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Original Article

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Abstract

The aims of this study were to demonstrate the effectiveness of fecal progesterone metabolite monitoring technique during estrus synchronization and to investigate fecal progesterone metabolite profile in Eld's deer (*Rucervus eldii thamin*) hinds for one year (February 2009-Janurary 2010). Fecal samples from five hinds were collected weekly, subjected to hormone extraction and subsequently progesterone analysis using enzyme immunoassay (EIA). Estrus synchronization and artificial insemination were performed during breeding season (June 2009). This included an intramuscular injection of Prostaglandin$F_{2\alpha}$ (5 mg) in combination with a vaginal insertion of a controlled internal drug-releasing device (CIDR type G) for 14 d. After CIDR-G removal for 70 h, all hinds were inseminated with frozen thawed semen by laparoscopic technique. This study found that one from five hinds responded to hormone stimulation. The fecal progesterone metabolite of the responded hind (no.11) was significantly highest compared with the non-responded hinds (no.7, no.42, no.43 and no.44) during the CIDR-G insertion (2,360±320 ng/g dry feces) (p<0.05), whereas the fecal progesterone concentrations of the four non-responded hinds during synchronization did not differ from basal progesterone concentrations (p>0.05). Mean±SE of progesterone metabolite concentrations during March to August (390.1±33.2 ng/g) were significantly higher than during September to February (291.4±20.1 ng/g) (p<0.05). The non-pregnant Eld's deer hinds exhibited multiple estrous cycles. Long estrous cycle (24.9±2.2 d) was observed throughout the year and mean number of estrous cycles was 14.0±0.4 cycles/animal/year. The concentration (mean±SE) of fecal progesterone during gestation was 816.9±60.0 ng/g in early pregnancy, 1,998.1±416.7 ng/g in middle pregnancy and 5,995.5±757.4 ng/g in late pregnancy. Consequently, fecal steroid analysis by EIA for monitoring of reproductive function and fecal steroid measurement can be used as an important breeding management in Eld's deer.

Keywords: Eld's deer, enzyme immunoassay, hormone, progesterone

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Introduction

Eld's deer (*Rucervus eldii*) is a subtropical cervid species of South and Southeast Asia. This deer species is listed as endangered by the IUCN (Timmins and Duckworth, 2008), and is one of fifteen species listed in the Thailand's National Wildlife Reservation and Protection Act since1992. Two subspecies of Eld's deer; the Thamin Eld's deer (*Rucervus eldii thamin*) and Siamese Eld's deer (*Rucervus eldii siamensis*) (Balakrishnan et al., 2003), historically existed in Thailand's dry forest and were extinct in the wild since 1980s. However, both populations are maintained in captivity. Because of a small population size and low heterozygozity, Siamensis population exhibits low fecundity and high incidence of stillbirths (Personal communication, Dusit Zoo, Thailand). From the joint conservation effort among Thai government agencies, Zoological Park Organization, Department of National Parks, Wildlife and Plant Conservation, academics and Smithsonian Conservation Biology Institute, the Thamin Eld'sdeer have been successfully bred and reintroduced into the wild (Buranapim et al., 2008; Prempree et al., 2013). At present, a total of 691 Eld's deer (641Thamin and 50 Siamensis) are being maintained in *ex-situ* conservation centers (unpublished data, Nikorn Thongtip).

Female Eld's deer attain puberty at 2 years of age (Myint et al., 2001). Data from behavioral (Wemmer and Grodinsky, 1988) and endocrine studies (Monfort et al., 1990[a]; Monfort et al., 1990[b]) indicate that the females are seasonally polyestrus with the length of estrous cycle ranging from 13.8 to 21.5 days (Hosack et al., 1997; Monfort et al, 1990[a]). Even though the animals were translocated to Northern temperate latitudes, estrous behaviors occur in early spring (January-March) and anestrus begin in autumn (August-October) (Monfort et al., 1990[b]). Gestational period of Eld's deer is 235-245 d (Monfort et al., 1993). In captivity, calves usually born between September and November in France (48°N latitude, Prescott, 1987) and the United States (38°N latitude, Wemmer and Grodinsky, 1998) and September to February in China (19.6°N latitude, Song and Zeng, 2003).

It is well recognized that captive breeding programs play important roles in conserving wildlife.These populations are important as a resource for public awareness programs in zoos and as a hedge in case of some catastrophic events (e.g., disease) occurring in nature. In many wildlife species that maintained *ex situ*, assisted reproductive technology has been widely utilized to maximize individual's reproductive performance and to maintain genetic diversity within the population. Non-invasive endocrine monitoring is one of the useful tools for breeding management. It has been used to generate information on species, reproductive biology, knowledge that is essential for successful establishment of assisted reproductive technologies such as artificial insemination. There has been a report on the monitoring of the urinary steroid pregnanediol-3α-glucuronide in captive of Eld's deer hinds. It indicates that Eld's deer has prolonged ovarian activity and exhibits serial reproductive cycle in one year at temperate latitudes. Currently, information about reproductive cycle of Eld's deer in native habitat is limited. Non-invasive monitoring of reproductive status has been studied in several deer species including white-tailed deer (*Odocoileus virginianus*) (Kapke et al., 1999), brown brocket deer (*Mazama gouazoubira*) (Pereira et al., 2006), Pere David's deer (*Elaphurus davidianus*) (Li et al.,2001) and Sika deer (*Cervus nippon*) (Yamauchi et al., 1999). Two objectives of this study were to utilize fecal progesterone monitoring in examining the response to an estrus synchronization protocol and to assess seasonal fluctuation of fecal steroid concentrations in the Eld's deer hinds.

Materials and Methods

Animals and sample collection: Thamin Eld's deer hinds (n=5, 3-6 years old; 35-45 kg body weight) were maintained at Kasetsart University Kampheng Sean Campus, Thailand (14°N latitude). All hinds were housed in stocking rate and exposed to natural photoperiod. In this study, there was no stag as a teaser animal. They were fed *ad libitum* fresh Paragrass (*Brachiaria mutica*), commercial pellets, mineral block and water supply. Fecal samples were collected weekly during February 2009 to January 2010. The fecal samples were collected at 8:30 weekly. All samples were stored at -20°C until steroid analysis.

Synchronization of estrus and artificial insemination: All Eld's deer hinds were synchronized with treatment consisting of 5 mg $PGF_{2\alpha}$ analogue ($PGF_{2\alpha}$5 mg/ml/hind; Lutalyse™, PfizerAnimal Health, U.S.A.) on day 0 and a CIDR-G (Eazi-Breed™ Sheep Insert, Pfizer Animal Health, New Zealand) was inserted intravaginally into each restrained hind for 14 d. CIDR-G was pulled out on day 14. After CIDR-G removal for 70 h (day 17), each hind was artificially inseminated by using laparoscopic procedures described for Eld's deer (Monfort et al., 1993). Total of post-thaw sperm number for insemination was 25 x 10^6 spermatozoa with >50% progressive motility.

Pregnancy diagnosis and fawning data: Pregnancy was diagnosed by progesterone measurement. Behavior and changing of physiology of all hinds were observed daily throughout the experiment.

Fecal steroid hormone extraction and enzyme immunoassay: Frozen fecal samples were thawed and dried at 60°C for 72 h. Dried fecal samples were ground. Steroid hormones were extracted with ethanol based procedure as described by Brown et al. (2004). Briefly, 0.1g feces was mixed with 5 ml of 90% ethanol in a 15 ml glass tube after vortexing. The tubes were then boiled in a water bath at 90°C for 20 min, and centrifuged at 1,500 rpm for 20 min. Supernatant was transferred into a new sample tube. A total of 5 ml of 90% ethanol was added to a pellet, vortexed for 30 seconds and recentrifuged for 15 min. The second supernatant was combined with the first one, dried and reconstituted in 1 ml of dilution buffer for frozen storage. Progesterone EIA was performed as described by Brown et al. (2004). Fecal samples were assayed in duplicate. Antibody for progesterone metabolite analysis (monoclonal pregnane CL no.425, 1:10,000

dilution) was obtained from Coralie Monro (University of California-Davis, CA, USA). Dosages of fecal extract samples from the Eld's deer were conducted according to Brown et al. (2004), by observation of parallelism between serial dilution (1:2-1:2,048) and standard curve (7.8-2,000 pg/ml). The samples were diluted to 1:200 for non-pregnant Eld's deer and 1:400 for pregnant Eld's deer in assay buffer. Sensitivity of assay was 7.8 pg/ml. Intra- and inter assay coefficients of variation were 6.77% and 6% (n=11 plates).

Statistical analysis: Data were presented as mean±SE. The effect of estrus synchronization with $PGF_{2\alpha}$ injection and CIDR-G insertion was evaluated by comparing fecal progesterone concentrations before, during CIDR-G insertion and after removal using paired samples T-test. The lowest progesterone concentrations of each cycle of each hind were defined as baseline progesterone and it was used for calculation. The baseline progesterone was calculated using an interactive process in which values that exceeded the mean plus 1.5 standard deviation were excluded. Average was then recalculated and the elimination process was repeated until no value exceeded the mean plus 1.5 standard deviation (Brown et al., 2001). If the values were more than the baseline progesterone value, they were considered as a luteal phase, whereas if the values were less than the baseline progesterone values, they were considered as an inter-luteal phase. The interval between progesterone concentration of luteal and inter-luteal phases was considered as duration of anestrous cycle (Pereira et al., 2006; Zanetti et al., 2010). Progesterone concentrations of luteal phase and inter-luteal phase were compared by paired samples T-test. Breeding season of Eld's deer in Thailand begins in March to August and non breeding season starts in September to February. Differences in fecal progesterone concentrations of the non-pregnant hinds during March-August and September-February were compared by using two samples T-test. Mean±SE of progesterone concentrations in early (1-11 weeks), middle (12-21 weeks) and late pregnancy (22-32 weeks) were determined by aligning the gestation day. Differences among the three gestation periods were determined using Kruskal-Wallis test, followed by Duncan's new multiple range test. Values of $p< 0.05$ were considered significant.

Results

Progesterone concentrations of estrous synchronized hinds are shown in Table1.The data from five estrous synchronized hinds in this study revealed that four hinds (no.7, no.42, no.43 and no.44) did not respond to CIDR insertion since there were no variations in fecal progesterone metabolites. For the remaining hind (no.11), the progesterone concentrations increased during CIDR insertion, but then declined to similar level before hormone treatment.

Fecal progesterone concentration ranged from 50 to 1,640 ng/g in non-pregnant hinds. Progesterone metabolite concentrations profiles of individual Eld's deer hinds are shown in Fig 1. Mean±SE of progesterone during March to August (390.1±33.2 ng/g of dry feces) was significantly higher than that during September to February (291.4±20.1 ng/g of dry feces) ($p<0.05$) (Fig 3). Mean estrous cycle duration was 24.9±2.2 d. Progesterone concentration of the luteal phase was higher ($p<0.05$) than the inter-luteal phase. Mean of progesterone concentration in the inter-luteal phase and the luteal phase were 176.8±12.8 and 391.0±23.6 ng/g, respectively. Mean number of estrous cycles in a year was 14.0±0.4 cycles (range, 13-15). After artificial insemination, 1 from 5 hinds (no.11) was pregnant. The pregnant hind increased her feed intake and was isolated from the other hind. Before the fawn was to be born, the pregnant hind searched a safe place to give birth and hide her fawn from predators. Gestation duration was 242 d (34.5 weeks). The female fawn was born alive unassisted. Fecal progesterone level for the pregnant hind is presented in Fig 3. Difference in progesterone concentrations among early pregnancy, middle pregnancy and late pregnancy were significant ($p<0.05$). Mean±SE of weekly progesterone profile in early pregnancy was 816.9±60.0 ng/g of dry feces, in middle pregnancy was 1,998.2±416.8 ng/g of dry feces and in late pregnancy was 5,995.6±757.4 ng/g of dry feces. Fecal progesterone metabolite levels were maintained between week 1 to 15 (average 659.3±78.0 ng/g of dry feces) before markedly increased during week 16-32 (4530.3±651.4 ng/g of dry feces). The highest peak was found on week 32 (12,160 ng/g of dry feces).

Table1 Mean± SE fecal progesterone metabolite concentration before, during and after CIDR-G removal in each Eld's deer hind

Events*	Mean ±SE of fecal progesterone metabolite concentration (ng/g of dry feces)				
	No.7	No.11	No.42	No.43	No.44
Before CIDR-G insertion (2 weeks: n = 2)	770±330[b]	640±220[a]	390±250	310±30	140±60
During CIDR-G insertion (2 weeks: n = 2)	1,070±130[b]	2,360±320[b]	160±20	490±90	180±20
CIDR-G removal (2 weeks: n = 2)	270±70[a]	550±150[a]	260±20	200±40	120±20

[a,b]Different subscripts within column are significantly different at the p<0.05 level.

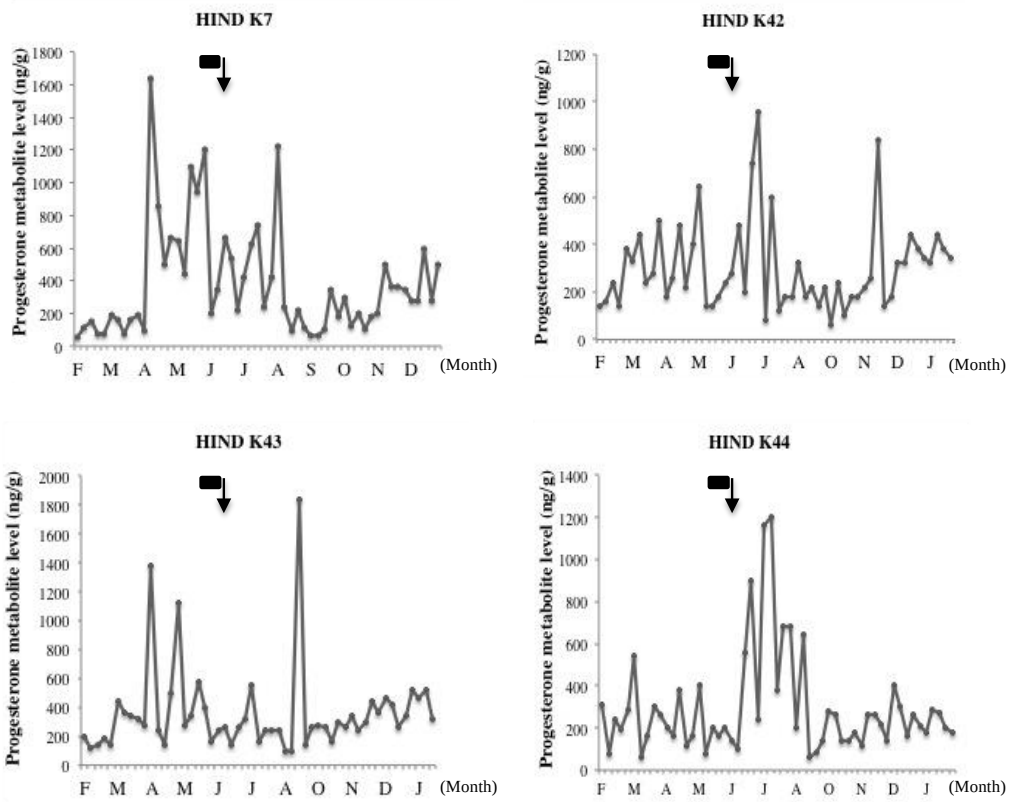

Figure1 Individual progesterone metabolite concentrations of non-pregnant Eld's deer hinds (no.K7, K42, K43, K44) (Black bars = CIDR insertion, arrow = time of artificial insemination)

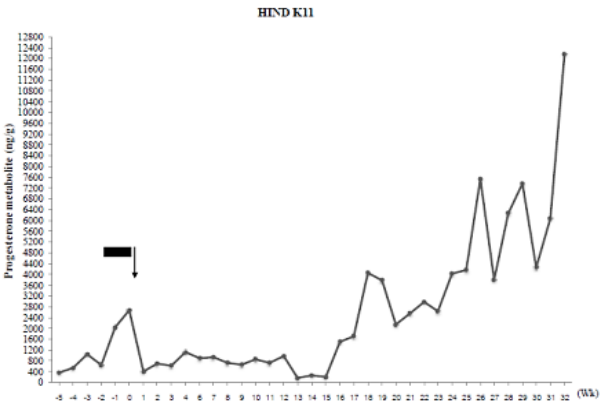

Figure 2 Mean fecal progesterone metabolite concentration throughout the year in the pregnant Eld's deer, no.K11 (Black bars = CIDR insertion, arrow= time of AI)

Discussion

In this present study, the evaluation of fecal progesterone metabolite concentrations during estrus synchronization and one year monitoring of fecal progesterone metabolite concentration in the non–pregnant and the pregnant Eld's deer hinds using enzyme immunoassay were done. The estrus synchronization using $PGF_{2\alpha}$ injection is associated with exogenous progesterone (CIDR devices) for CL regression. The CIDR maintain exogenous progesterone in the blood circulation and excretes into feces. After the removal of devices, normal estrous cycle restarts. The CIDR devices has been successfully performed in follow deer (*Dama dama*) (8/10; 80%) (Asher et al., 1990; Morrow et al., 1995), Brown brocket deer (*Mazama gouazoubira*) (3/3; 100%) (Zanetti et al., 2010). The obtained data in this present study represented the values of fecal progesterone concentrations during estrus synchronization. In deer no.7, no.42 no.43 and no.44, the concentrations did not differ from the normal luteal phase concentrations. The reason for poor response inducing of estrous might be caused by the ovarian activity at the time of CIDR insertion. The response of ovary on CIDR depended on the stage of estrous cycle at CIDR insertion and duration of treatment (Macmillan and Peterson, 1993). Macmillan et al (1991) reported that CIDR insertion in cattle during metestrus and diestrus might compromise endogenous progesterone production by the newly corpus luteum. Therefore, progesterone absorption from CIDR device may be varied (Thompson and Monfort, 1999; Rathbone et al., 2002). Two studies reported that plasma progesterone concentration of red deer hinds (*Cervus elaphus*) receiving single CIDR-G/CIDR-S insertion within the first 6 d (2-3 ng/ml) (Asher et al., 1993) was higher than plasma progesterone concentration of single CIDR-G insertion for 14 d (1.0 ng/ml) (Jopson et al., 1990).

Our data indicated that the Eld's deer hinds had multiple estrous cycles throughout the year. The average fecal progesterone concentration during March to August was higher than during September to February. Moreover, the fecal progesterone concentration patterns of this study were similar to the

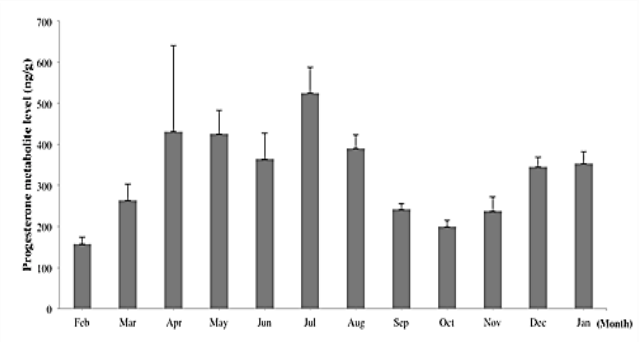

Figure 3 Average of fecal progesterone metabolite concentrations during February 2009 to January 2010 in 4 Eld's deer hinds (K7, K42, K43, K44)

urinary pregnanediol-3α-glucuronide (PdG) patterns of a previous report in the northern temperate latitudes (38°N latitude) (Hosack et al., 1997). Therefore, due to the higher progesterone concentration, it might be assumed that in Thailand during March to August was the breeding season. This was in accordance with a previous study reporting that the oocyte quality from the Eld's deer hinds in the breeding season had higher quality than during the non-breeding season in terms of number of follicles, follicle size, number of oocytes recovered and oocyte quality (Siriaroonrat, 2006).

The data of fecal progesterone profiles in this study coincided with the expression of endogenous rhythms in the Eld's deer reproductive hormones in northern temperate latitudes. It was not related to historical pattern of habitat resources, local food and photoperiod (Hosack et al., 1997). In this study, the mean of continuous estrous cycle (14.0±0.4 cycles) throughout 12 months was similar to a previous report in North America (13.8±0.5 cycles) (Hosack et al., 1997). However, in this present study, they had longer estrous cycles (24 d) than previous data (17.4-21.5 d for *thamin*; Monfort et al., 1990[a]; 1990[b] and 16.3 d for *hainanus*; Song and Zeng, 2003). However, these results were similar to the brown brocket deer (26.9 d) (Krepschi et al., 2013). The reason of longer estrous in this study remains unclear, probably the effect of stag absence on ovarian irregularity (Pereira et al., 2006). Therefore, we assumed that the longer estrous cycle of Eld's deer in this study might be related to the stag absence. However, further studies need to be done. The changes of fecal progesterone metabolite during pregnancy of the Eld's deer in this study were similar to previous data of urinary PdG concentrations pattern in pregnant Eld's deer hinds (Monfort et al.,1990[b]; Monfort et al., 1993) and other deer species (Elk deer; White et al., 1995, Red brocket deer; Krepschi et al., 2013, Reindeer; Bubenik et al., 1997, Sika deer; Matsura et al., 2004, White tailed deer; Kapkeet al.,1999). The fecal progesterone level of pregnant hind was markedly higher than hinds that failed to conceive. The progesterone level slightly elevated in the initial stage of gestation because the *corpus luteum* is the only source of progesterone released in blood circulation for maintenance of pregnancy. In mid and late pregnancy, progesterone level is clearly much increased due to the supplement source of progesterone by placenta (Flood et al., 2005).

In summary, fecal hormone analysis by using enzyme immunoassay could be useful for monitoring reproductive status in the Eld's deer such as estrous cycle, baseline hormone concentration, estrous synchronization and pregnancy diagnosis. Hormone concentration of individual female Eld's deer can be important information for Eld's deer conservation program.

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บทคัดย่อ

การตรวจวงรอบการเป็นสัดด้วยการวิเคราะห์ปริมาณฮอร์โมนโปรเจสเตอโรนจากอุจจาระของละมั่งเพศเมียสายพันธุ์พม่า *(Rucervus eldii thamin)*

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การศึกษานี้มีวัตถุประสงค์เพื่อตรวจหาระดับฮอร์โมนโปรเจสเตอโรนจากอุจจาระในช่วงเหนี่ยวนำการเป็นสัดเพื่อการทดลองผสมเทียมและศึกษาการเปลี่ยนแปลงของปริมาณฮอร์โมนโปรเจสเตอโรนที่พบในอุจจาระของละมั่งพันธุ์พม่าเพศเมีย (*Rucervus eldii thamin*) จำนวน 5 ตัวเป็นระยะเวลา 1 ปี (ก.พ. 2552-ม.ค. 2553) โดยเก็บตัวอย่างอุจจาระละมั่ง สัปดาห์ละ 1 ครั้ง จนกระทั่งเสร็จสิ้นการทดลอง แล้วนำมาวิเคราะห์ปริมาณฮอร์โมนโปรเจสเตอโรนด้วยวิธี Enzyme immunoassay (EIA) ขั้นตอนการเหนี่ยวนำการเป็นสัดและการผสมเทียมดำเนินการในเดือนมิถุนายน ละมั่งทั้งหมดได้รับการฉีดฮอร์โมน $PGF_{2\alpha}$ (5 มก./ตัว) พร้อมกับสอดแท่งโปรเจสเตอโรนสังเคราะห์ (CIDR-G) ไว้ในช่องคลอดเป็นเวลา 14 วัน จึงถอดแท่งโปรเจสเตอโรนสังเคราะห์ออก ทำการผสมเทียมด้วยน้ำเชื้อแช่แข็งผ่านกล้องลาปาโรสโคป หลังจากถอดแท่งโปรเจสเตอโรนออกเป็นระยะเวลา 70 ชั่วโมง พบว่าปริมาณโปรเจสเตอโรนที่วิเคราะห์ได้ขณะที่มีการเหนี่ยวนำการเป็นสัดในละมั่งหมายเลข 11 มีค่าความเข้มข้นของฮอร์โมนโปรเจสเตอโรน (2,360±320 นาโนกรัม/กรัม) สูงกว่าละมั่งตัวอื่น (หมายเลข 7, 42, 43 และ 44) อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ขณะเดียวกันไม่พบความแตกต่างของความเข้มข้นของฮอร์โมนโปรเจสเตอโรนในละมั่งทั้ง 4 ตัวระหว่างที่มีการเหนี่ยวนำการเป็นสัด ($p>0.05$) ละมั่งสามารถแสดงวงรอบการเป็นสัดซ้ำหลายวงรอบ (multiple estrous cycle) ต่อปี มีความยาวของวงรอบการเป็นสัด 24.9±2.2 วัน คิดเป็น14.0±0.4 รอบต่อปี ซึ่งค่าเฉลี่ยของฮอร์โมนโปรเจสเตอโรนในช่วงเดือนมีนาคมถึงเดือนสิงหาคม (390.1±33.2 นาโนกรัม/กรัม) มีค่าสูงกว่าช่วงเดือนกันยายนถึงเดือนกุมภาพันธ์ (291.4±20.1 นาโนกรัม/กรัม) อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ส่วนละมั่งที่ตั้งท้องมีค่าเฉลี่ยของฮอร์โมนโปรเจสเตอโรนแบ่งออกเป็น 3 ระยะ ได้แก่ ระยะต้น (816.9±60.0 นาโนกรัม/กรัม), ระยะกลาง (1,998.1±416.8 นาโนกรัม/กรัม) และระยะท้ายของการอุ้มท้อง (5,995.6±757.4 นาโนกรัม/กรัม) ดังนั้นการใช้วิธีวิเคราะห์ปริมาณโปรเจสเตอโรนด้วยเทคนิค EIA สามารถประเมินการทำงานสรีรวิทยาของระบบสืบพันธุ์และเป็นเครื่องมือที่สำคัญในการจัดการด้านการสืบพันธุ์ละมั่งได้

คำสำคัญ: ฮอร์โมน โปรเจสเตอโรน ละมั่ง enzyme immunoassay

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