ml for more than 2 consecutive weeks. The luteal phase included those days from the initial rise in P_4 until concentrations returned to baseline. Spearman's correlation was used to analyze the correlation between serum P_4 and faecal progestins concentration. Analysis of variance (ANOVA) was used to evaluate differences among serum P_4 and faecal progestins during the oestrus cycle. Differences between groups were determined by the Student's *t*-test. The statistical significance level was defined as $P<0.05$.

CHAPTER 4

RESULTS

On an average, the oestrus cycle of the non-pregnant female Asian elephant based on the serum P_4 profile was 14.1±2.4 weeks (range 10-17 weeks). The follicular phase was 7.0±2.3 weeks (range 4-11 weeks) and the luteal phase was 7.0±2.7 weeks (range 3-11 weeks) in duration (Table 6). The females showed the oestrus behavior in the early follicular phase 52.63%. During a normal oestrus cycle, serum P_4 and faecal progestins concentrations varied considerably among and within animals (see below).

Table 4.1 Individual oestrous cycle based on serum P4 and faecal progestins

Female's ID	Cycle	Length of the oestrous cycle	Serum P_4 (follicular:luteal phase) (week)	Faecal progestins (follicular:luteal phase) (week)
NP1	1	16	8 : 8	10 : 6
	2	17	7 : 10	9 : 8
NP2	1	15	9 : 6	8 : 7
	2	15	6 : 9	NA
NP3	1	14	11 : 3	NA
	2	15	4 : 11	NA
NP4	1	12	8 : 4	NA
	2	10	5 : 5	NA
	3	10	4 : 6	
NP5	1	15	10 : 5	NA
NP6	1	16	6 : 10	NA
P1	1	16	10 : 6	NA
Mean±SD		14.1±2.4	7.0±2.3 : 7.0±2.7	NA

NA=data not available

4.1 Serum P_4 profiles in pregnant and non-pregnant female Asian elephants

Concentrations of serum P_4 varied considerably among individuals and the stage of the oestrus cycle. The individual concentration of serum P_4 determined in the present study ranged from 0.03 up to 1.82 ng/ml in boths group. On an average, serum P_4 varied between 0.03 to 1.82 ng/ml in pregnant elephants and between 0.03 to 0.54 ng/ml in non-pregnant elephants (Fig 4.1). Regardless of the stage of the oestrus cycle, the overall mean serum P_4 concentration differed significantly between pregnant and non-pregnant elephants (0.56 versus 0.08 ng/ml, $P<0.001$). Mean serum P_4 was elevated for 5-10 weeks in non-pregnant elephants (Fig. 4.1). In pregnant elephants, the mean serum P_4 was increased significantly from the 4th week after mating and remained elevated throughout the evaluation period. The levels of serum P_4 in pregnant elephants were significantly higher than non-pregnant elephants after the 13th week post oestrus/mating (0.89 versus 0.03 ng/ml, $P=0.05$).

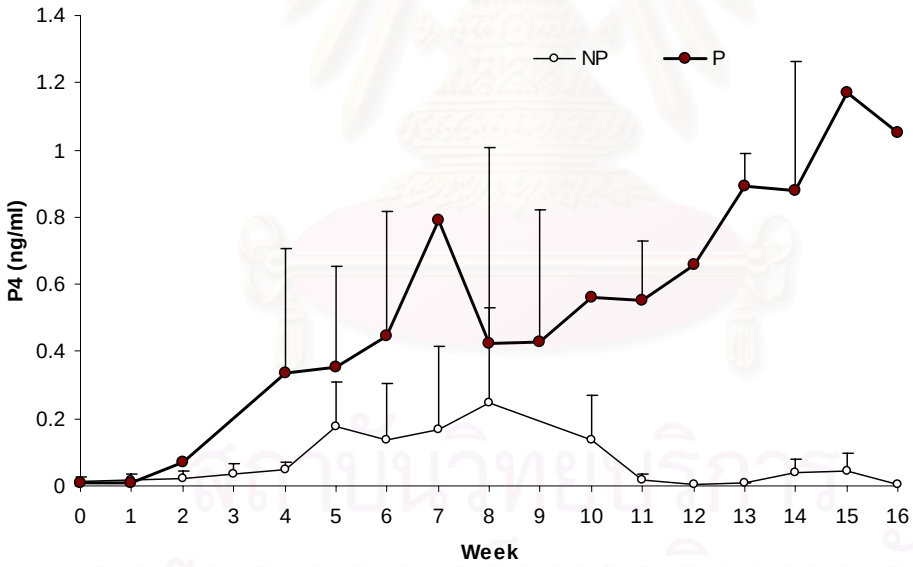

Fig 4.1 Mean (±SD) serum P_4 profiles in pregnant (P, n=4) and non-pregnant (NP, n=4) Asian elephants (*Elephas maximus*). Week 0 is designated as oestrus/mating based on the bull sniff test.

4.2 Relationship between serum P_4 and faecal progestins in non-pregnant female Asian elephants

During the oestrus cycle, overall mean serum P_4 varied from 0.03-1.04 ng/ml (0.125±0.20 ng/ml, means±SD) and faecal progestins varied from 176-896 ng/g (478.99±152.60 ng/g, means±SD). On average, the level of serum P_4 dramatically increased from the 3rd to the 4th week of the oestrus cycle, reached the peak concentration at week 6 and then decreased. Mean faecal progestins increased slowly from the first week and reached peak concentrations at week 7th after standing oestrus and fluctuated at a high level until week 15 (Fig 4.2). Both serum P_4 and faecal progestins were high during week 4 -8 of the oestrus cycle. Concentrations of serum P_4 between week 4th -8th of the oestrus cycle were significantly different from week 0 (P<0.05) (Fig. 4.2). Concentrations of faecal progestins at the 4th, 5th, 7th, 8th, 11th, 13th and 15th week of the oestrus cycle were significantly different from week 0 (P<0.05) (Fig. 4.2). The overall mean concentrations of serum P_4 and faecal progestins in non-pregnant female elephants were significantly correlated (r=0.32, P=0.004).

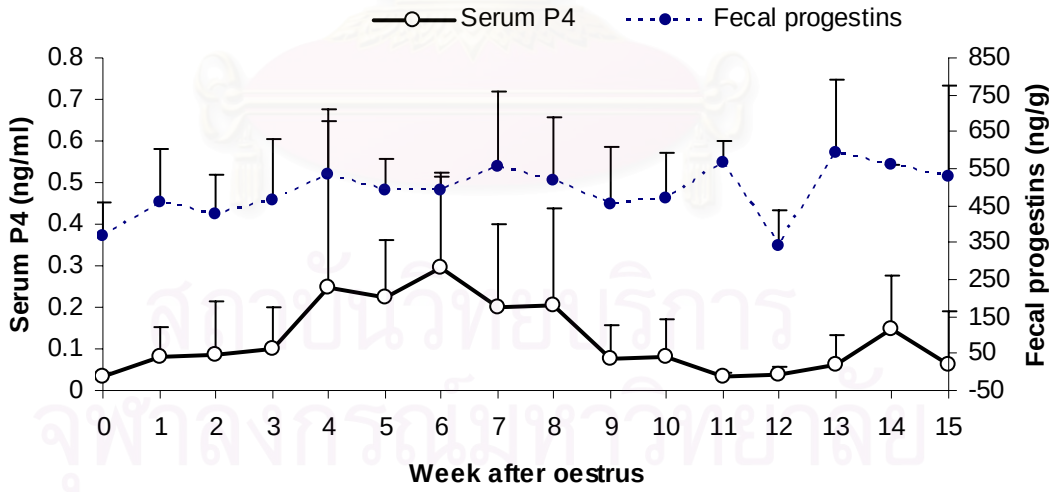

Fig. 4.2 Mean (±SD) serum P_4 and faecal progestins profiles during the oestrus cycle from 6 non-pregnant female Asian elephants. Week 0 is designated as the beginning of standing oestrus (n=13 oestrus cycle) based on the bull sniff test. Superscripts (a and A) indicate significant differences from week 0 within hormone (P<0.05).

4.3 Relationship between serum P_4 and faecal progestins in pregnant female Asian elephants

During the early stage of pregnancy (≤35 weeks), overall mean serum P_4 varied from 0.03-1.67 ng/ml (0.22±0.30 ng/ml) and faecal progestins varied from 208-910 ng/g (466.03±169.90 ng/g). On average, the concentration of serum P_4 dramatically increased from the 4th to the 5th week of the oestrus cycle and remained elevated throughout gestation, whereas faecal progestins increased from the first week and reached peak concentrations at about 12-14 weeks after standing oestrus onwards and remained high throughout the first 35 weeks of gestation (Fig 4.3). In total, the overall mean concentrations of serum P_4 and faecal progestins in pregnant Asian elephants were significantly correlated (r=0.33, *P*<0.001). However, the correlation coefficient varied between animals from r= -0.086 to r= 0.88 (see part 4.4).

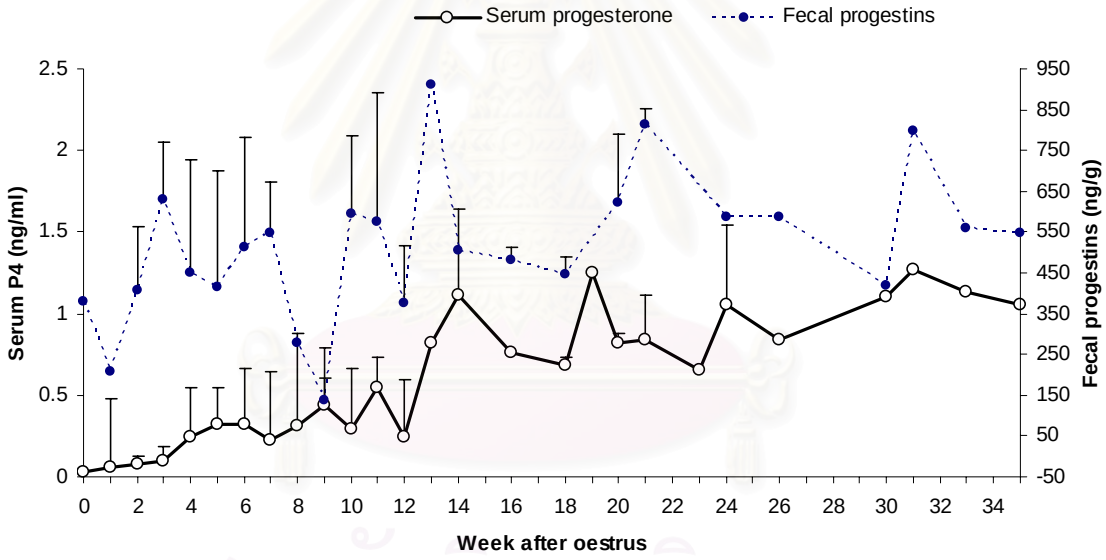

Fig. 4.3 Mean (±SD) serum P_4 and faecal progestin profiles during the early stage of pregnancy in female Asian elephants (n=3). Week 0 is designated as the beginning of standing oestrus based on the bull sniff test and mating.

4.4 Individual serum and faecal progestin profiles

For NP1 (Jumpee), during the normal oestrus cycle, serum P_4 varied from 0.03-0.58 ng/ml (0.186±0.20 ng/ml) and faecal progestins varied from 252-574 ng/g (385.70±91.24 ng/g). On average, the level of serum P_4 increased from the 4th to 5th week of the oestrus cycle, whereas faecal progestins increased slowly from the first

week and reached the peak concentrations at week 10 after standing oestrus (Fig 4.4). The concentrations of serum P_4 and faecal progestins were significantly correlated (r=0.80, *P*<0.001).

For NP2 (Jamjuree), during oestrus cycle, the serum P_4 varied from 0.03-1.04 ng/ml (0.269±0.280 ng/ml) and faecal progestins varied from 330-882 ng/g (553.4±122.7 ng/g). On average, the level of serum P_4 increased from the 4^{th} to 5^{th} week of the oestrus cycle, whereas faecal progestins increased slowly from the first week and reached the peak concentrations at weeks 7^{th} after standing oestrus (Fig 4.5). The concentrations of serum P_4 and faecal progestins were significantly correlated (r=0.88, *P*<0.001).

For NP3 (Namphet), during the oestrus cycle, serum P_4 varied from 0.03-0.12 ng/ml (0.045±0.030 ng/ml) and faecal progestins varied from 176-729 ng/g (463.7±151.3 ng/g). On average, the level of serum P_4 fluctuated as did the faecal progestins (Fig 4.6). This animal seemed to have a rather short oestrus cycle (about 9-10 weeks), and overall lower concentrations of hormones. The concentrations of serum and faecal progestins were not correlated (r = -0.086, *P*=0.735).

For NP4 (Karawek), during the oestrus cycle, serum P_4 varied from 0.03-0.14 ng/ml (0.053±0.033 ng/ml) and faecal progestins varied from 343-644 ng/g (457.80±103.02 ng/g). The level of serum P_4 fluctuated as well as the faecal progestins. This animal seemed to have a rather short oestrus cycle (about 8-12 weeks), and overall lower concentrations of hormones (Fig 4.7). The concentrations of serum P_4 and faecal progestins were not correlated (r= -0.17, *P*=0.605).

For NP5 (Doksano), during the oestrus cycle, serum P_4 varied from 0.03-0.17 ng/ml (0.05±0.05 ng/ml) and faecal progestins varied from 308-504 ng/g (424.7±598.7 ng/g). On average, the level of serum P_4 fluctuated with low concentrations as did the faecal progestins (Fig 4.8). The concentrations of serum P_4 and faecal progestins were not correlated (r=0.13, *P*=0.76).

For NP6 (Sopha), during the oestrus cycle, serum P_4 varied from 0.03-0.29 ng/ml (0.14±0.10 ng/ml) and the faecal progestins varied from 238-896 ng/g (637.5±200.7 ng/g). The level of serum P_4 fluctuated with low concentrations as well as the faecal progestins (Fig 4.9). The concentrations of serum P_4 and faecal progestins were not correlated (r = -0.16, *P*=0.64). The serum P_4 and faecal samples began to be collected close to the time of calving with the record. It was found that the oestrus symptoms occurs 3 months after calving.

For P1 (Mayuree), during early pregnancy, serum P_4 varied from 0.03-1.67 ng/ml (0.13±0.31 ng/ml) and faecal progestins varied from 350-770 ng/g (548.63±539.66 ng/g). On average, the level of serum P_4 dramatically increased after mating and remained elevated, whereas faecal progestins fluctuated more randomly (Fig 4.10). The concentrations of serum P_4 and faecal progestins were significantly correlated (r=0.62; *P*=0.003).

For P2 (Chommanat), during early pregnancy, serum P_4 varied from 0.03-1.40 ng/ml (0.47±0.49 ng/ml) and faecal progestins varied from 134-840 ng/g (397±219 ng/g). On average, the level of serum P_4 dramatically increased from the 4^{th} week after mating and remained elevated, whereas faecal progestins increased slowly with a similar pattern (Fig 4.11). The concentrations of serum P_4 and faecal progestins were significantly correlated (r=0.77; *P*=0.0001).

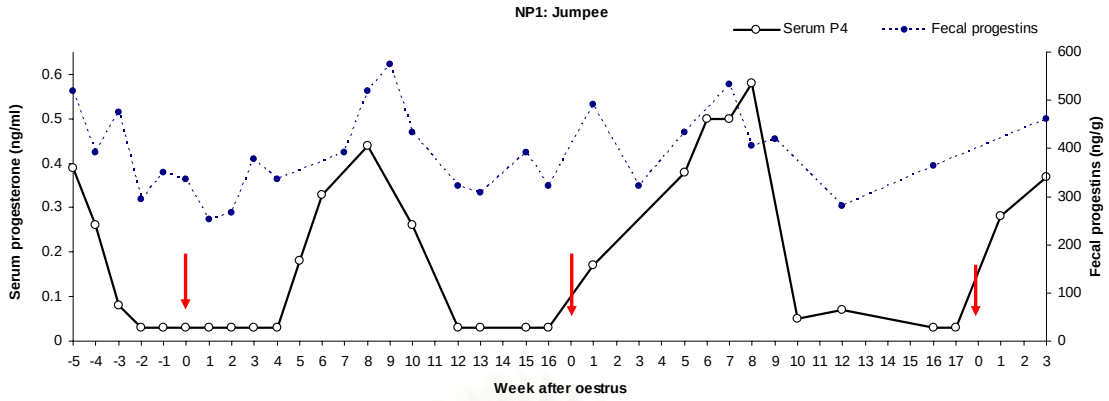

Fig. 4.4 Serum P_4 and faecal progestins profiles during the oestrus cycle in NP1. Week 0 is designated as the beginning of standing oestrus (arrow)

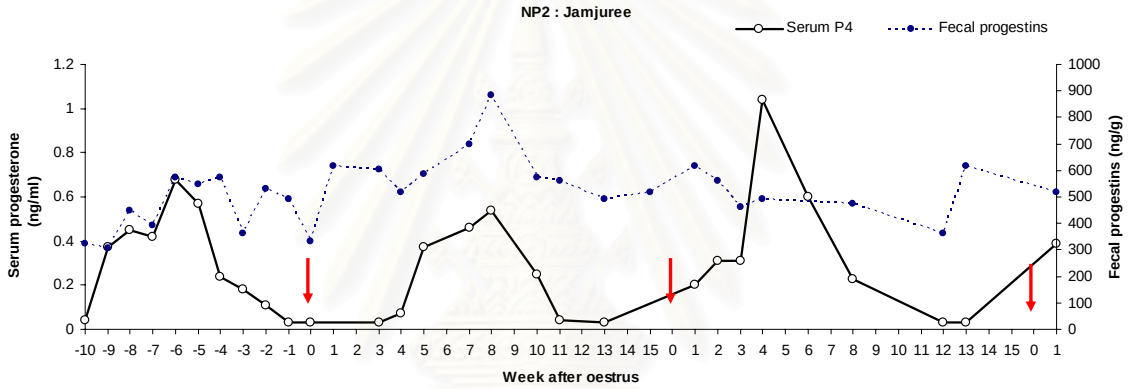

Fig. 4.5 Serum P_4 and faecal progestins profiles during the oestrus cycle in NP2. Week 0 is designated as the beginning of standing oestrus (arrow)

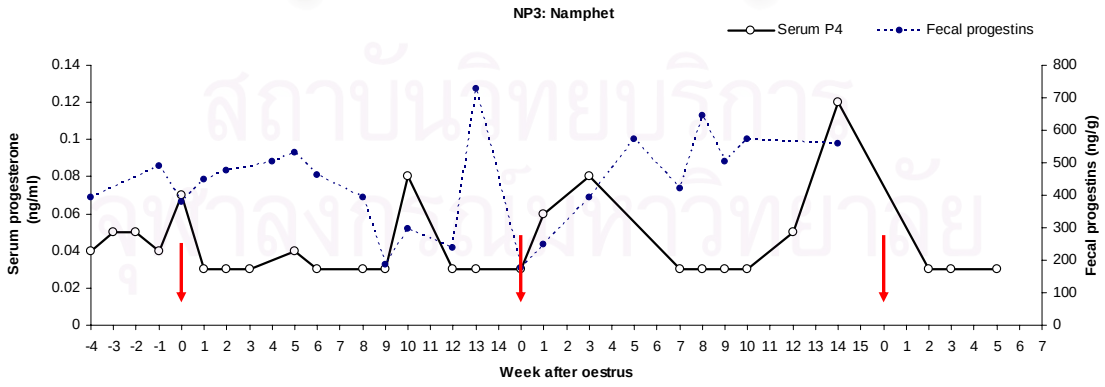

Fig. 4.6 Serum P_4 and faecal progestins profiles during the oestrus cycle in NP3. Week 0 is designated as the beginning of standing oestrus (arrow)

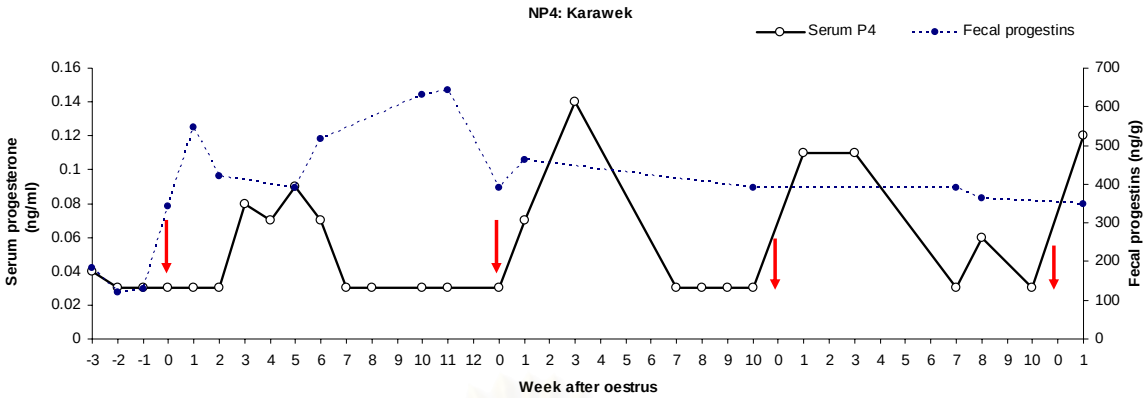

Fig. 4.7 Serum P_4 and faecal progestins profiles during the oestrus cycle in NP4. Week 0 is designated as the beginning of standing oestrus (arrow)

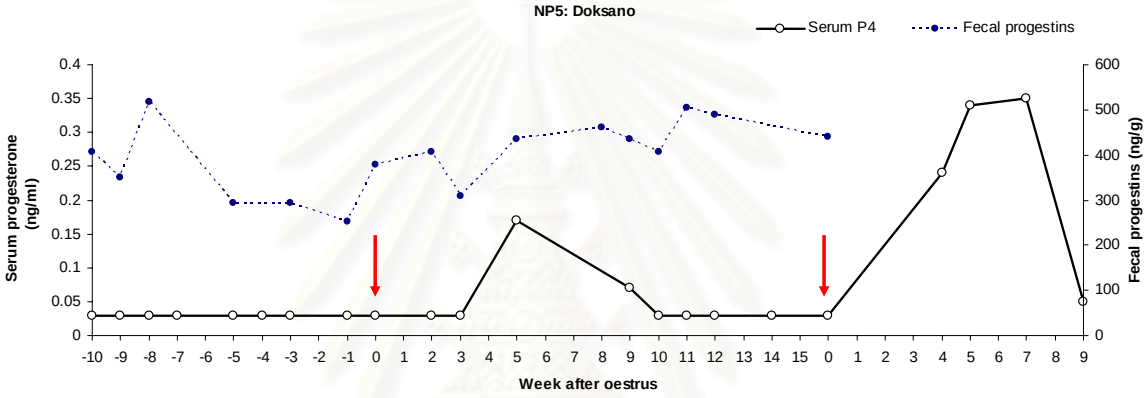

Fig. 4.8 Serum P_4 and faecal progestins profiles during the oestrus cycle in NP5. Week 0 is designated as the beginning of standing oestrus (arrow)

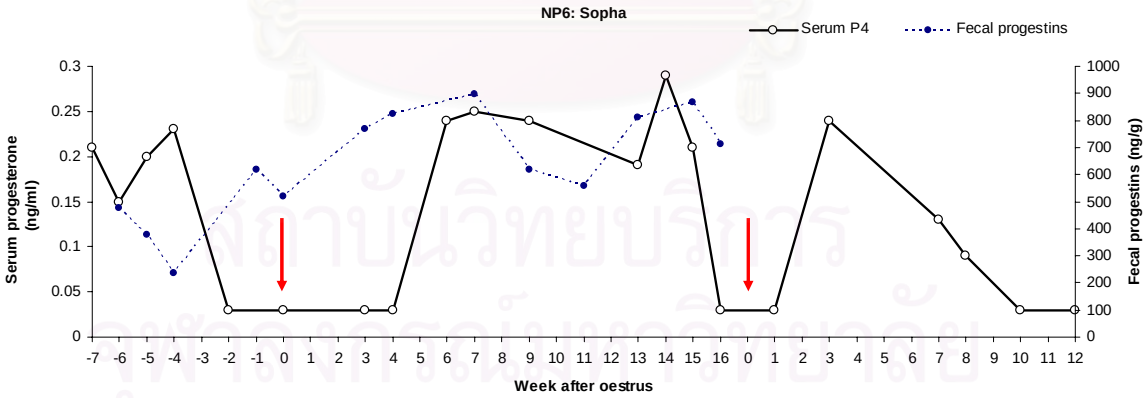

Fig. 4.9 Serum P_4 and faecal progestins profiles during the oestrus cycle in NP6. Week 0 is designated as the beginning of standing oestrus (arrow)

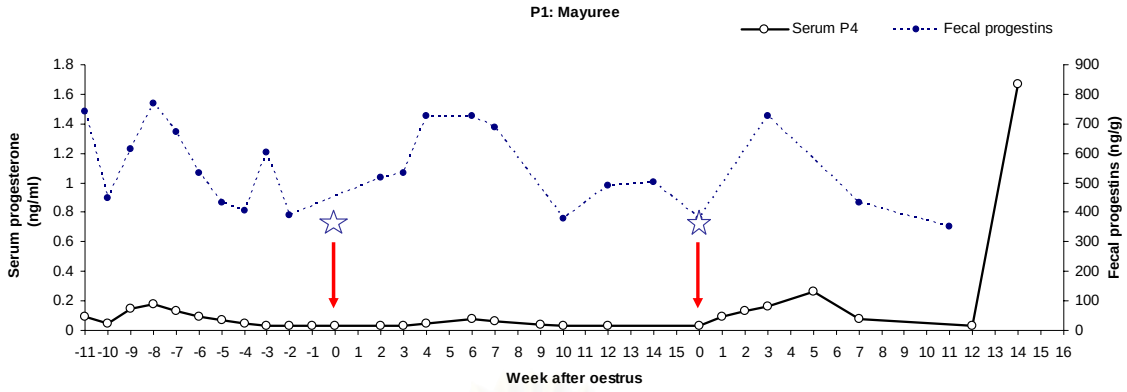

Fig. 4.10 Serum P_4 and faecal progestins profiles during the early pregnancy in P1. Week 0 is designated as the beginning of standing oestrus (arrow), ☆: mating

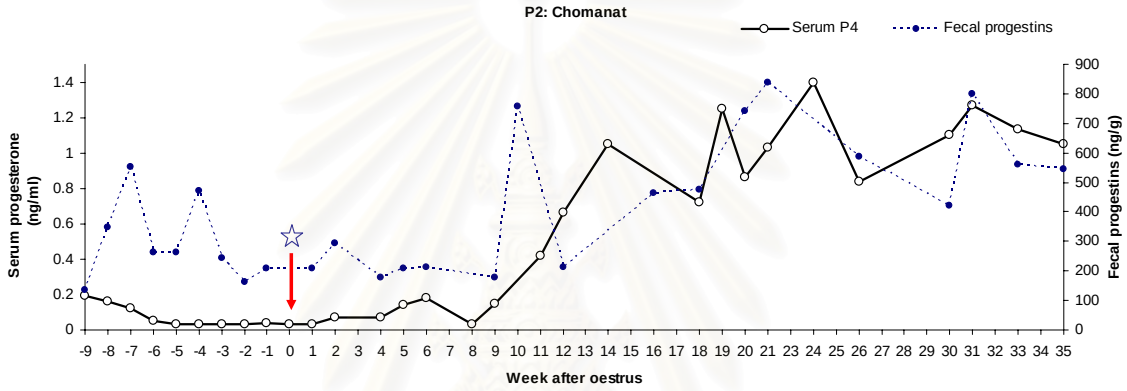

Fig. 4.11 Serum P_4 and faecal progestins profiles during the early pregnancy in P2. Week 0 is designated as the beginning of standing oestrus (arrow), ☆: mating

CHAPTER 5

DISCUSSION

The present study attempted to establish valid immunoassay techniques for measuring serum P_4 and faecal progestins in Thai elephants. The goal was to characterize hormonal profiles during the oestrus cycle and early pregnancy in domestic female Asian elephants managed under hot and humid climate conditions. Historically, two methods have been used to measure the serum P_4 and faecal progestins in elephants i.e., RIA and EIA (Hess et al., 1983, Olsen et al., 1994; Brown et al., 1991; Taya et al., 1991; Kapustin et al., 1996). In the present study, serum P_4 were measured by an RIA technique, while the faecal progestins was measured by the EIA method. The RIA was used to measure serum P_4 because it has good crossreactivity with relevant progestin metabolites in circulation and is the most common technique used for measurement of serum P_4 in elephants, as well as in other mammals (Brown et al., 1991, 1995a, 1997, 1999, 2004a, Kapustin et al., 1996, Carden et al, 1998, Tummaruk et al. 2004b). The EIA technique was used to measure the faecal progestins (Wasser et al., 1996,, Brown et al., 2005).

5.1 Serum P_4 profiles in pregnant and non-pregnant Asian elephants

The present study confirmed that serum P_4 concentrations in female Asian elephants vary throughout the oestrus cycle, being highest during the luteal phase of the cycle. The concentration of serum P_4 detected in normal oestrus cycle of the present study (range 0.03-0.54 ng/ml) was in agreement with previous findings (Hess *et al*., 1983; Plotka *et al*., 1988; Mainka and Lothrop, 1990; Taya *et al.* 1991; Kapustin *et al*., 1996). Based on serum P_4, the overall length of the oestrus cycle in general and the luteal phase specifically appeared shorter than previous reports of elephants in western zoos (Hess *et al*., 1983; Plotka *et al*., 1988; Mainka and Lothrop, 1990; Taya *et al.* 1991, Brown et al., 1991). This might be due to individual differences in social behavior or reproductive tract pathologies, such as leiomyomas (fibroids) (Montali et al., 1997). It could also be a reflection of normal variation among elephants, with these animals being

on the low end of normal. The facts that all had conceived previously at least once suggests they are potentially fertile.

The present study also demonstrated significant differences in the serum P_4 and faecal progestins profile between pregnant and non-pregnant Asian elephants. Other studies have reported that progestins concentrations may (Hess et al., 1983; McNeilly et al., 1983; Olsen et al., 1994; Brown and Lehnhardt, 1995) or may not (Mainka and Lothrop, 1990) be higher during gestation, but overlap enough so that serial samples are required for accurate diagnosis. In this study population, it was found that after the 13^{th} week of pregnancy, the P_4 concentration was significantly higher in pregnant than in non-pregnant elephants. Thus, serum P_4 can be used for pregnancy diagnosis in the Asian elephant after this time period, but only if longitudinal samples are collected. This appears to be true regardless of what assay is used. For example, in a 20-oxo-P assay, P_4 metabolite concentrations in the pregnant and non-pregnant elephants overlapped enough to prevent using single sample analysis as a pregnancy diagnosis tool (Schwarzenberger et al., 1997).

5.2 Relationship between serum P_4 and faecal progestins in non-pregnant female Asian elephants

It was unclear why the faecal progestin was not significantly different between the luteal and the follicular phases. Earlier studies in wild African elephants showed a significant increase in the level of faecal progestins concentrations in dry season due to quality of food, water availability, body condition and stress (Foley et al., 2001). We do not know yet whether the Asian elephants produced different progestins compare to African elephants that CL425 monoclonal antibody were not suitable in our experiment (Foley et al., 2001). However, the significant correlation between faecal progestins and serum P_4 was encouraging and indicates this technique might be useful for monitoring the reproductive status of captive and wild elephants as in African elephant. The high variation among individual elephants in the faecal progestin data suggest more individuals need to be examined, preferably for longer periods of time.

5.2 Relationship between serum P_4 and faecal progestins in pregnant female Asian elephants

In pregnant elephants, the serum P_4 and faecal progestins had a significant correlation, which is in agreement with previous report in African elephants (Graham, 2000, Foley et al., 2001). However, the high variation among individual elephants for the faecal progestin suggest that more individuals data need to be examined, preferably for longer periods of time.

5.4 Individual serum P_4 and faecal progestins profiles

The present study demonstrated significant differences in the serum P_4 and faecal progestins concentrations between the luteal and the follicular phases. However, in 4 out of 8 elephants, the serum P_4 and faecal progestins were not correlated. It should be noted that those females that did not have a significant correlation also were those with unclear oestrus cycles. Some elephants seem to be non-cycling especially NP3, NP4 and NP5.

In the NP6, the serum P_4 and faecal samples were collected close to the time of calving. It was found that the oestrus symptoms occurs 3 months after calving. This is similar to earlier reports (Dehnhard et al., 2001).

In the present study, we found that the male elephants exhibited the highest courseship behavior during the first half of the follicular phase (52.6%), which is earlier than reports on when natural mating (Brown et al., 1997, Olsen et al., 1994, Carden et al., 1998). However, there are anecdotal tales of females displaying false oestrus, including eliciting bull interest, about 3 weeks before oestrus (Brown et al., 1999). It would be interesting to determine the proportion of females that exhibit sexual receptivity at the time of the anLH surge. Understanding the function of the first follicular wave is of interest. Elephants have the longest calving interval compared to other land mammals, which can be varied from 3 to 7 years. Oestrus is a rare event, and so females would benefit by evolving a mechanism to advertise impending fertility and attract bulls. Elephants have been observed in 'false behavioural oestrus' several weeks

before conceptive mating occurs. It was found that female Asian elephants excrete a urinary pheromone, (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac), that stimulates male breeding behaviour (Dehnhardt et al., 2001; Rassmussen, 2001). After luteal progestagens decline to baseline, *Z*7-12:Ac becomes detectable in the urine, increasing in a linear fashion throughout the follicular phase. This pattern suggests that follicles from both waves are capable of mediating this pheromonal signal. However, *Z*7-12:Ac concentrations are highest just before ovulation; thus, production is greatest during the second wave in conjunction with dominant follicle selection. Considering the long distances bulls travel in search of oestrus females in nature, this strategy would be highly conducive to species survival. Studies are needed to determine if other physiological changes, such as temporal gland or urinary pheromone excretion accompany the anLH surge. It has been reported in the Asian elephants that during the first week of the luteal phase, the oestrus behavior and the endocrine changes had a significant 1-2 day transient decrease in progestin secretion (Carden et al., 1998; Brown et al., 1999). It has been suggested that the initial increase in the serum P4 come from the follicular origin, while the secondary rise resulted from ovulation (Carden et al., 1998). The mechanism by which bull elephants recognize this peri-ovulatory period has been shown to be regulated by pheromone signals from the urine (Rasmussen et al., 1998). In addition, behavioral interest of the male elephant in the females sniff tests occurred just prior to the time of peak urinary total E_2 (Mainka et al., 1990).

Further studied are needed to evaluate the profiles with other sex hormones in relation to the oestrus behaviors. The next step should use suitable and competent ultrasound instruments to determine the reproductive organs in association with the hormonal profiles. Given that few, if any, captive populations of Asian elephants are self-sustaining, understanding the relationship between endocrine status, behavior, abnormal function and fertility is critical to help elephant camps develop reliable natural breeding program to maximize reproductive efficiency.

CONCLUSIONS

In the present study, it could be concluded that:

- Serum P_4 in Asian elephants could be measured using an RIA technique and the level of P4 varied according to the stage of the oestrus cycle
- The concentrations of serum P_4 in pregnant elephants were significantly higher than non-pregnant elephants after 13^{th} weeks of mating
- Faecal progestins and serum P_4 were significantly correlated in some, but not all females. This indicates that the technique might be useful for monitoring the reproductive status of captive and wild elephants, but assays need to be refined to make this a definitive test for assessing ovarian status
- The faecal progestins could be apply for wild elephants but has a high variation among individual elephants. We suggested that more individuals needed to be examined

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