Chapter VI

Effect of *Pueraria mirifica* on Urinary Gonadotropin and Sex Steroid Hormone Levels in Female Monkeys

Abstract

The study was investigate the effect of *Pueraria mirifica* (PM) on urinary hormone levels in female cynomolgus monkeys, both cyclic and aged menopause after a single or long-term feeding. In each experiment, the monkeys were divided into 3 groups. Each group was fed with 10, 100, and 1,000 mg/day of PM, respectively. There were three experiment in this study. Experiments 1 and 2 using adult cyclic monkeys for a single and long-term feeding of PM and experiment 3 using aged menopausal monkeys for a long-term feeding of PM. The experimental schedule was separated into 2 periods, the pre-treatment and post-treatment periods for experiment 1, separated into 3 periods, the pre-treatment, treatment, and post-treatment periods for experiments 2 and 3. Urinary FSH, LH, and estradiol levels were determined in those monkeys, while the adult cyclic monkeys were additionally determined the urinary progesterone levels. The results showed that a single feeding of all doses of PM and long-term feeding of PM-10 did not change pattern of urinary FSH, LH, estradiol, or progesterone in adult cyclic monkeys compared to those in the pre-treatment period. Long-term feeding of PM 100 and PM-1,000 induced the decrease in urinary FSH, LH, and estradiol in adult cyclic monkeys and decrease in urinary FSH and estradiol in aged menopausal monkeys during the treatment period compared to those in the pre-treatment period. However, change of urinary FSH was clearly observed more than that of other hormones. So, urinary FSH is considered as a good indicator of estrogenic effect of PM on hormonal levels in monkeys.

Key words: *Pueraria mirifica*, phytoestrogen, urine, gonadotropin, estradiol, progesterone, female monkey

6.1 Introduction

Pueraria mirifica (PM), a Thai medicinal plant, is classified into the family Leguminoceae. Its tuberous root contains many kinds of phytoestrogens such as genistein, daidzein, coumestrol (Ingham et al., 1986, 1989), miroestrol (Pope et al., 1958), deoxymiroestrol, and kwakhurin (Chansakaow et al., 2000a, 2000b). The previous reports showed conflicting results on the influence of phytoestrogens on hormone-related ovarian function (Cassidy et al., 1995; Duncan et al., 1999; Lu et al., 2000). Daily consumption of soy containing a high amount of isoflavones depressed a midcycle surge of both follicle stimulating hormone (FSH) and luteinizing hormone (LH) in premenopausal women (Cassidy et al., 1995; Duncan et al., 1999). Other report showed, however, that a daily consumption of soy diet in premenopausal women lowered estradiol levels bud did not FSH or LH levels (Lu et al., 2000). The previous studies clearly showed that a long-term treatment of PM could suppress the levels of gonadotropins and sex steroid hormones in both of adult cyclic and aged menopausal monkeys (Trisomboon et al., 2002a, 2002b, 2004). A single feeding of PM on the day of menstruation did not change the levels of FSH, LH, estradiol, or progesterone in adult cyclic monkeys (Trisomboon et al., 2004).

The long-term study on changes in serum levels of hormones is posed the limitations by the frequent sampling. Subject monkeys would suffer from loss of high amount of blood and injury from frequent venipuncture, which may disturb the homeostasis of physiological system. To avoid these problems, the assay of hormonal level in urine, a non-invasive method, should be considered. The present study was investigated the efficacy of urine assay to find out the PM effect on

reproductive hormones through three kinds of experiments, a single and the 90-day treatments of PM on either cyclic adult or aged menopausal cynomolgus monkeys. From the previous findings on the effect of PM on serum hormone levels, we considered that the 90-day treatment of PM is long enough to disturb the hormonal patterns that would be reflected in the urinary excretion.

6.2 Materials and Methods

6.2.1 Animals

Adult cyclic and aged menopausal cynomolgus monkeys (*Macaca fascicularis*) were used in this study. This study used eighteen adult cyclic female monkeys with regular menstrual cycle for at least 4 consecutive cycles and nine aged menopausal cynomolgus monkeys with a complete cessation of menstrual cycle for at least 1 year before the onset of the study. They were randomly selected from candidate monkeys. The monkeys were housed in the individual cages at the Primate Research Unit, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. The menstruation was monitored daily by vaginal swabbing method. Lighting conditions of the animal room were controlled (12 : 12 h light to dark cycle). Temperature and humidity fluctuated slightly depending on the season. The monkeys were fed daily with monkey chow (Pokaphan Animal Feed Co., Ltd., Bangkok, Thailand) in the morning (09:00 – 10:00 h) and supplemented with fresh fruits in the afternoon (14:00 – 15:00 h). The experimental protocol was approved by the ethics committee in accordance with the guide for the care and use of laboratory animals prepared by the Primate Research Unit, Faculty of Science, Chulalongkorn University.

6.2.2 Experimental Designs

Experiment 1: Effect of a single feeding of PM on urinary gonadotropin and sex steroid hormone levels in adult cyclic monkeys.

Nine adult cyclic female monkeys were divided into three groups. Subjects of these groups (n = 3) were fed with the suspension of 10, 100, and 1,000 mg/day of *P. mirifica*, abbreviated as PM-10, PM-100, and PM-1,000, at 08:00-08:30 h. The experimental schedule was separated into the pre-treatment and post-treatment periods. The duration for the pre-treatment and post-treatment periods was 1 and 2 menstrual cycles, respectively. The monkeys were fed with PM after a cycle of the pre-treatment period. The first day of the menstrual bleeding was designed as day 1. During these two periods, urinary samples were collected everyday and assayed for FSH, LH, estradiol, and progesterone levels.

Experiment 2: Effect of long-term treatment of PM on urinary gonadotropin and sex steroid hormone levels in adult cyclic monkeys.

Nine adult cyclic female monkeys were divided into three groups. Subjects of these groups (n = 3) were fed daily with PM-10, PM-100, and PM-1,000, respectively, at 08:00 - 08:30 h. The experimental schedule was separated into the pre-treatment, treatment, and post-treatment periods. During the pre-treatment and post-treatment periods, the monkeys were fed daily with 5 ml of distilled water at 08:00-08:30 h for 1 and 2 menstrual cycles, respectively. During the treatment period, the monkeys were fed with the suspension of PM for 3 menstrual cycles. If the monkeys showed an amenorrhea symptom after PM treatment, the duration of experiment was performed 90 and 60 days for the treatment and post-treatment periods, respectively. The first day of the menstrual bleeding was designed as day 1. Urinary samples was collected everyday throughout the study period and assayed for FSH, LH, estradiol, and progesterone levels.

Experiment 3: Effect of long-term treatment of PM on urinary gonadotropin and sex steroid hormone levels in aged menopausal monkeys.

Nine aged menopausal monkeys were divided into three groups. Subjects of these groups (n = 3 in each) were fed daily with PM-10, PM-100, and PM-1,000. The experimental schedule was separated into the pre-treatment, treatment, and post-treatment periods for 30, 90, and 60 days, respectively. The monkeys were fed daily with 5 ml distilled water during the pre-treatment and post-treatment periods and with the suspension of PM during the treatment period at 08:00 - 08:30 h. Urinary samples were collected every 5 days and assayed for FSH, LH, and estradiol levels.

6.2.3 Collection of Urinary Samples.

The tray was inserted and kept under the monkey's cage between 18:00 – 08:00 h. The 14-h urinary samples were collected from the tray with plastic syringes at 08:00 h and then centrifuged at 1,700 xg, 4 ° C for 20 minutes. The supernatant was separated and stored at –20 ° C until hormonal assays.

6.2.4 Preparation of the Suspension of *Pueraria mirifica*

The fresh tuberous roots of PM were obtained from the same lot. The roots were sliced, dried in hot air oven at 70 ° C, and subsequently ground into a powder at size of 100 Mesh. The stock of powder was kept in the dark desiccator before suspended. PM powder was suspended with distilled water and kept in a dark bottle, at 4 ° C, until the feeding time.

6.2.5 Hormonal Analyses

The urinary samples were analyzed for FSH and LH levels using the heterologous RIA system described previously (Hodgen et al., 1976). Iodinated preparations were rat NIDDK-rat FSH -I-5 and rat LH-I-5. The anitisera were anti-

ovine FSH (NIDDK-H-31) and anti-ovine LH (YM#18). Antiserum against ovine LH (YM#18) was kindly provided by Dr.Y.Mori (University of Tokyo, Tokyo, Japan).

Urinary estradiol and progesterone levels were determined by a double-antibody RIA system using ^{125}I-labeled radioligands as described previously (Taya, et al., 1985; Gibori, Anrczak, and Rothchild, 1977). Antisera against estradiol (GDN#244) and progesterone (GDN#377) were kindly provided by Dr.G.D. Niswender (Animal Reproduction and Biotechnology, Colorado State University, Fort Collins, CO, U.S.A.).

Creatinine (Cr) level of each urinary sample was measured by the Jaffe method using an Autoanalyzer to compensate for differences in urine concentration and volume. Urinary hormone levels were calculated as a milligram of creatinine.

6.2.6 Statistical Analyses

Data are expressed as mean ± S.E.M. The student *t*- test was used to determine the difference of hormonal levels in urine between adult cyclic and aged menopausal monkeys. Analysis of variance (ANOVA) followed by the LSD test was applied to determine the significance of differences of urinary levels among each period. The differences of means were considered significant at $P < 0.05$.

6.3 Results

6.3.1 Patterns of Urinary Hormones in Normal Menstrual Cycle of Adult Cyclic Monkeys.

The normal length of menstrual cycle of adult cyclic monkeys during the pre-treatment period was 29.29 ± 0.79 days (n = 18). There were an individual variation in urinary hormonal levels depending on the length of each menstrual cycle in subject

monkeys. The profiles of hormonal levels in urine were adjusted according to the day of peak level of LH, which was assigned as day 0. It separated into 2 phases: the late follicular and early luteal phases, respectively. As shown in Figure 6.1, urinary FSH and LH levels were elevated simultaneously during the late follicular phase and attained the peak on the same day on day 0. The increase in urinary estradiol levels during the late follicular phase coincided with those in urinary FSH and LH levels. All of urinary FSH, LH, and estradiol levels declined during the early luteal phase. Urinary estradiol and progesterone levels showed a high fluctuation throughout the late follicular and early luteal phases. However, urinary progesterone levels trended to be high during the early luteal phase.

To calculate the correlation between hormonal levels in serum and urine of normal adult cyclic monkeys, data of hormonal levels in urine were correlated with levels in serum from the previous report. There was a significant correlation between FSH, LH, and progesterone levels in urine and serum ($r = 0.61$, $P = 0.05$ for FSH, $r = 0.84$, $P = 0.001$ for LH, $r = 0.67$, $P = 0.02$), and no significant correlation between those levels of estradiol ($r = 0.26$, $P < 0.44$).

6.3.2 Experiment 1: Effect of a Single feeding of PM on Urinary FSH, LH, Estradiol, and Progesterone levels in adult cyclic monkeys.

After a single feeding of PM-10 and PM-100, the menstrual cycle length of adult monkeys are kept in the normal range (for PM-10, 30.97 $\pm$ 2.91 and 33.33 $\pm$ 3.84 days for the first and second cycle; for PM-100, 33.33 $\pm$ 3.84 and 35.67 $\pm$ 6.33 days for the first and second cycle, respectively). Adult monkeys treated with PM-1,000 had the prolongation of menstrual cycle length compared to the cycle length during the pre-treatment period (42.00 $\pm$ 4.04 days, $P < 0.01$ for the first cycle and 39.67 $\pm$ 0.67 days, $P < 0.01$ for the second cycle). Comparing the pattern of urinary hormone levels in all PM treated groups (Figures 6.2 – 6.4) to those patterns of

normal monkeys in Figure 6.1, it was shown a similar pattern. Peak levels of urinary FSH and LH are occurred at the mid phase of menstrual cycles or day 0 of Figure 6.1 in all monkeys treated with PM-10, PM-100, and PM-1,000. After the declining of urinary FSH and LH levels, urinary estradiol and progesterone trended to increase in some monkeys.

6.3.3 Experiment 2: Effect of Long-term Treatment of PM on Urinary FSH, LH, Estradiol, and Progesterone Levels in Adult Cyclic Monkeys.

Changes of urinary FSH, LH, estradiol, and progesterone levels in adult cyclic monkeys treated daily with different dose of PM for 3 menstrual cycles or 90 days are shown in Figures 6.6 – 6.8, respectively. As shown in Figure 6.5, PM-10 prolonged the menstrual cycle length in all 3 monkeys. The length of 3 consecutives menstrual cycles were prolonged to 50, 81, and 52 days for monkey no. 601, to 40 and > 90 days for monkey no. 627, and to > 90 days for monkey no. 619. However, all adult monkeys treated with PM-10 had the peak levels of FSH and LH when they showed menstrual cycles. There were no changes in pattern of urinary FSH, LH, estradiol, or progesterone levels throughout the treatment and post-treatment periods when compared to the pre-treatment period.

As shown in Figure 6.6, adult monkey nos. 616 and 801 showed a shorten menstrual cycle at the beginning of the treatment period (15 and 21 days, respectively) and stopped the menstruation afterward. There was no peak of urinary FSH level in these two monkeys and only monkey no. 801 showed a small peak of urinary LH level. Monkey no. 108 completely stopped menstruation throughout the 90-day of treatment and 60-day of post-treatment periods, and the peak levels of FSH and LH could be found only at the beginning of the treatment period. All adult monkeys in this group showed the low basal level of urinary FSH at the early

follicular phase. Urinary estradiol and progesterone levels fluctuated throughout the treatment and post-treatment periods.

As shown in Figure 6.7, all monkeys treated with PM-1,000 stopped menstruation throughout the 90-day of treatment and 60-day of post-treatment periods. There were no peak levels of urinary FSH or LH in monkey nos. 624 and 77 throughout the treatment period, while monkey no. 633 showed a small peak of urinary FSH level. Urinary estradiol level in monkey nos. 77 and 624 decreased throughout the treatment and post-treatment periods compared to the pre-treatment levels.

6.3.4 Experiment 3: Effect of Long-term Treatment of PM on Urinary FSH, LH, and Estradiol Levels in Adult Cyclic Monkeys.

The menopausal state of aged monkeys was confirmed by the high levels of urinary FSH (8.00 $\pm$ 2.08 ng/mg Cr, P = 0.01) and LH (17.80 $\pm$ 4.71 ng/mg Cr, P = 0.23) and by the low levels of urinary estradiol (0.18 $\pm$ 0.08 ng/mg Cr, P = 0.01) during the pre-treatment period compared to those levels in the early follicular phase, days 1-3, of adult cyclic monkeys (1.32 $\pm$ 0.31 ng/mg Cr for FSH, 10.70 $\pm$ 1.68 ng/mg Cr for LH, and 4.44 $\pm$ 1.41 ng/mg Cr for estradiol).

Means of urinary levels of FSH, LH, and estradiol in aged menopausal monkeys treated daily with PM-10, PM-100, and PM-1,000 for 90 days are shown in Figure 6.8. The daily treatment of PM seems to induce, but not significantly, the decrease in urinary FSH levels in a dose dependent manner. In PM-10, urinary FSH levels decreased for the first 15 days during the treatment period and then recovered to the pre-treatment levels afterward. Urinary FSH levels rebounded significantly on day 20 during the post-treatment period. Urinary FSH levels in monkeys treated with PM-100 and PM-1,000 tended to decrease between days 5 – 90 during the treatment

period and remained low for the first 10 days of the post-treatment period before the increase and significant rebound on days 20 and 25, respectively.

There were high fluctuations in urinary LH levels throughout the study period in all aged menopausal monkeys. During PM treatment, there was no significant difference in urinary LH levels compared to those during the pre-treatment period in all groups. However, urinary LH levels significantly increased on some days in monkeys treated with PM-100 and PM-1,000. Urinary LH levels tended to increase in general and rebounded after the first 20 days of the cessation of PM treatment in all monkey groups.

Daily treatment of PM seems to insignificantly decrease the urinary estradiol levels in monkeys treated with PM-10 and PM-100. There was no significant difference in urinary estradiol levels during the treatment period compared to those during the pre-treatment period in monkeys treated with PM-1,000.

6.3.5 The Correlation between Hormonal Levels in Serum and Urine in Female Monkeys Treated with PM

The data of hormonal levels in both serum and urine at the same points from adult cyclic and aged menopausal monkeys throughout the study period (n = 27) were selected and pooled together. As shown in Figure 6.9, there was a significant positive correlation between gonadotropins (FSH and LH) and estradiol levels in serum and urine of the monkeys ($r = 0.46$, $P < 0.01$ for FSH; $r = 0.13$, $P < 0.01$ for LH; $r = 0.16$, $P < 0.01$ for estradiol). There was, however, no correlation between progesterone level in serum and urine ($r = 0.05$, $P = 0.24$).

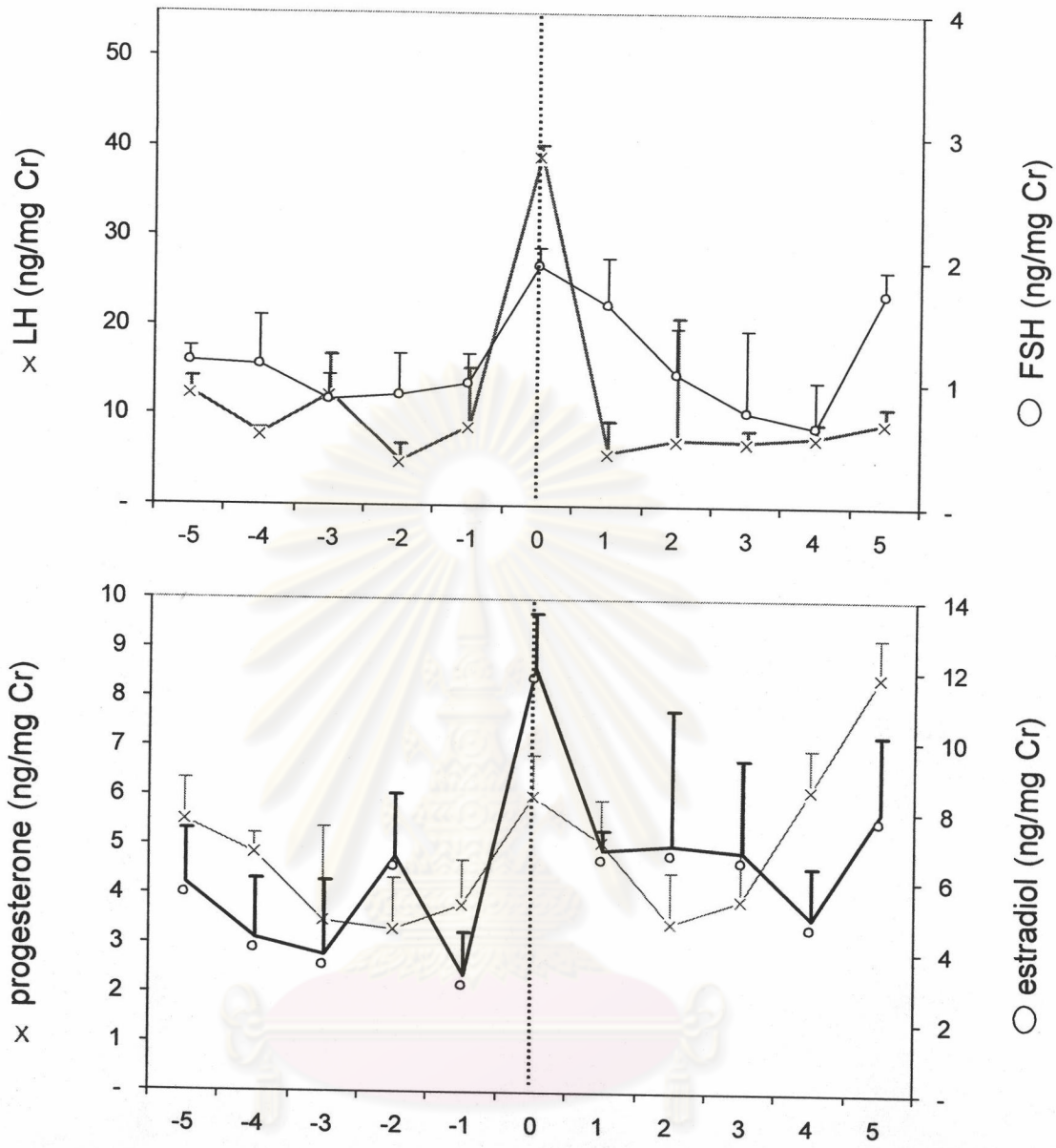


Figure 6.1 Pattern of urinary of FSH, LH, estradiol, and progesterone levels in normal menstrual cycle of adult female monkeys.

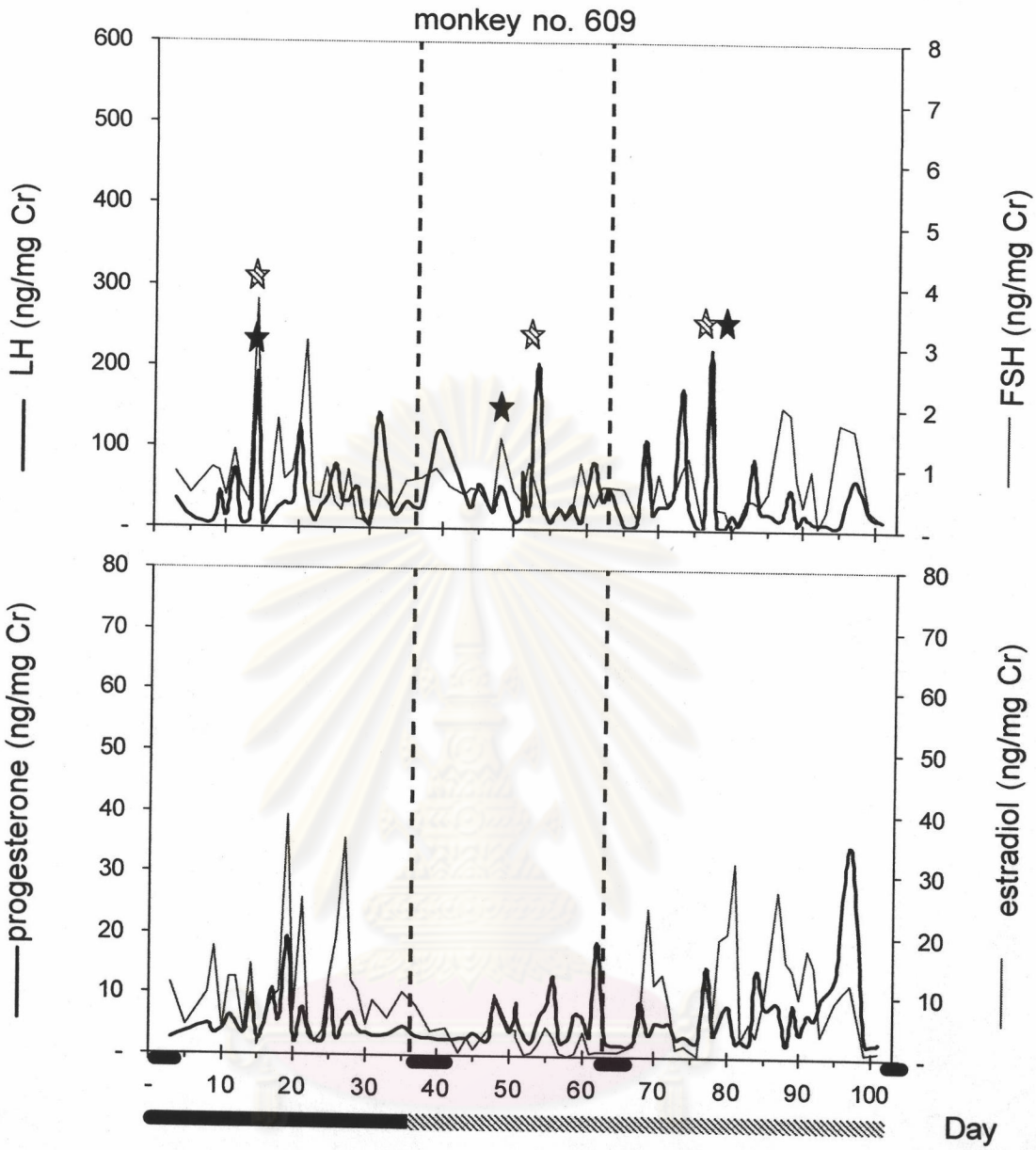
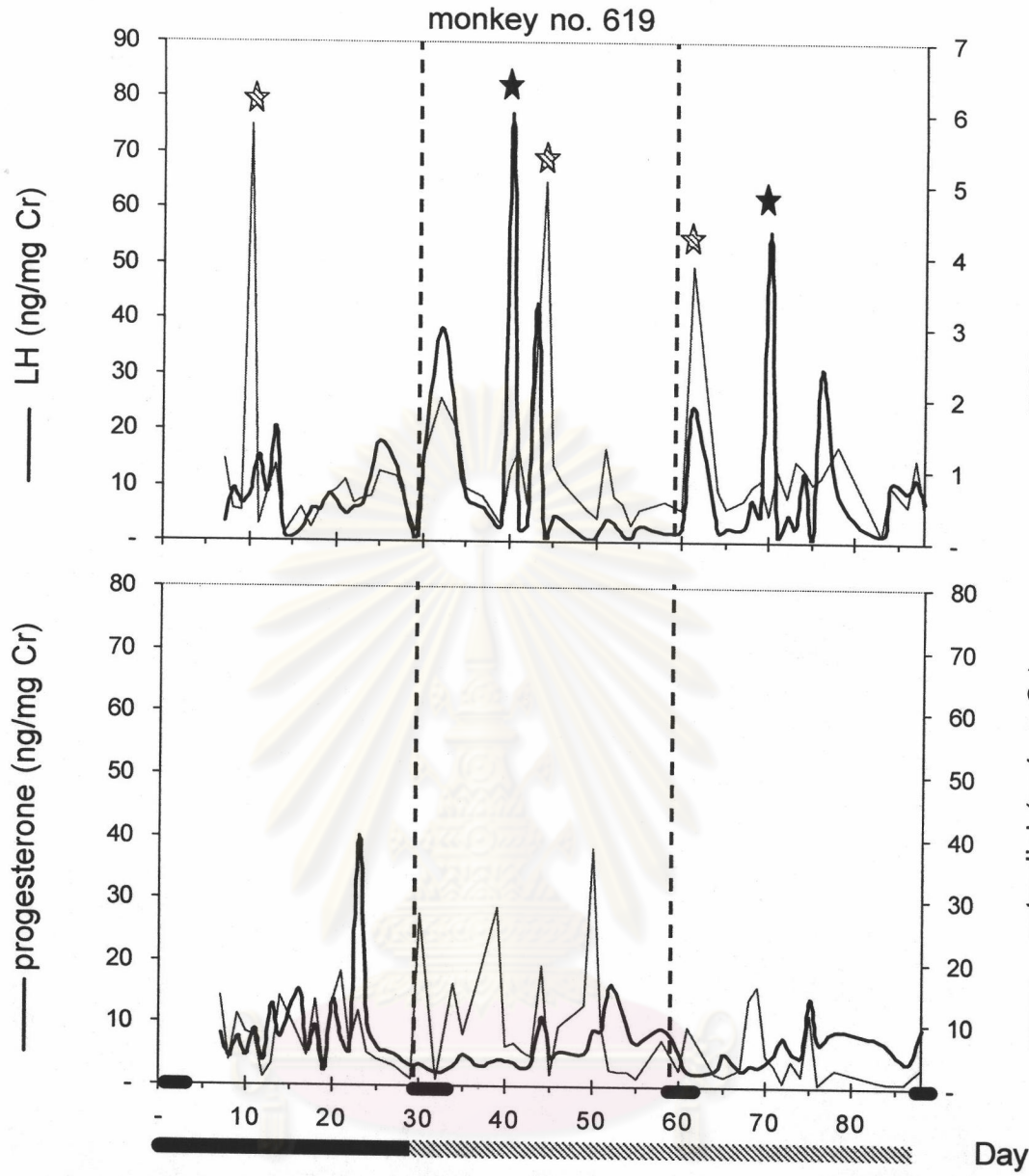


Figure 6.2 Changes of urinary FSH, LH, estradiol, and progesterone levels in adult cyclic monkeys on a single feeding of PM-10. The black and stripe lines indicate the pre-treatment and treatment periods, respectively. The dash-vertical lines separated each of cycles. The star indicates the highest peak of urinary FSH and LH levels in each menstrual cycle.



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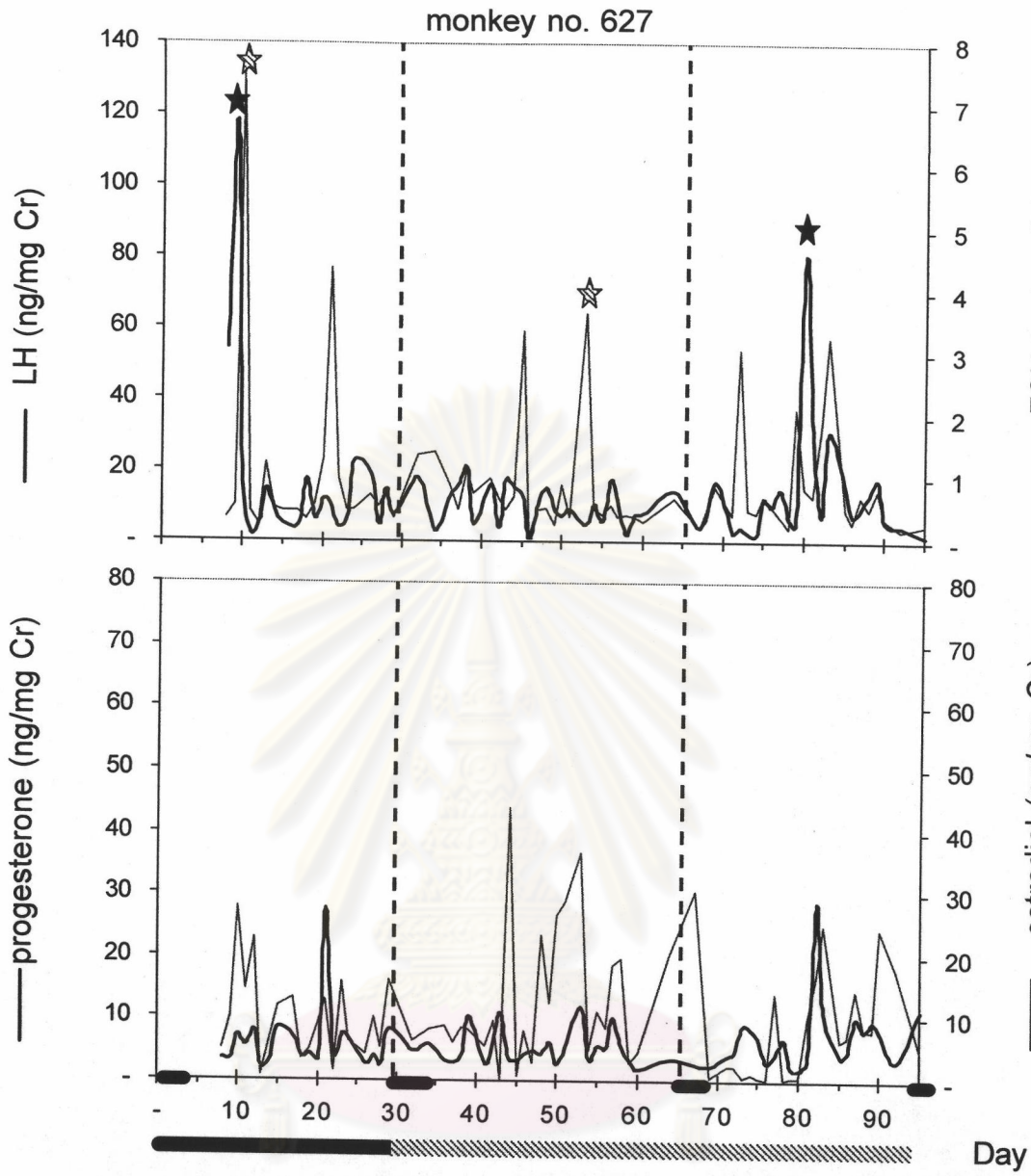

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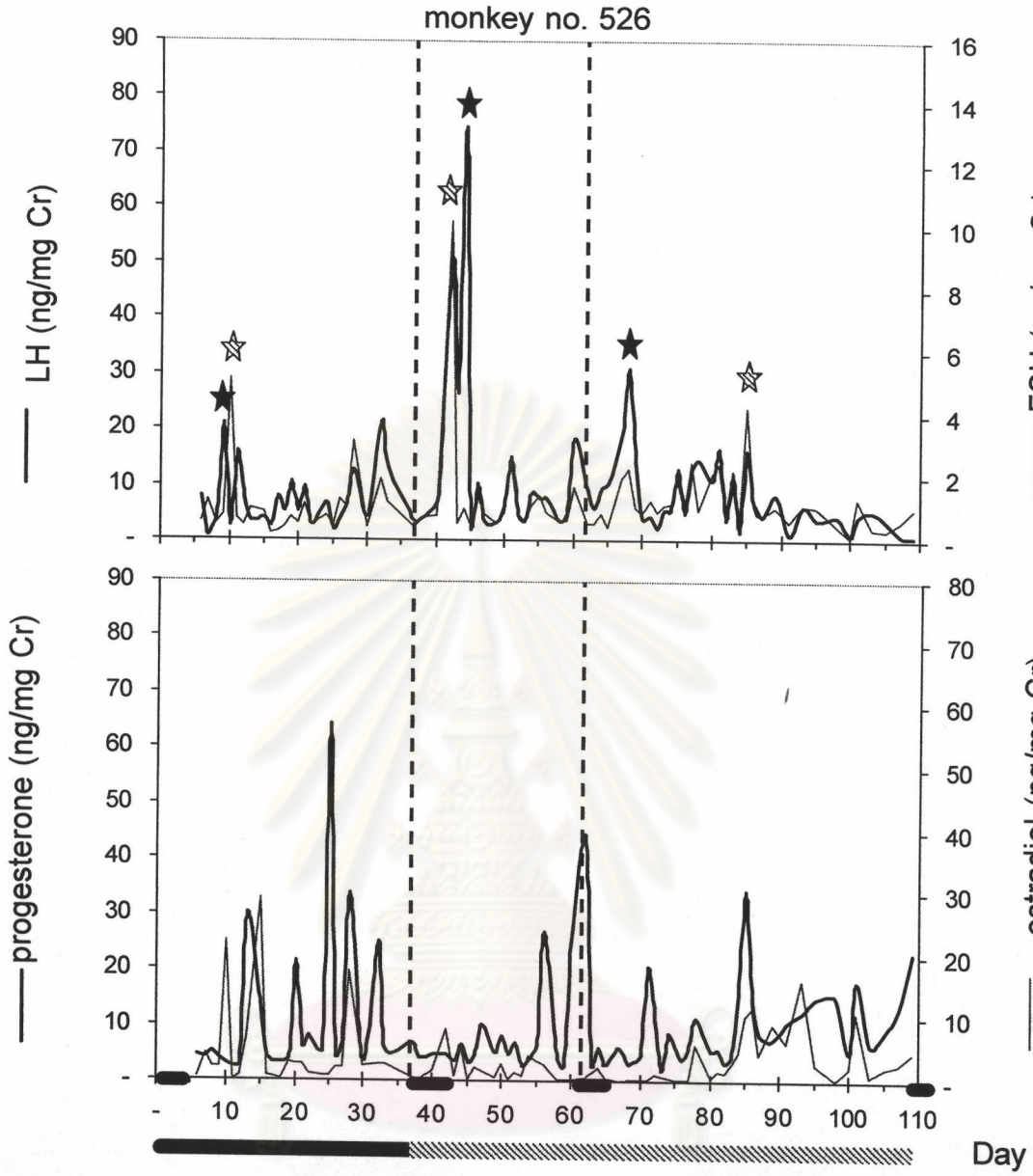
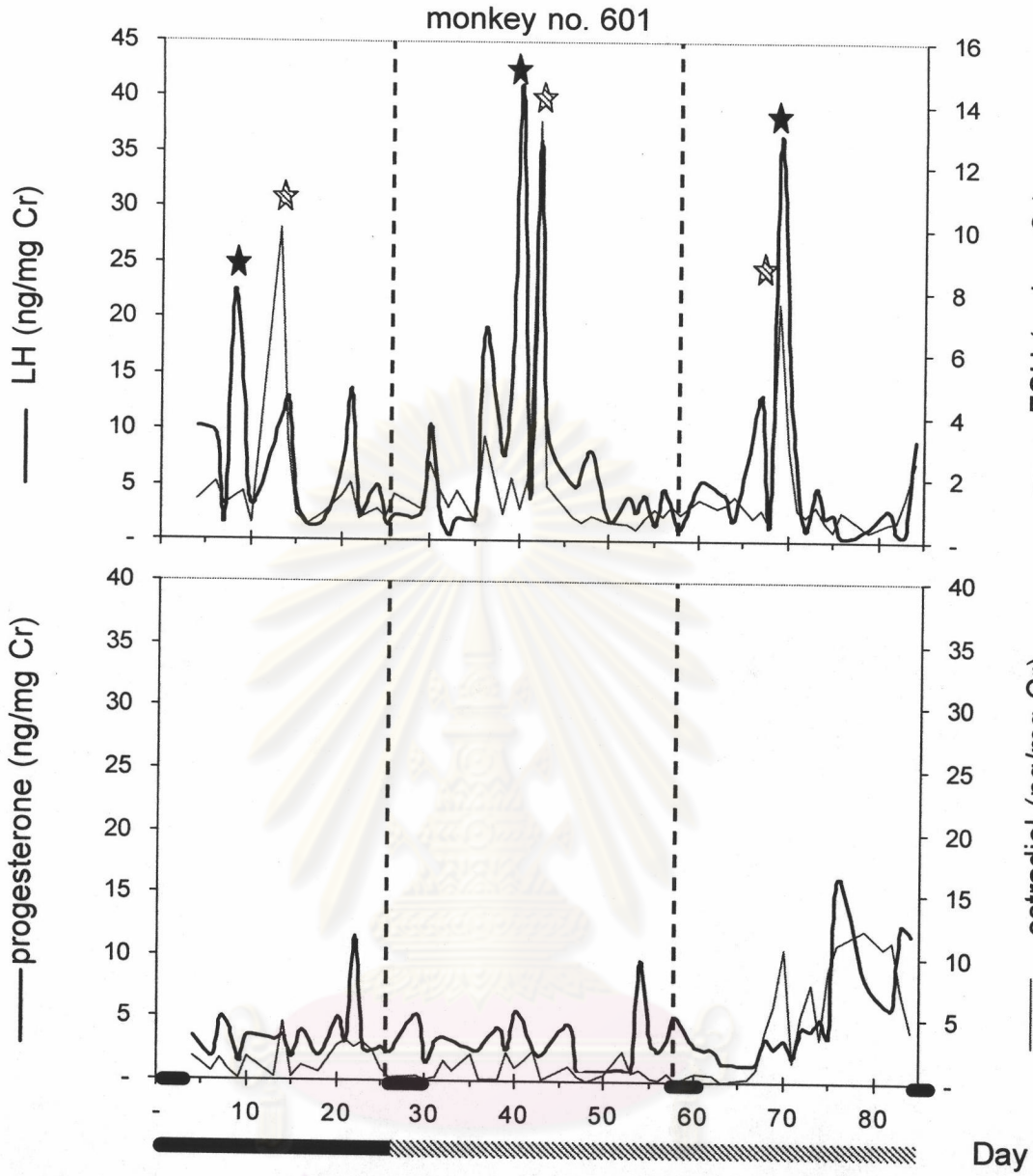


Figure 6.3 Changes of urinary FSH, LH, estradiol, and progesterone levels in adult cyclic monkeys on a single feeding of PM-100. The meanings of the black and stripe lines and dash-vertical line are the same as explained in Figure 6.2. The star indicates the highest peak of urinary FSH and LH levels in each menstrual cycle.



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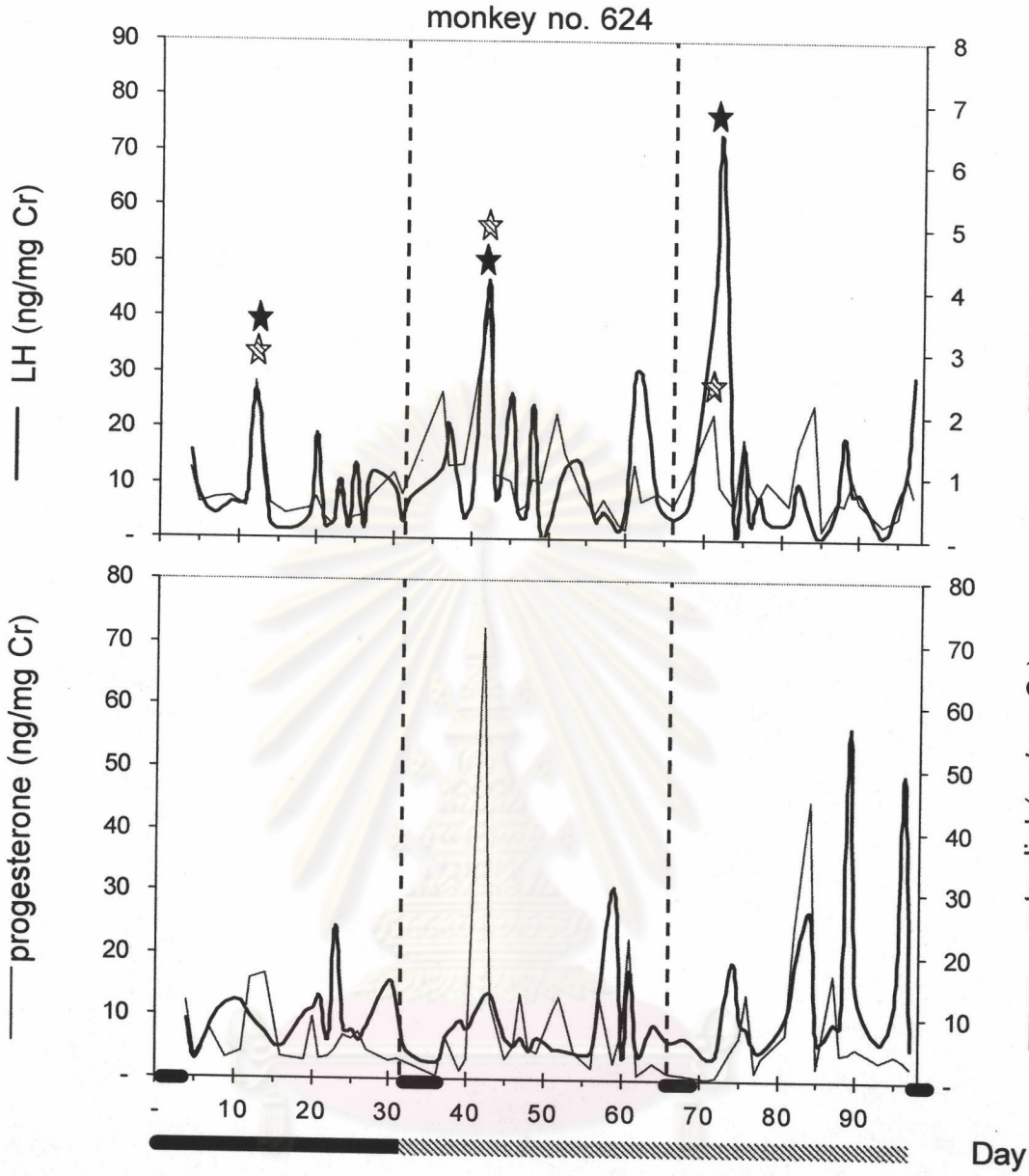

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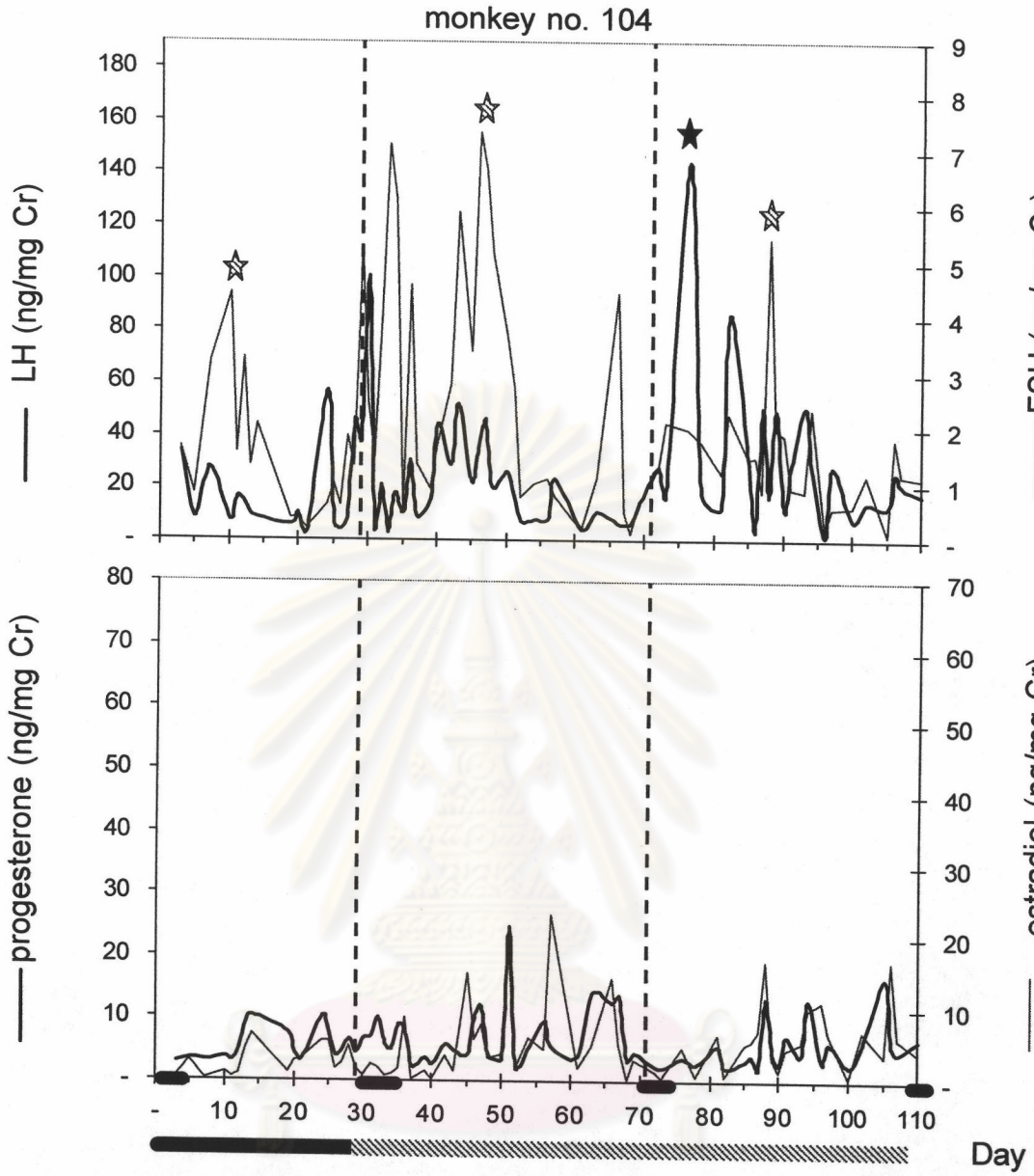
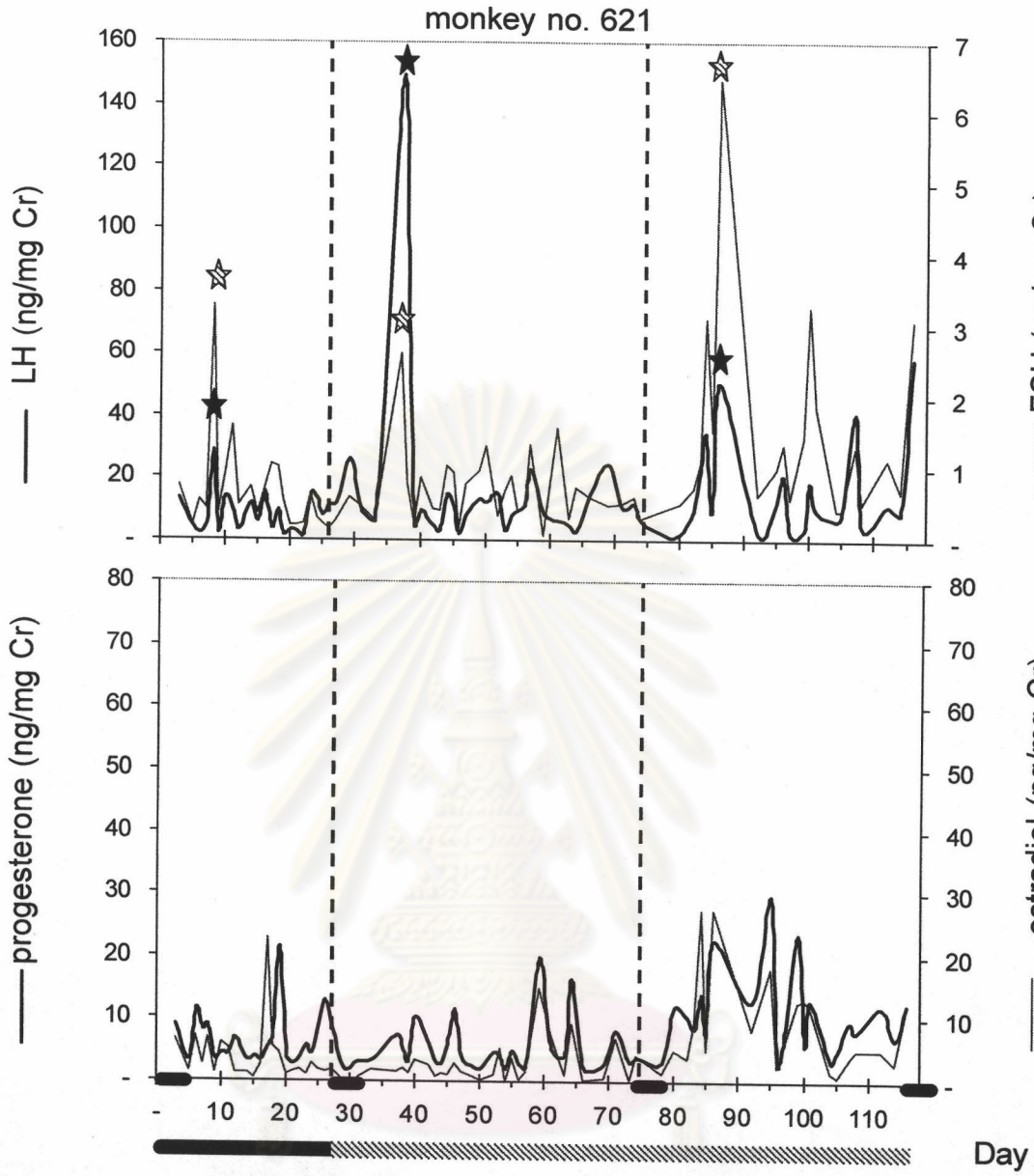


Figure 6.4 Changes of urinary FSH, LH, estradiol, and progesterone levels in adult cyclic monkeys on a single feeding of PM-1,000. The meanings of the black and stripe lines and dash-vertical line are the same as explained in Figure 6.2. The star indicates the highest peak of urinary FSH and LH levels in each menstrual cycle.



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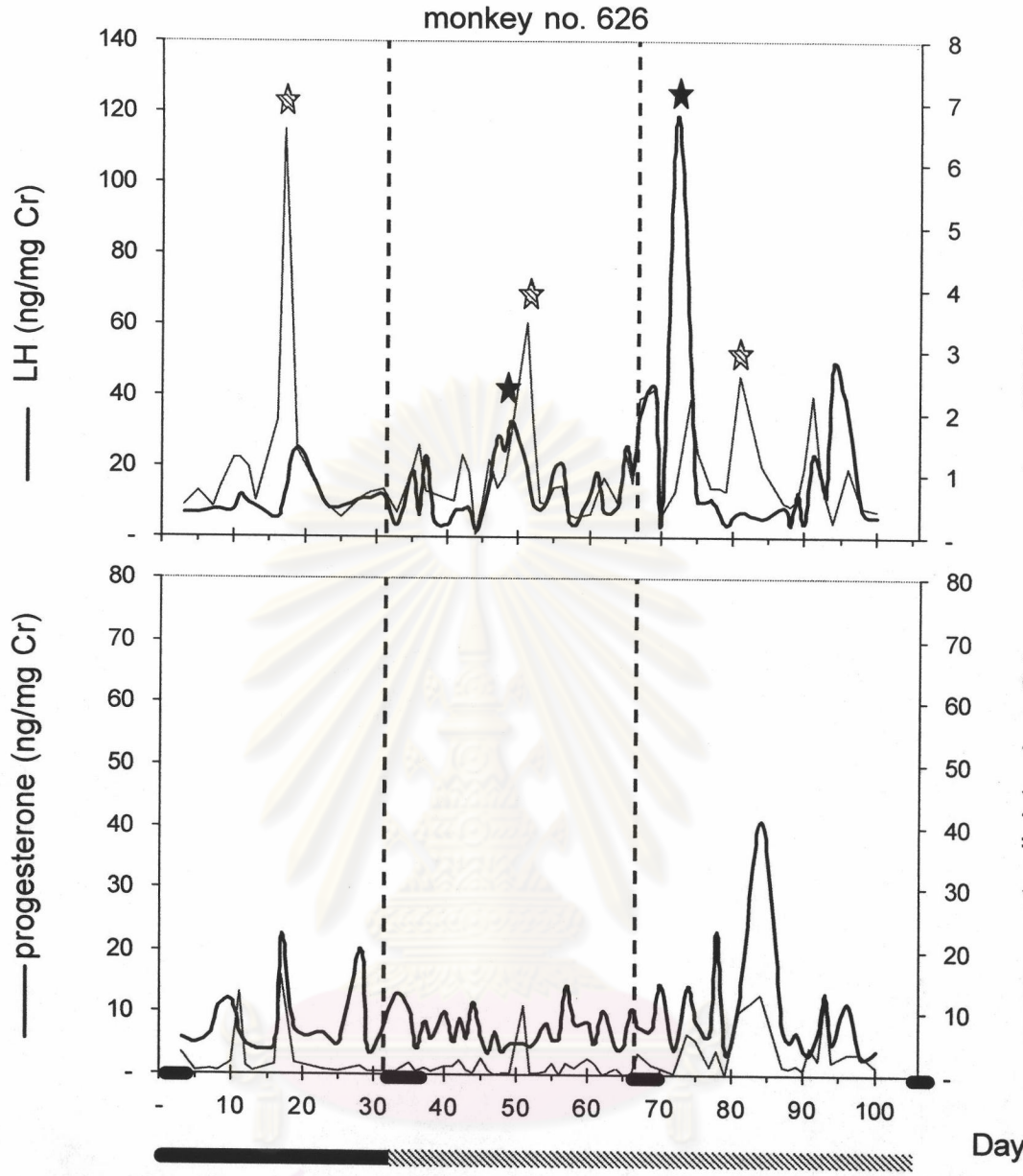

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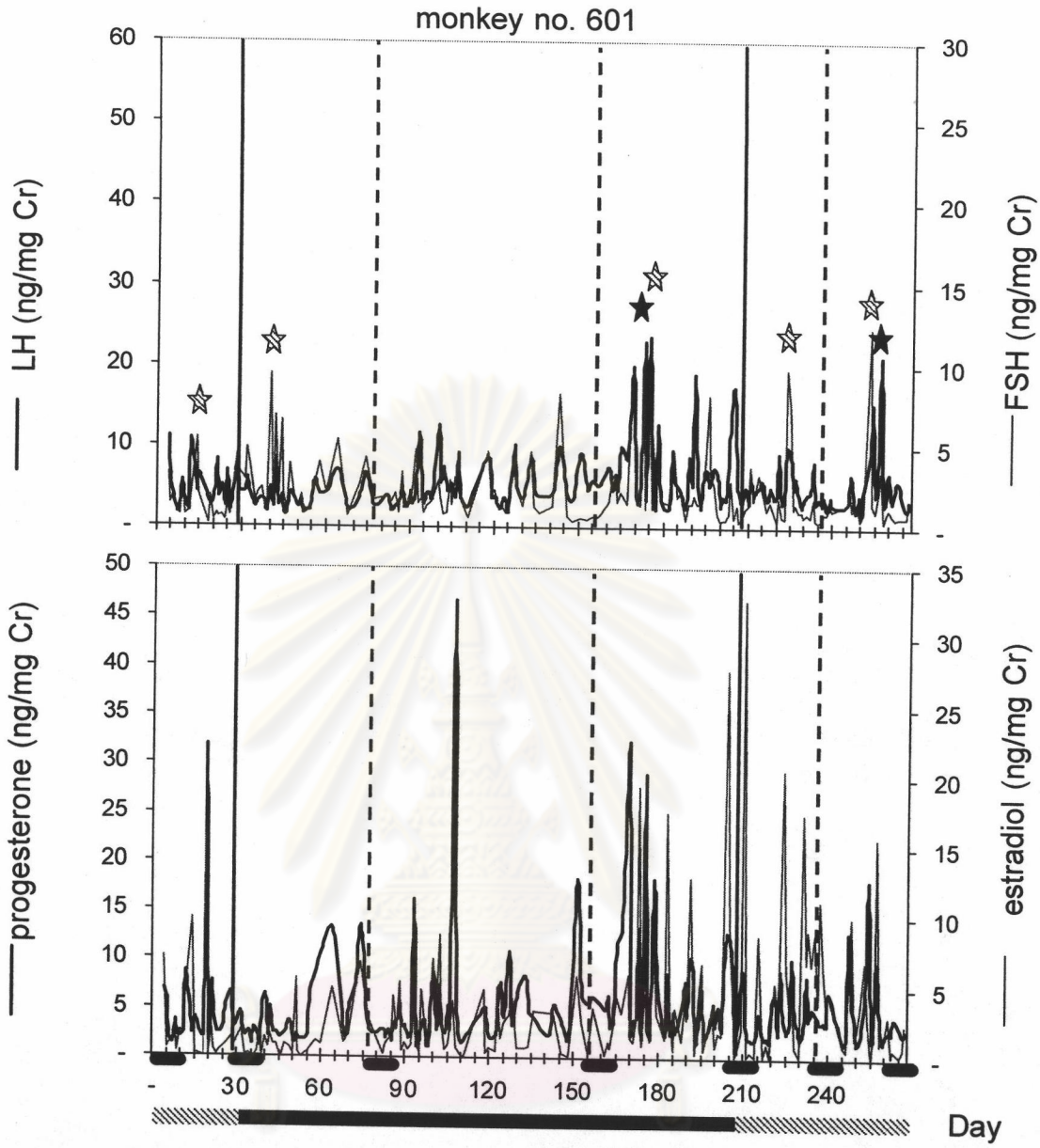
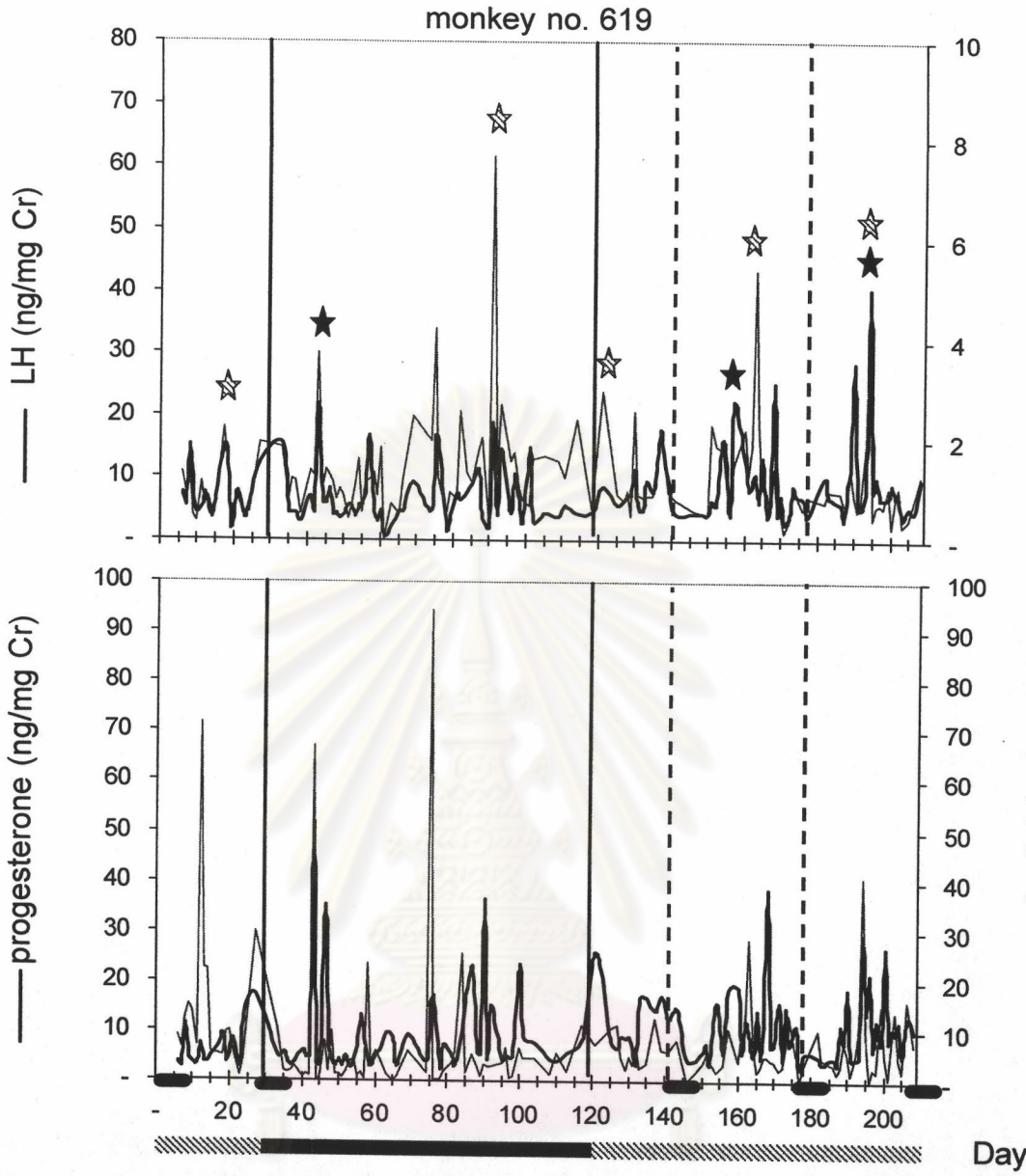


Figure 6.5 Changes in the levels of urinary FSH, LH, estradiol, and progesterone in adult cyclic monkeys fed daily with PM-10 for 90-day. The black horizontal line indicates the treatment period. The vertical lines separated the menstrual cycles. The short horizontal bars at the abscissa represent the day of menses. The star indicates the highest peak of urinary FSH and LH levels in each menstrual cycle.



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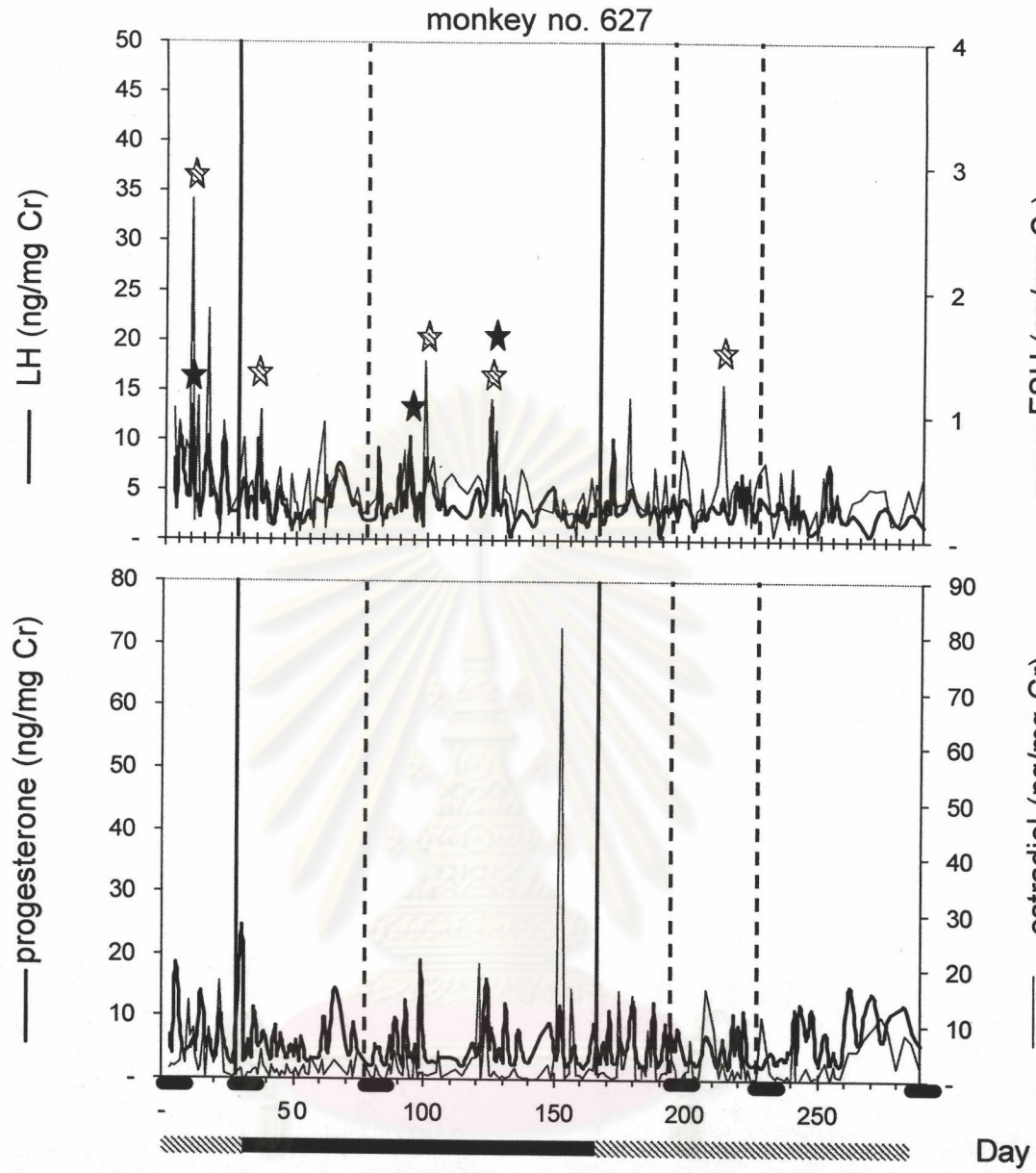

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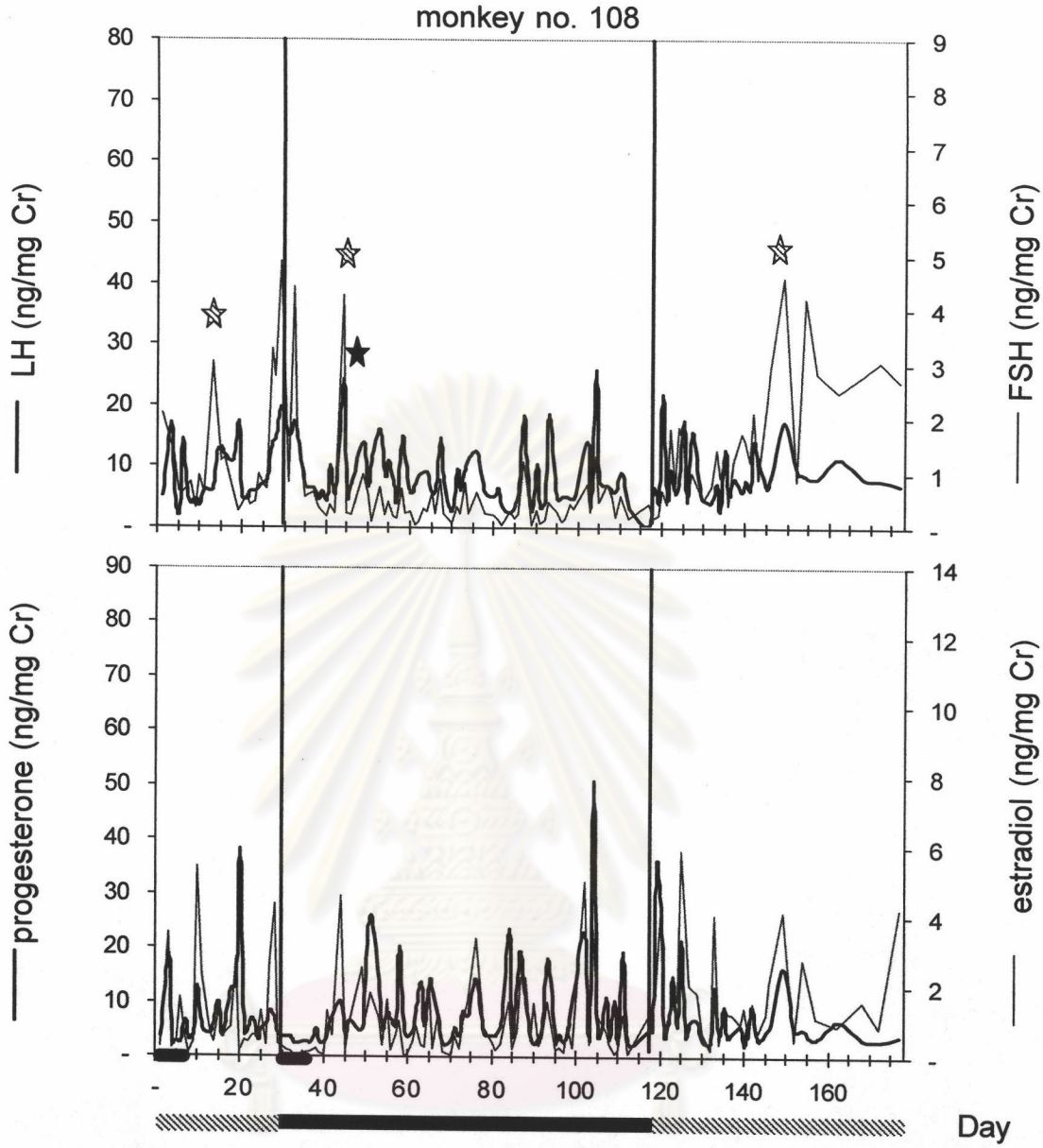
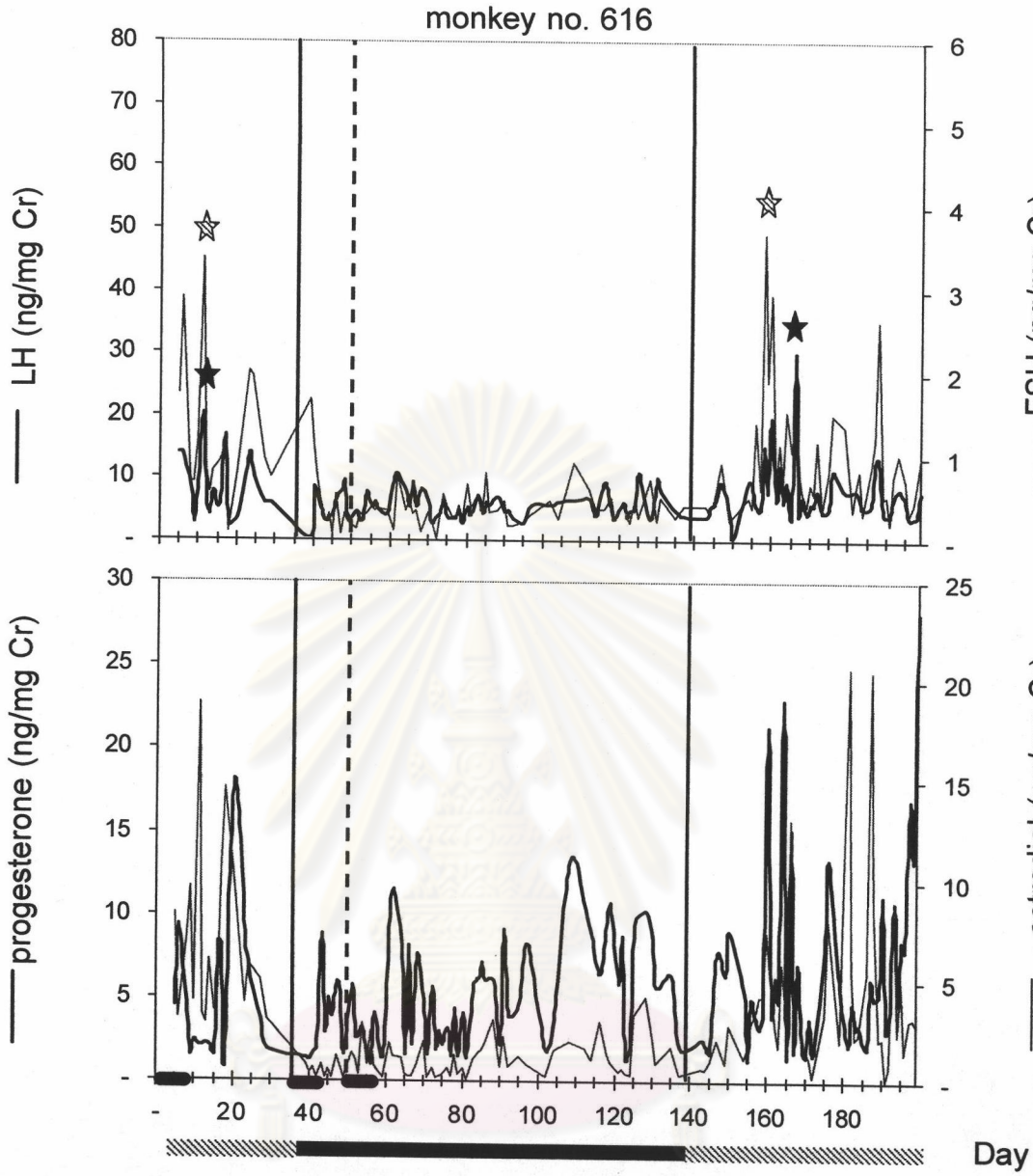


Figure 6.6 Changes in the levels of urinary FSH, LH, estradiol, and progesterone in adult cyclic monkeys fed daily with PM-100 for 90-day. The meaning of the black horizontal and vertical lines, horizontal bars, and stars are the same as explained in Figure 6.5.



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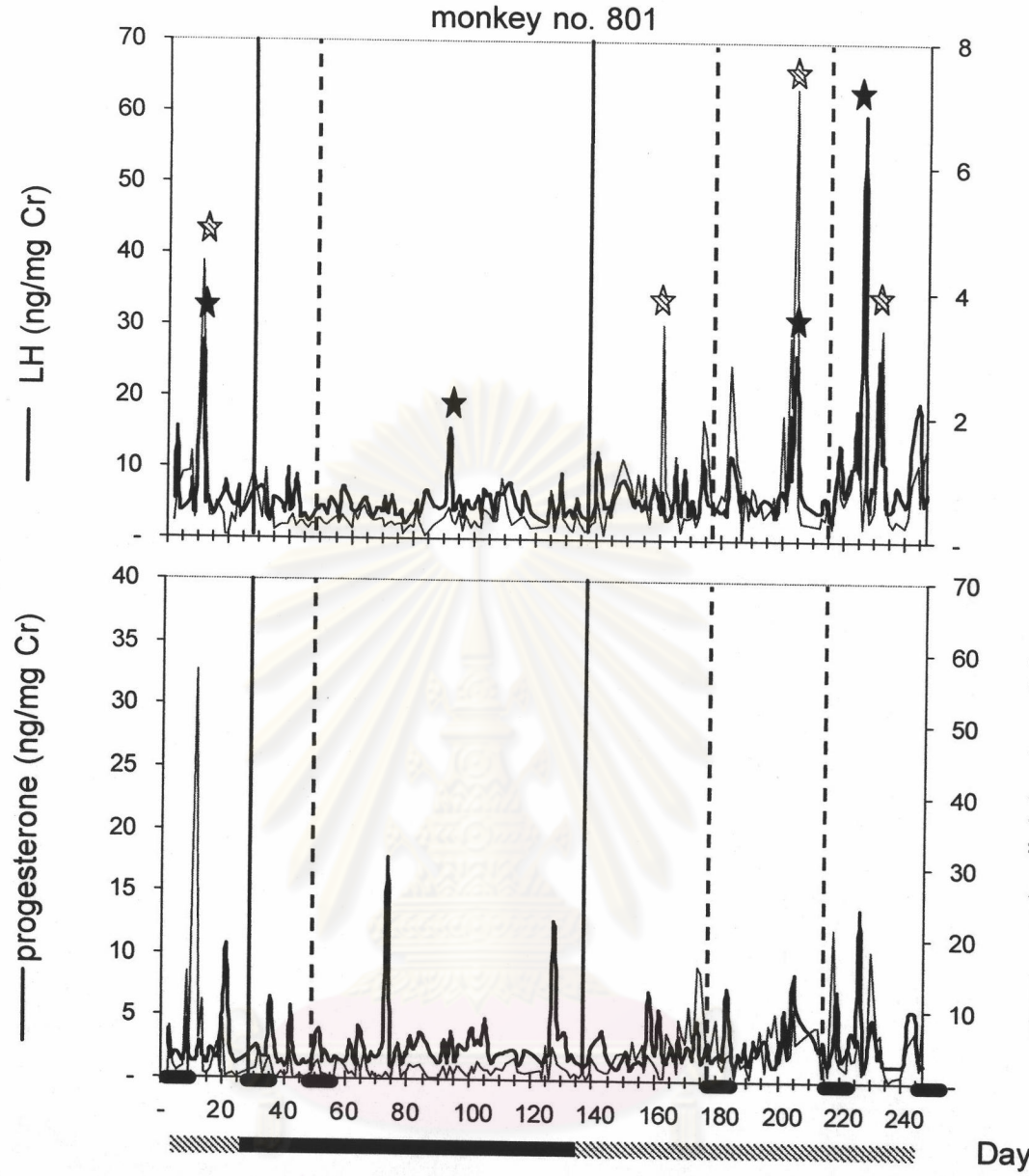

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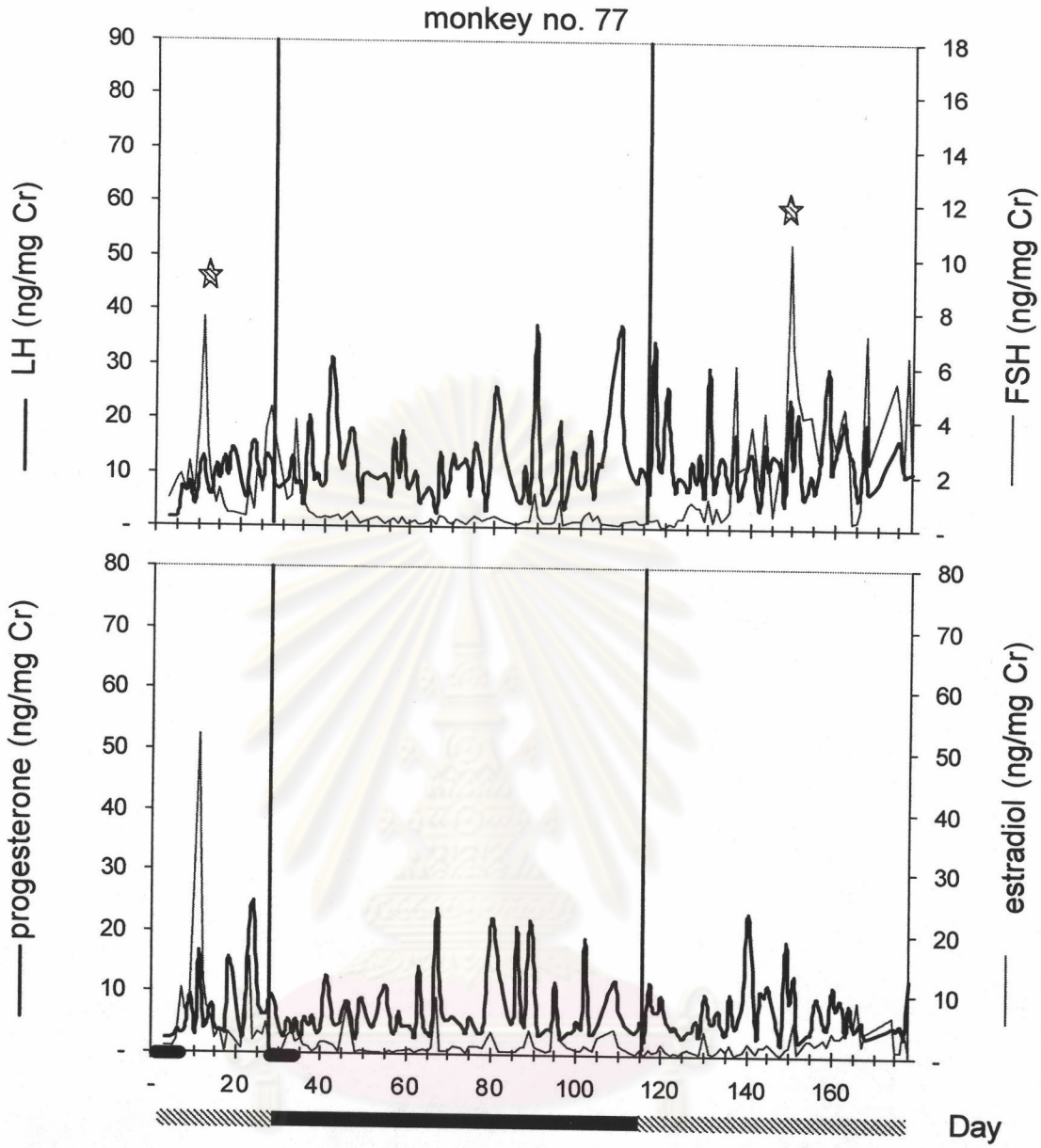
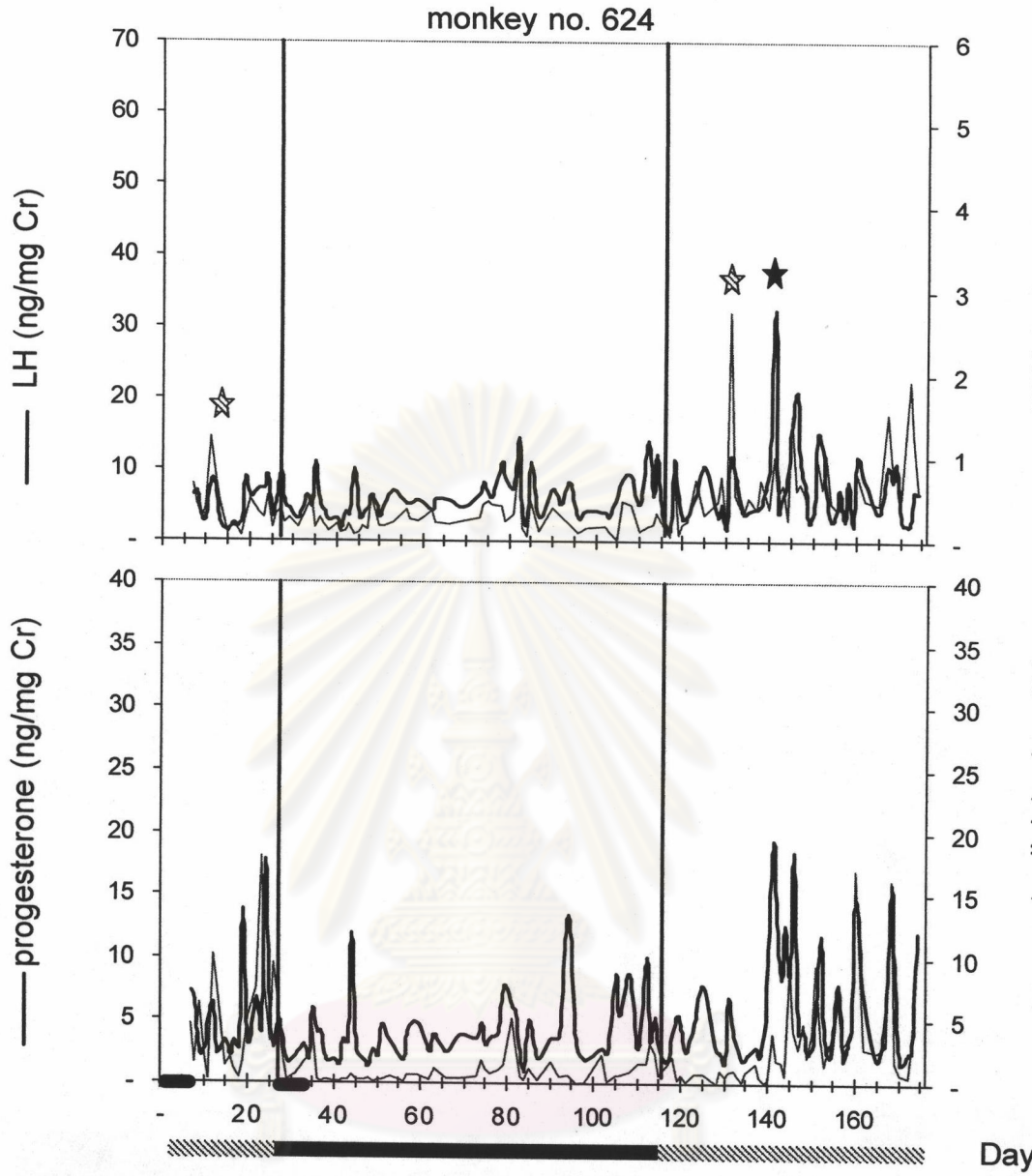


Figure 6.7 Changes in the levels of urinary FSH, LH, estradiol, and progesterone in adult cyclic monkeys fed daily with PM-1,000 for 90-day. The meaning of the black horizontal and vertical lines, horizontal bars, and stars are the same as explained in Figure 6.5.



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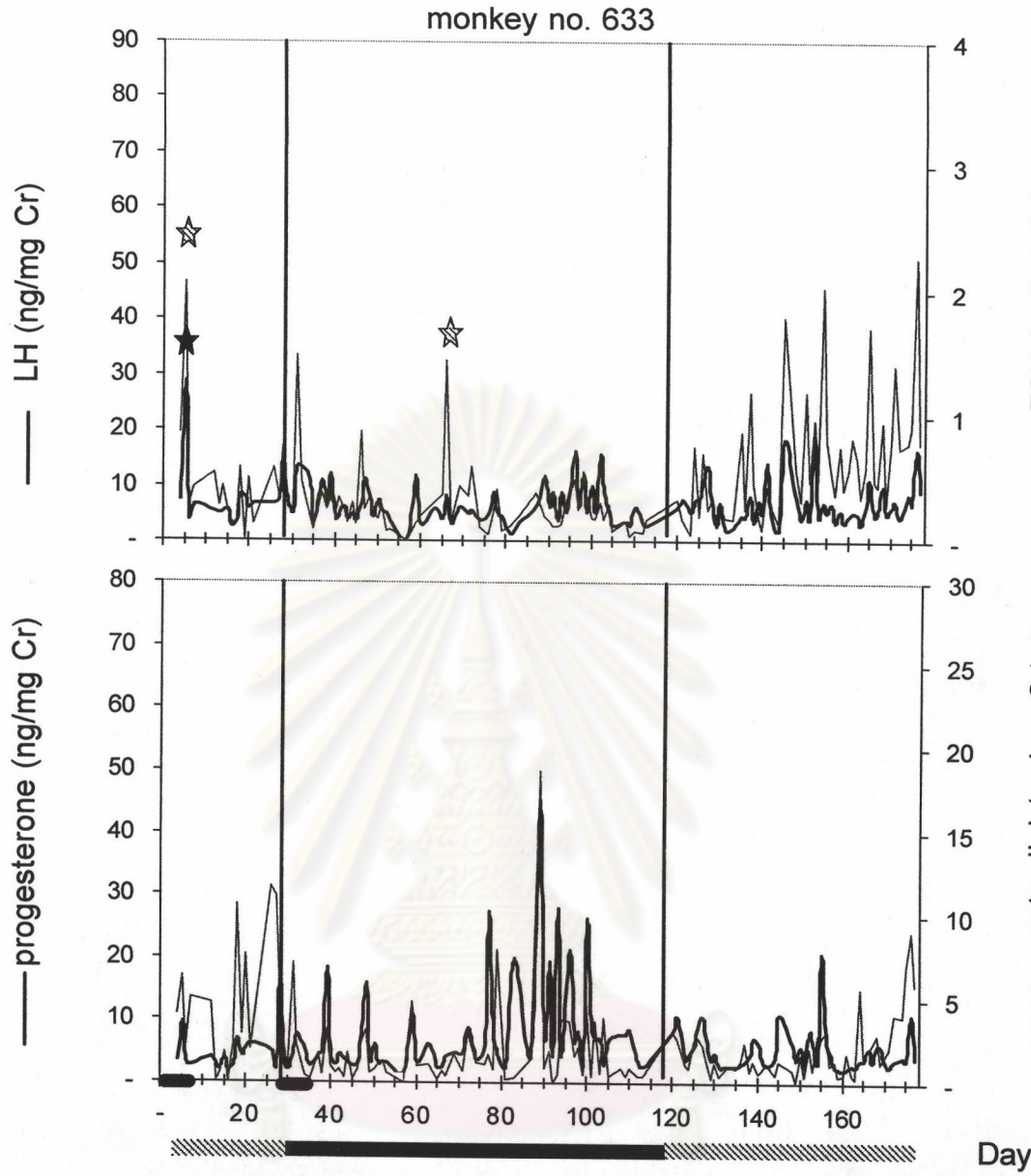

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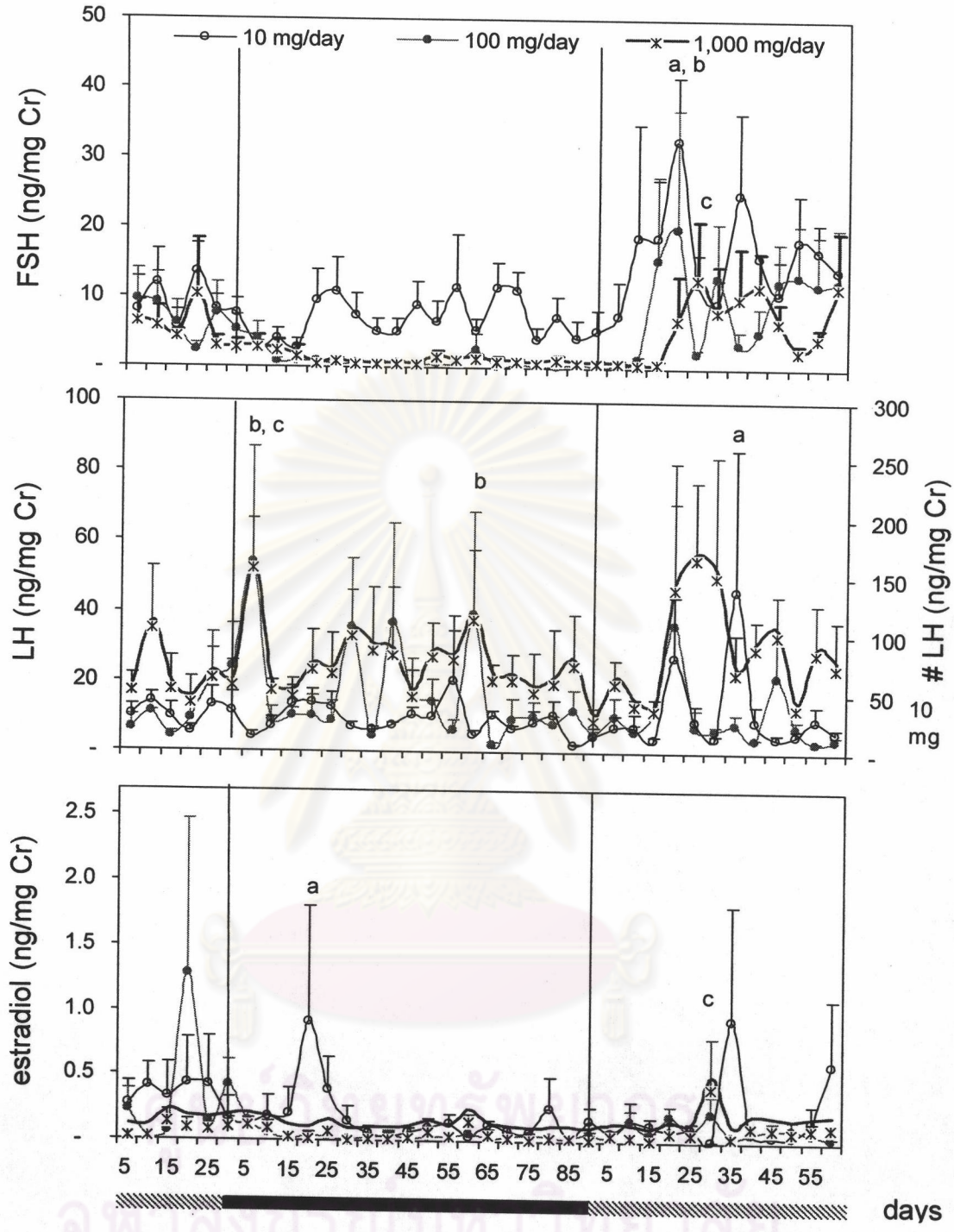

Figure 6.8 Mean levels of urinary FSH (panel A), LH (panel B), and estradiol (panel C) in aged menopausal monkeys treated with PM-10, PM-100, and PM-1,000. The black horizontal and stripe lines indicates the treatment period. The symbols of *a, b,* and *c* indicated the significant difference with $P < 0.05$.

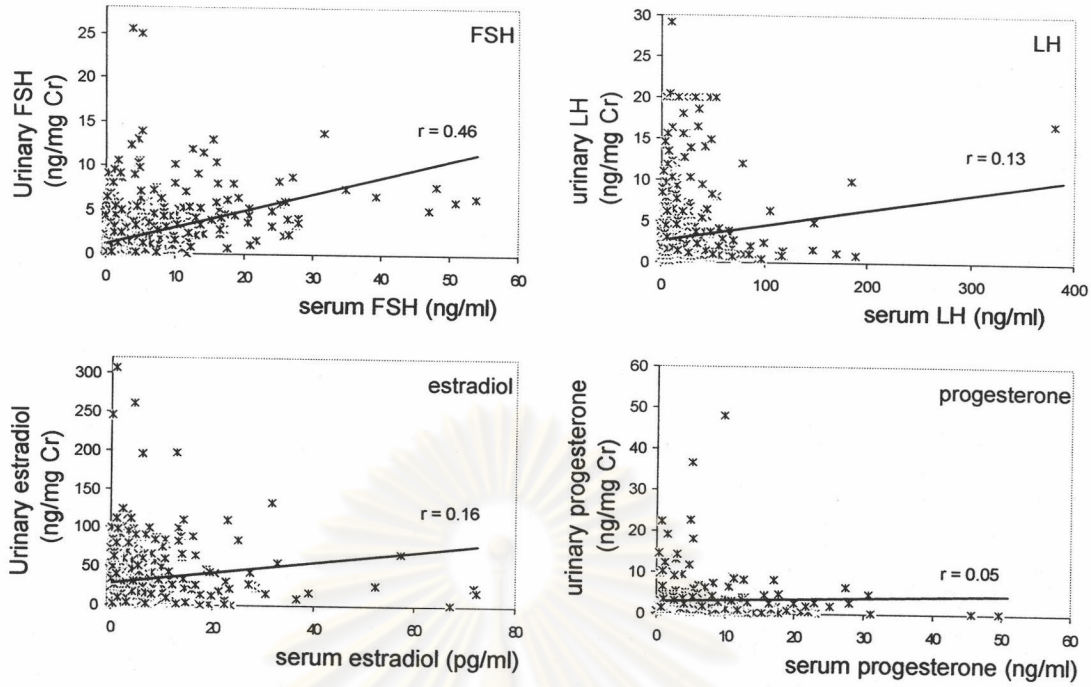

Figure 6.9 Correlation between FSH, LH, estradiol, and progesterone levels in serum and urine of adult cyclic and aged menopausal monkeys throughout the study period.

6.4 Discussion

The patterns of urinary excretion of gonadotropins and estradiol during the menstrual cycle in female cynomolgus monkeys in the present study are similar to those secretion patterns in serum, which were previously reported (Trisomboon et al., 2004). Urinary estradiol and progesterone levels showed highly fluctuation throughout the menstrual cycle, although progesterone levels was a trend toward the increase during the early luteal phase. The analysis of correlation coefficient revealed that there was a significant positive correlation in FSH, LH, and progesterone between serum and urine, but no correlation in estradiol levels. The results are in agreement with those form previous studies showing that there are the

similar patterns of gonadotropins (Cano and Aliaga, 1995) between in serum and urine of women. Urinary gonadotropin levels can therefore provide a useful index of serum gonadotropin secretion (Frohman, 1995).

The present study also investigated the estrogenic effect of PM on the secretion of urinary gonadotropin and sex steroid hormones in both adult cyclic and aged menopausal monkeys. We hypothesized that the pattern of urinary gonadotropins and sex steroid hormones were parallel with those of the respective hormones in serum, which was revealed by our previous reports (Trisomboon et al., 2002a, 2002b, 2004). A single treatment of PM in the dosages of PM-10, PM-100, and PM-1,000 did not change the patterns of urinary gonadotropin, estradiol, or progesterone throughout the menstrual cycles in adult cyclic monkeys. The result was coincide with the previous report determining no changes of serum FSH, LH, estradiol, or progesterone levels from the same monkeys (Trisomboon et al., 2004). Furthermore, the previous studies showed that the daily feeding of PM for 90 days significantly suppressed serum gonadotropin and sex steroid hormone levels in both adult cyclic and aged menopausal monkeys in a dose dependent manner (Trisomboon et al., 2002a, 2002b). The similar pattern of changes on those hormones in urine were found, especially for FSH and in aged menopausal monkeys.

Li et al., (2002) found that the peak of urinary FSH level was observed within 1 day of follicular collapse in 96.92% of the menstrual cycle in premenopausal women, indicating that urinary excretion of FSH is a useful biomarker for estimating the day of ovulation. Not only the prominent decrease in basal level but also the absence of peak urinary FSH levels were found in adult monkeys fed daily with PM-100 and PM-1,000 for 90 days. It implies that the daily feeding of PM can disturb the folliculogenesis through the suppression of FSH menstrual cycle. This was partly proved by the fact that the menstrual cycle was either prolonged or stopped in adult monkeys treated with PM-100 and PM-1,000.

Effect of PM on urinary LH level in both adult cyclic and aged menopausal monkeys could not be clearly observed because of the high fluctuation of urinary LH levels throughout the study period. The reason of this fluctuation could not be explained in this study.

Changes in urinary estradiol levels also reflected the daily dose of PM in both adult cyclic monkeys and aged menopausal monkeys. Up to now, no reports showing the effect of phytoestrogen consumption on metabolism of sex steroid hormones. From the previous study, it suggested that PM phytoestrogens act as estrogen and suppressed estradiol through the decrease of gonadotropins in adult cyclic monkeys as well as a direct action on peripheral conversion of androstenedione to estradiol in aged menopausal monkeys (Trisomboon et al., 2002b). It is considered that the decrease in urinary estradiol levels of adult cyclic and aged monkeys determined in the present study are reflects the suppression of estradiol production.

The present study demonstrated that patterns of levels of gonadotropins and estradiol in urine of female cynomolgus monkeys treated with PM are closely related with those of respective hormones in serum in female cynomolgus monkeys. Within these hormones, urinary FSH is considered to be a good indicator on the study of the estrogenic effect of PM on the disturbances of reproductive system.

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